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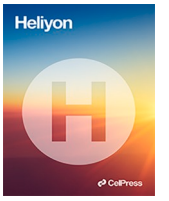

Review article

Targeting sine oculis homeoprotein 1 (SIX1): A review of oncogenic roles and potential natural product therapeutics

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ABSTRACT

Sine oculis homeoprotein 1 (SIX1), a prominent representative of the homeodomain transcription factors within the SIX family, has attracted significant interest owing to its role in tumorigenesis, cancer progression, and prognostic assessments. Initially recognized for its pivotal role in embryonic development, SIX1 has emerged as a resurgent factor across a diverse set of mammalian cancers. Over the past two decades, numerous investigations have emphasized SIX1's dual significance as a developmental regulator and central player in oncogenic processes. A mounting body of evidence links SIX1 to the initiation of diverse cancers, encompassing enhanced cellular metabolism and advancement. This review provides an overview of the multifaceted roles of SIX1 in both normal development and oncogenic processes, emphasizing its importance as a possible therapeutic target and prognostic marker. Additionally, this review discusses the natural product agents that inhibit various pro-oncogenic mechanisms associated with SIX1.

1. Introduction

SIX1 is a member of the mammalian Six family of homeobox genes, which are homologous to the sine oculis (*so*) gene in *Drosophila*. These genes produce transcription factors that play crucial roles in the proliferation and survival of progenitor cells during the process of organ development [1]. Initially discovered in *Drosophila* as essential regulators of eye formation, SIX1 proteins are known to play a role in the expansion and differentiation of cell populations in various organs, such as the retina, craniofacial structures, auditory system, brain, lungs, muscles, kidneys, and gonads, among others [2–9].

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SIX genes are broadly expressed throughout vertebrate embryogenesis, indicating their involvement in a variety of differentiation processes. A notable homeobox gene is the SIX1 homeoprotein, which plays a crucial role in the proliferation of progenitor cell populations during early embryogenesis and is essential for the development of various organs [10]. Beyond its role in muscle development, SIX1 is also necessary for the organogenesis of the kidneys, thymus, inner ear, nose, and lacrimal and salivary glands. These organ defects are associated with SIX1 expression in embryonic primordium structures and arise from the absence of the SIX1 homeoprotein during the initial stages of organ development [11].

Genetic disorders emanating from dysfunctional SIX1 have also highlighted the significance of SIX1 in the development of tissues and organs in humans. One notable disorder is branchio-oto-renal syndrome, characterized by abnormalities of the kidney and urinary tract, as well as hearing loss. The primary cause of this disease is mutations in the SIX1 gene that disrupt its ability to interact with EYA1, as well as some mutations that disrupt SIX1's DNA binding domain [12]. Another SIX1-linked developmental disorder is craniosynostosis, which features premature fusion of cranial sutures, leading to malformation of the cranium. This disease also arises due to missense and nonsense alterations in the SIX1 gene, resulting in a loss-of-function in this transcription factor [13].

In humans, the SIX family members (SIX1-6) are homologs of the *Drosophila* sine *oculis* (*so*) gene. Human SIX1 was originally identified based on its sequence similarity to murine SIX1 [14]. All SIX proteins consist of two conserved domains: the Six-type homeodomain (HD) and the SIX domain (SD) [15]. The HD mainly mediates DNA binding, whereas the SD facilitates protein-protein interactions. SIX1, as well as other members within its family, lacks an inherent activation domain and necessitates interaction with the eyes of absent family members EYA1-4 facilitates transcriptional activation to regulate gene expression [1,16]. SIX1 and EYA members are frequently overexpressed together in various cancers, such as, though not limited to, breast, pancreatic, ovarian, cervical, and colorectal cancer [17–21]. SIX1 overexpression was first observed in 1998 in primary and metastatic breast cancers, suggesting its involvement in tumor progression [22]. Later, numerous studies have validated that elevated levels of SIX1 are associated with the onset and advancement of many other forms of cancer [2,23–25].

SIX1 is recognized for promoting tumor development and metastasis by influencing various cancer cell functions, including maintaining genome stability, modulating response to apoptotic signals, driving cell proliferation, and controlling epithelial differentiation [25]. Notably, high levels of SIX1 have been closely linked to aggressive and metastatic cancers, as well as to poor patient outcomes [26].

Multiple studies have recognized elevated SIX1 expression as a new prognostic indicator in breast, ovarian, pancreatic, colorectal, and hepatocellular cancers. It is a promising biomarker for stratifying patients into various risk categories. Additionally, targeting SIX1 represents a potential innovative treatment strategy for cancer patients [27].

1.1. SIX1' role in tumor progression

SIX1 serves a critical role in biological development as a transcription factor influencing a wide array of gene expression patterns. However, these very functions also contribute redundantly to tumor promotion. The involvement of SIX1 in cancer development and progression has been demonstrated via a multitude of mechanisms. For example, SIX1 showed a strong pro-tumorigenic effect on murine tumors and fibroblast cells via the induction of an undifferentiated phenotype that maintains stemness and represses senescence-related gene signatures, characteristics commonly observed in cancer cells. SIX1 elevates the stem-like features of cancer cells by promoting SOX2 (sex-determining region Y-box 2), a key pluripotency factors [28]. SIX1 also helps in the epigenetic control of p16INK4A (cyclin-dependent kinase inhibitor 2A, CDKN2A), a protein that slows cell cycle progression and is frequently nullified in multiple cancers. Additionally, SIX1 assists in the epigenetic control of a multitude of genes implicated in tissue growth and specialization [29]. These mechanisms enhance tumorigenic capacities by bypassing intrinsic cell cycle checkpoints and retaining stemness in cancer cells, which augments their resiliency and ability to transition into more aggressive malignancies.

SIX1 transcriptional activity induces cell proliferation by modulating the expression of crucial cell cycle gene. Overexpression of SIX1 decreases the fraction of tumor cells in the G_0/G_1 phase and increases the fraction in the S phase, while SIX1 knockdown produces the opposite effect [30]. Further, SIX1 overexpression enhanced the mRNA transcripts of cyclin A1, a stimulator of cell cycle progression, while knockdown of SIX1 exerted the opposite effect on cyclin A1 [31]. In addition, SIX1 has been demonstrated to regulate transcriptionally mediate the presence of cyclin D1, as confirmed by a cyclin D1 promoter reporter assay that demonstrated increased luciferase activity. ChIP analysis further validated the interaction of SIX1 with the promotor section of cyclin D1, and silencing of SIX1 decreases cyclin D1 protein and reduces phosphorylation process of retinoblastoma (Rb) [32].

SIX1 also plays a role in tumor immune cell infiltration. While immune cells can have dual roles in tumor progression, they generally contribute to tumor surveillance and eradication. Consequently, their infiltration is typically associated with reduced tumor burden. Patient datasets revealed that an increased level of SIX1 in human tumor tissues was negatively associated with immune cell infiltration within the tumor microenvironment (TME) and overall survival rates [33]. Mechanistically, this is believed to be due to the association of SIX1 with immunosuppressive TGF-β (transforming growth factor-beta) signaling cascade (Fig. 1) [34], which requires SIX1 to express various collagen genes through the Smad2/3 activation pathway dependent on TGFBR2. SIX1 facilitates TGF-β-induced collagen accumulation in the TME and significantly impairs immune cell invasion and activation [33], leading to a poorer prognosis and increased tumor burden.

In coordination with TGF-β signaling, SIX1 further enhances tumor progression by facilitating lymphangiogenesis, a process frequently utilized by cancer cells to facilitate metastasis. Specifically, SIX1 expression promotes TGF-β pathway activation to enhance the expression of Vascular Endothelial Growth Factor-C (VEGF-C), which induces the formation of new lymphatic endothelial cells. This process facilitates tumor cell migration into fresh lymphatic tissue, thereby promoting both *in vitro* and *in vivo* metastasis. Consequently, targeting SIX1 is suggested as a promising strategy to inhibit metastasis [35].

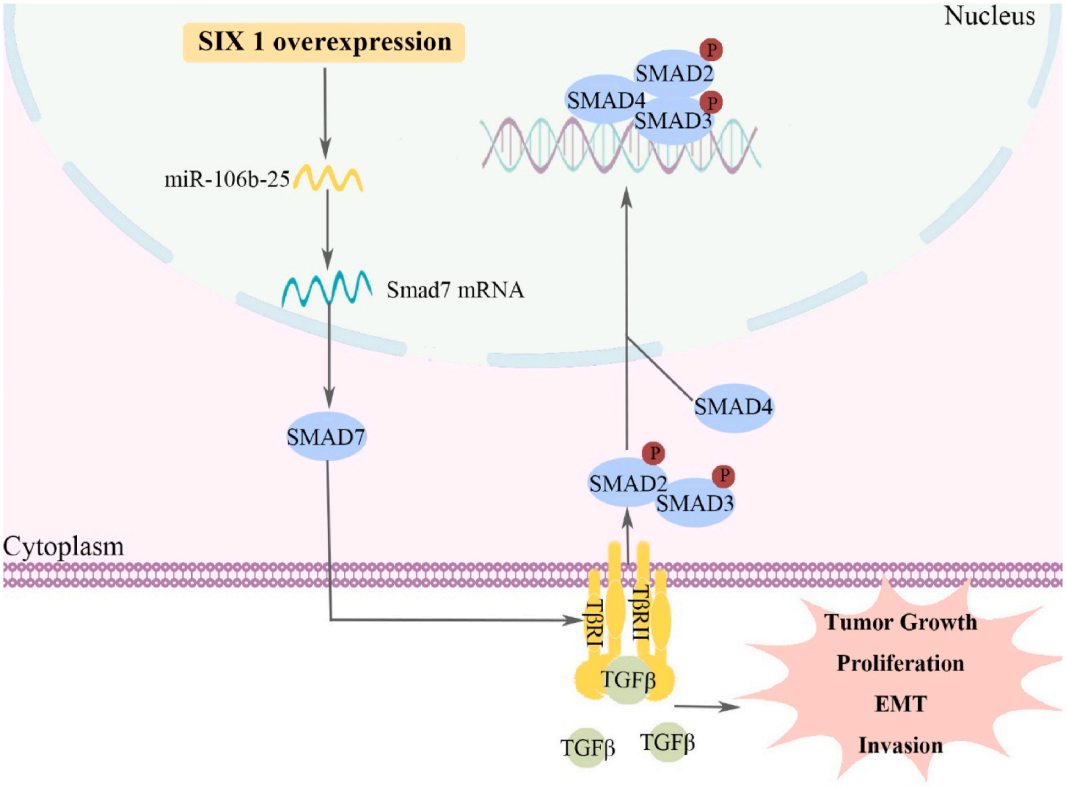

Fig. 1. TGF-β signaling and its interaction with SIX1. SIX1 activates the tumor-promoting arm of TGF-β signaling by upregulating the miR-106b-25 microRNA cluster. This microRNA cluster targets the inhibitory Smad7 protein, leading to increased levels of the TGF-β type I receptor (TβRI) and the subsequent activation of downstream TGF-β signaling.

There are numerous key signaling pathways linked to driving proliferation, cell growth, and cell survival, all of which SIX1 influences. For example, SIX1 overexpression elevates ERK (extracellular signal-regulated kinase) and AKT (serine/threonine kinase) pathways [36]. It has been shown the transfection of SIX1 led to an upregulation matrix metalloproteinase (MMP)-2 expression, and treatment with ERK inhibitors counteracted the impact of SIX1 on MMP-2 expression. This points to the involvement of the ERK/MMP-2 signaling pathway. Furthermore, treatment with AKT inhibitors inhibited the SIX1-mediated levels of the anti-apoptotic protein Bcl-2 [36]. These instances highlight the interaction and interplay of SIX1 with key tumor-promoting pathways. As a crucial transcription factor in tissue and organ development, SIX1's regulation of diverse gene signatures can facilitate tumorigenesis across various types of cancer, as elaborated in subsequent sections. Fig. 2 illustrates the multiple signaling pathways through which SIX1 contributes to tumorigenesis, largely reflecting mechanisms outlined in this section across different cancer types.

1.2. SIX1 contributes to cancer metastasis

Cancer cell dissemination from its origin site to distinct body organs, known as metastasis, is the primary reason for cancer-related fatalities. SIX1 contributes to the phenotypic transition of the tumor cells in the TME [33]. The transformation from epithelial to mesenchymal phenotype is central to the metastatic cascade, where overexpression of SIX1 was observed to induce a shift towards the mesenchymal phenotype in cancer [37].

To promote metastatic events, SIX1 is known to coordinate with Ezrin, a protein associated with multiple cellular functions. SIX1 binds to the Ezrin promotor and regulates Ezrin gene transcription. The activity of Ezrin on metastasis in tumor cells is essential and was validated through shRNA-mediated suppression of Ezrin, which ablated tumor metastasis to the lungs of nude mice from the tail region [38]. Thus, SIX1 modulates metastasis via transcriptional control of Ezrin.

SIX1 also promotes metastasis through angiogenesis. Histopathological analysis of tumors overexpressing SIX1 in mice presented elevated blood vessels, as indicated by CD31 and α-SMA. Furthermore, *in vitro* assays showed that SIX1 overexpression increased cellular motility and infiltration. Immunoblot analysis revealed elevated levels of VEGF, MMP9, and MMP2 with SIX1 overexpression, implying that these elements might play a role in the heightened metastatic potential associated with SIX1 [39].

Inhibition of the SIX1/EYA2 interaction also sheds light on its epithelial-mesenchymal transition (EMT) and metastatic-promoting capabilities. TGF-β is known to promote EMT and metastasis in cancer. Previous studies have demonstrated that SIX1 augments EMT and metastatic progression by enhancing TGF-β signaling [40–44]. Treatment by NCGC00378430 "compound 8430", a small synthetic

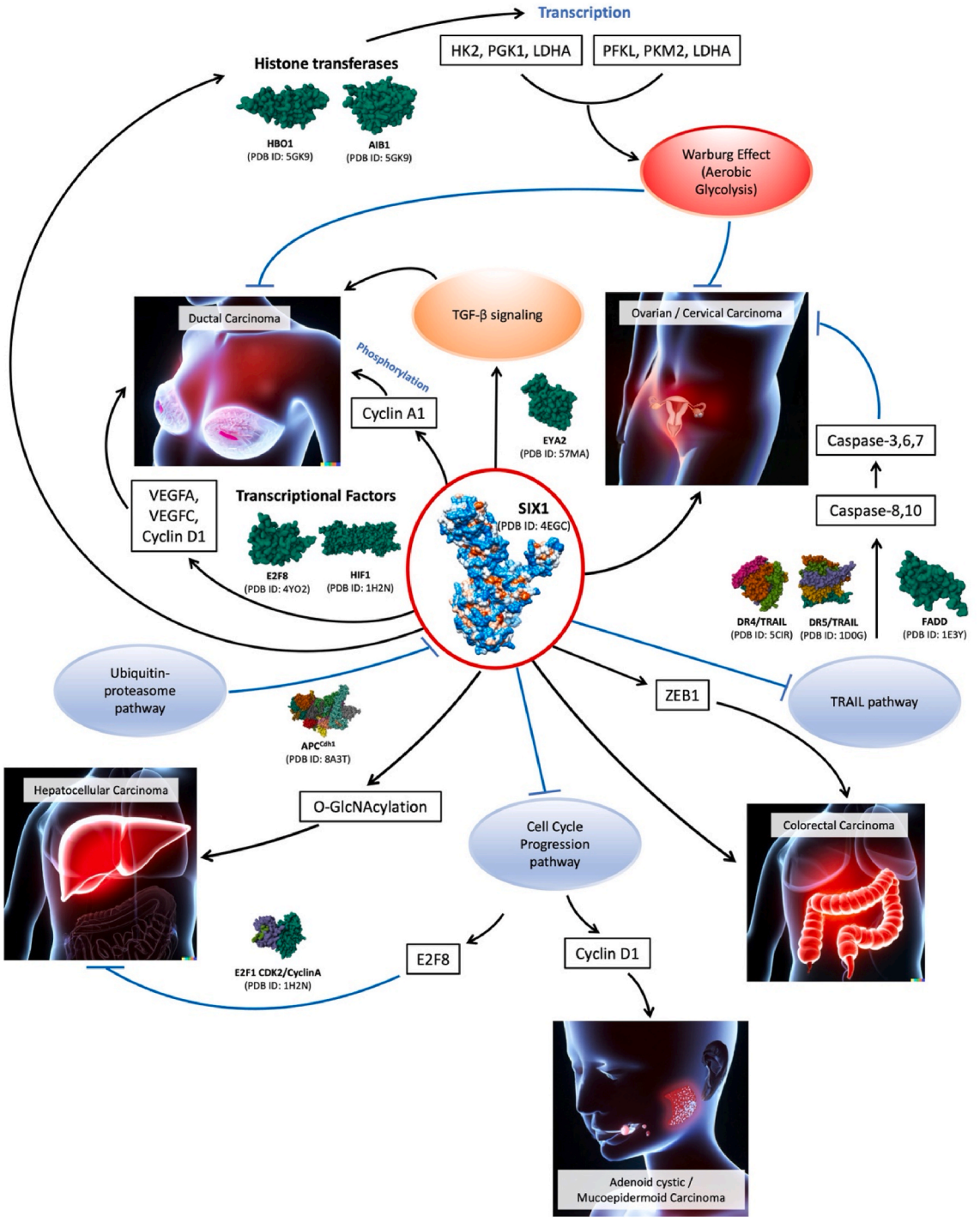

Fig. 2. SIX1 mediates tumorigenesis in various cancer types through several general mechanisms. It can drive cell cycle progression by upregulating cyclin D1 and A1, and promote metastasis and immune evasion by enhancing TGF-β signaling and ZEB1 expression. SIX1 also prevents cell death by negatively regulating apoptotic proteins. Additionally, SIX1's transcriptional control over glycolytic genes boosts metabolism in a way that favors tumorigenesis. Black arrows: Direction of mechanism; Blue lines: Signaling pathways. 3D protein models obtained from the RCSB PDB. Cancer models generated with the assistance of artificial intelligence (AI) via OpenAI's DALL·E image modeling program- https://openai.com/dall-e-2. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

molecule, disrupts the interplay among EYA2 and SIX1 via blocking TGF-β activation of Smad3. In parallel, compound 8430 increased E-cadherin expression, a common marker of cells that have not undergone EMT. Furthermore, treatment of this compound blunted metastasis in mice while primary tumor growth remained unchanged [45]. These results demonstrate SIX1's role in EMT and metastasis and how it is intertwined with TGF-β signaling and correlated with the downregulation of E-cadherin.

1.3. SIX1 interaction with p53

In addition to its involvement in enhancing tumor-promoting pathways, SIX1 is also implicated in tumor-suppressive pathways. SIX1 exerts epigenetic control of the tumor-inhibiting factor p16INK4A and modulates the expression of another cancer suppressor, p53 (TP53). p53 is crucial in cancer, with mutations occurring in ~50 % of all human cancers, while its function is often compromised even in non-mutated cases. A prior study has demonstrated that increased p53 expression levels correlate with improved survival rates among cancer patients [46]. Notably, the tumor-promoting and metastasis-inducing SIX1 homeoprotein emerges as a significant factor in modulating p53 levels through a distinctive mechanism independent of the established negative regulator of p53, MDM2 (mouse double minute 2) [47]. One of the hallmark consequences of this interaction is the imposition of notable resistance to therapeutic interventions targeting the p53-MDM2 interaction [48]. The engagement of SIX1 unveils a parallel avenue through which cancer cells adeptly evade therapies aimed at restoring p53's tumor suppressive functions.

Western blot analysis demonstrated that sustained overexpression of SIX1 results in reduced nuclear p53 protein levels and its downstream target p21. In comparison to control cells, MCF7- SIX1 cells have approximately 50 % less p53 protein attached to both the strong distal-binding site (2,283) and the weaker proximal binding site (1,391) on the p21 promoter. qRT-PCR shows that overexpression of SIX1 in MCF7 cells reduces the mRNA expression of p53 target genes induced by 10 μM etoposide, compared to control cells. Relative gene expression of p21, HDM2, PIG3, 14-3-3σ, DDB2, BAX, GDF15, and DR5 decreased about 50, 30, 50, 55, 40, 25, 35, and 20 %, respectively. SIX1 controls p53 through the increased expression of microRNA-27a and the simultaneous downregulation of ribosomal protein L26 (RPL26). Furthermore, the capacity of SIX1 to govern p53 via a novel, MDM2-independent pathway may hold clinical significance. This is particularly noteworthy as increased SIX1 expression has the potential to confer resistance to therapies aimed at MDM2, which are currently in clinical trials. This intricate dual mechanism finely tunes p53 levels, contributing to the altered cellular landscape characteristic of cancer driven by SIX1 [48]. Interestingly, in another study, increased expression of SIX1 was found to enhance tumor growth by reducing p53 expression, achieved through the suppression of Dachshund homolog 1 (DACH1) expression. SIX1 overexpression suppressed DACH1 expression by approximately 60 % and decreased p53 protein levels by around 50 %, while inducing MDM2 expression (~2-fold) [49].

2. SIX1 in carcinomas

Considering the pro-tumorigenic mechanisms enhanced by SIX1, it is unsurprising that this transcription factor is implicated in a broad spectrum of cancers, including carcinomas. In this section, we explore the intricate associations of SIX1 with various types of carcinoma (Table 1). Furthermore, we present the comparative mRNA and protein expression levels of SIX1 in these carcinoma subtypes (Table 2).

Hepatocellular carcinoma (HCC) stands as a prime example of SIX1 overexpression. SIX1 mRNA is elevated in approximately 85 % of liver tumor tissues, while absent in 91.7 % of non-tumor liver tissues [50]. The mRNA expression levels of SIX1 in comparison to HCC are about 5-fold higher than in surrounding non-tumor liver tissue tissues [51]. Upregulation of SIX1 is prominently detected in

Table 1
SIX1 role in carcinoma subtypes.

carcinoma subtypes	pathway	protein/genes
Hepatocellular carcinoma	*O*-GlcNAcylation	MMP-9, ABCC2, SOX2
Endometrial cancer	ERK, AKT	
Adenoid cystic carcinoma		cyclin D1
Mucoepidermoid carcinoma		cyclin D1
Colorectal cancer	Wnt/β-catenin	TEAD4
Papillary thyroid carcinoma	TGF-β/Smad2/3, EMT	TGFβ1, *p*-Smad2/3
Esophageal squamous cell carcinoma	TGF-β, ERK, AKT	TGFB1, TGFB2, TGFBR2, Bcl-2, cyclin E
Ovarian carcinoma		TRAIL, EYA2
Melanoma	Glycolysis	miR-150-5p, miR-489-3p
Gastric tumors	ERK, EMT	Bcl-2, caspase-7, cyclin D1, MMP2, E-cadherin
Cervical cance	TGFβ-SMAD, MAPK, EMT	MCM2, MCM3, MCM6, POLA1, PRIM1, PRIM2, RFC3, RFC4, RFC5, POLD3, POLE2, α5β1
Pancreatic ductal adenocarcinomas	EMT	CDH1, vimentin, LDHA
Prostate tumors		miR-30a, GRP75, USP1
Breast cancer	TGF-β, Hh, EMT	cyclinA1, cyclin D1, c-Myc, Ezrin, VEGF-C, EYA, miR-204-5p
Non-small cell lung cancer	EMT, Notch	
Glioma		SOX2
Glioblastoma multiforme		CTGF
Rhabdomyosarcoma		MYOD1, MYOG
Osteosarcoma	PTEN/PI3K/AKT	cyclin D1, VEGF-C, caspase-3
Acute myeloid leukemia	Glycolysis	

Table 2
Relative SIX1 mRNA and protein expression in cancer tissues than in normal tissues.

carcinoma subtypes	mRNA level/fold change	protein level/fold change
Hepatocellular carcinoma	~5	n.a.
Colorectal cancer	~6	n.a.
Papillary thyroid carcinoma	~3.5	n.a.
Esophageal squamous cell carcinoma	~5.5	n.a.
Ovarian carcinoma	~3	n.a.
Gastric tumors	~3.5	n.a.
Cervical cancer (HKc/DR *vs.* HKc/HPV16 lines)	200 to over 600	~2
Pancreatic cancer	3~5	n.a.
Prostate tumors	~5	n.a.
Breast cancer	~1.5	n.a.
Non-small cell lung cancer	~3	n.a.
Glioblastoma multiforme	~2.5	n.a.
Osteosarcoma	~3	~3

n.a. – not available.

female patients with hepatitis C virus-positive HCC and correlates with increased tumor growth kinetics and decreased survival within this subgroup [52]. SIX1 overexpression significantly correlates with tumor dimensions, pathological tumor-node-metastasis (pTNM) staging, and vascular invasion. The distinct influence of SIX1 is underscored by markedly lower survival rates at 5 years for patients with increased SIX1 expression, identifying it as a standalone prognostic indicator of poor outcomes in HCC [51].

The tumorigenic potential of SIX1 is further accentuated by its facilitation of HCC growth both *in vitro* and *in vivo*. SIX1's role involves O-GlcNAcylation, which not only drives HCC proliferation but also regulates glucose metabolism [53]. Furthermore, SIX1 promotes tumor growth and the endometrial carcinoma phenotype in HCC cells, where macrophage-derived SIX1 escalates cancer cell invasion through heightened MMP-9 expression [54]. The regulatory axis is further highlighted by the direct interaction between SIX1 and the SOX2 promoter, culminating in increased SOX2 expression and transcriptional activity. This underscores its roles in both stemness regulation and chemoresistance, which is postulated to be achieved by SOX2-mediated transcriptional activation of ABCC2 expression, a molecular transporter known to enhance cancer cell chemoresistance [55,56].

SIX1 is involved in endometrial carcinogenesis in both mice and humans, potentially acting as an indicator for abnormal estrogen response and a subtype of endometrial cancer (EC) [57]. Overexpression of SIX1 is commonly detected in EC and emerges as an independent factor impacting prognosis [58]. Functionally, SIX1 overexpression increases cancer cell growth and the formation of colonies in EC, possibly mediated through ERK- and AKT-mediated pathways [59]. SIX1 also governs the intricate process of normal endometrial epithelial differentiation, impacting the behavior of $CK14^{+}/18^{+}$ cells that function as cancer progenitors via transformation and promotion into neoplastic lesions. Intriguingly, SIX1 mitigates diethylstilbestrol and promotes basal differentiation within $CK14^{+}/18^{+}$ cells, contributing to (DES)-induced endometrial carcinogenesis cell populations [60].

In malignancies of the salivary glands, both adenoid cystic carcinoma (AdCC) and mucoepidermoid carcinoma (MEC), elevated SIX1 expression coincides with increased levels of cyclin D1, a pivotal proliferation-associated factor and an early initiator of transitioning from G_0 to G_1 phase in the cell cycle, spotlighting SIX1's potential in modulating growth of AdCC and MEC [61].

In summary, the complexities of SIX1 in carcinoma extend across various dimensions, from HCC to endometrial carcinoma and salivary gland malignancies, emphasizing its multifaceted influence in shaping tumorigenic behavior across diverse cancer contexts.

2.1. SIX1 in colorectal cancer

SIX1 overexpression triggers advanced-stage colorectal cancer (CRC) and metastasis by orchestrating the restructuring of the tumor stroma, promoting angiogenesis, and attracting tumor-associated macrophages (TAMs) to support tumor growth microenvironment [39]. Subsequent quantitative RT–PCR evaluation of a range of CRC cell lines revealed that SIX1 mRNA expression is commonly detected (8 out of 18 lines, 44 %). The relative mRNA expression of SIX1 in CRC is approximately 6-fold higher compared with normal human colon tissue [25]. Indeed, elevated SIX1 expression emerges as an autonomous prognostic indicator in stage I-III CRC [37]. The transcription factor TEAD4, also known as TEA domain transcription factor 4, which correlates with EMT, metastasis, and poor CRC prognosis, directly governs SIX1 expression at the transcriptional level. This newly established link underscores SIX1 as a direct objective of TEAD4, and concurrent elevated levels of nuclear TEAD4 and SIX1 indicate an unfavorable prognosis for CRC patients [62]. SIX1 also upregulates the Wnt/β-catenin signaling pathway, driving the proliferation and migration of CRC cells [63]. Genetic suppression of SIX1 using shRNA reduced the motility and invasion of CRC lines, as shown in cellular assays, inhibited colorectal cancer growth *in vitro*, and inhibited tumor proliferation in CRC xenotransplantation models, demonstrating a promising therapeutic avenue for CRC treatment [64].

2.2. SIX1 in thyroid cancers

SIX1 is pivotal in shaping tumor progression in a subset of thyroid cancer called papillary thyroid carcinoma (PTC). SIX1 mRNA expression was increased in cytological specimens from PTC patients compared to those from non-toxic nodular goiter (NTG) patients. The relative mRNA expression of SIX1 in PTC is approximately 3.5-fold higher compared to NTG [44]. In PTC tissues, upregulation of

SIX1 is associated with the extent of extrathyroidal extension (ETE), pathological T (pT) stage, tumor-node-metastasis (TNM) staging, lymph node metastasis (LNM) spread, and distant metastasis among patients with PTCMoreover, SIX1 triggers EMT and bolsters key biological traits such as invasion and migration in PTC cells. This effect is orchestrated by the upregulation of TGFβ1 and *p*-Smad2/3 when SIX1 is overexpressed, thereby revealing a complex molecular mechanism that involves the TGF-β/Smad2/3 pathway as a driving force behind SIX1's influence in PTC [44]. Furthermore, SIX1 upregulates EYA1 expression and stabilizes the protein, which activates the canonical STAT3 signaling pathway and promotes PTC [65]. SIX1 expression modulates glucose metabolism and cell invasion. Intriguingly, these effects are mediated by increased expression of critical factors like Snail, MMP2, and GLUT3 [66].

2.3. SIX1 in throat cancer

SIX1 overexpression is linked to heightened metastasis and unfavorable prognosis in esophageal squamous cell carcinoma (ESCC). Ectopic expression of SIX1 induced the elevated expression of transforming growth factor beta (TGFB1 and TGFB2) and its receptor (TGFBR2) [67]. It was found that SIX1 protein was upregulated in 36.9 % (44/119) cases [36]. The relative SIX1 mRNA expression was significantly higher (5.5-fold) in lymph nodes with metastasis compared to those without metastasis in ESCC specimens [67]. Crucially, SIX1 significantly accelerated the self-regeneration characteristics of cancer stem cells within ESCCs, contributing to the tumor's aggressiveness and resilience [67].

Furthermore, ectopic expression of SIX1 upregulated both ERK and AKT pathways in ESCC. Genes associated with anti-apoptosis, such as Bcl-2, were upregulated in ESCC through AKT signaling, and expression was abolished with the AKT inhibitor LY294002. Concomitant to elevated Bcl-2 expression, SIX1 overexpression also downregulated the pro-apoptotic protein Bim, propagating ESCC resistance to radiotherapy. Consequently, SIX1 overexpression in ESCC cell lines promoted tumor growth in xenograft models as well as *in vitro* proliferation assays. Cell cycle promotor cyclin E was upregulated in SIX1, overexpressing ESCC, which partially explains the strong proliferative effects of SIX1 in ESCC [36].

2.4. SIX1 in ovarian cancer

In ovarian cancer, overexpression of SIX1 is a defining feature. Notably, SIX1 exhibited overexpression in 50 % of early-stage (stage I) and 63 % of late-stage (stages II, III, and IV) ovarian carcinomas analyzed. On average, late-stage tumors demonstrated approximately three times higher levels of SIX1 mRNA compared to early-stage tumors [68]. SIX1 promotes ovarian cancer cell proliferation, as deemed in proliferation assays with SIX1 stably transfected ovarian cancer cell lines compared to their respective control counterparts [68]. Additionally, SIX1 inhibits apoptosis of ovarian carcinoma cells induced by tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), highlighting its role in evading cell death mechanisms [50].

The interplay of SIX1 within the TME is revealed by its correlation with intratumoral stroma (ITS) in malignant cases. The SIX1/EYA2 complex is linked to an unfavorable prognosis in ovarian cancer. The expression of SIX1 within ITS is a prognostic indicator for stromal invasion in borderline ovarian tumors, thus carrying implications for diagnostic precision and treatment strategies. Ultimately, the presence of SIX1 within the ITS can potentially influence patient survival in ovarian cancer [69].

2.5. SIX1 in skin cancer

The aggressiveness of melanoma cells also revealed a high expression of SIX1 [70]. The current understanding of SIX1's involvement in skin cancer stems from microRNAs that target SIX1 expression, which ameliorated melanoma tumorigenic capacities *in vitro*. MicroRNA-150-5p (miR-150-5p) [71] and miR-489-3p [72] inhibit direct targeting of the 3′-UTR of SIX1 expression in melanoma cells. These microRNAs inhibited melanoma cell growth, migration, and infiltration by inhibiting SIX1 mRNA transcripts. Mechanistically, both miR-150-5p and miR-489-3p dampen reducing glycolysis through decreased glucose uptake, lactate production, ATP synthesis, and extracellular acidification rate (ECAR), while enhancing oxygen consumption rate (OCR), by targeting the transcription of SIX1-mediated glycolytic genes [71,72]. Thus, SIX1 also offers a potential avenue for therapeutic intervention in targeting skin cancer.

2.6. SIX1 in gastric (stomach) cancer

While SIX1 expression is downregulated in adenocarcinoma gastric tumors compared to normal healthy tissue surrounding the tumor, the expression of SIX1 is notably increased in diffuse-type and grade III gastric cancer tumors [73]. qRT-PCR analysis also verified elevated levels of SIX1 mRNA expression in gastric adenocarcinomas (~3.5-fold) compared to normal gastric mucosa in fresh samples [74]. This distinction suggests a potential oncogenic role for SIX1 in the development of specific gastric cancer subtypes. One of the mechanisms through which SIX1 influences gastric cancer is by promoting mitochondrial apoptosis. Silencing the expression of SIX1 can induce mitochondrial apoptosis, characterized by the repression of Bcl-2 and activation of executor caspase-7 [75]. This mechanism underscores the role of SIX1 in inhibiting apoptosis, which is often a hallmark of cancer cell survival.

Furthermore, the upregulation of SIX1 in a subtype of gastric cancer tissues is associated with its ability to enhance several critical aspects of cancer biology. SIX1 overexpression promotes gastric cancer promotes cell proliferation through the regulation of cyclin D1, a key cell cycle regulator. Additionally, SIX1 contributes to induce invasion by activating the ERK signaling pathway and promoting EMT by targeting MMP2 and E-cadherin [76]. These functions collectively indicate the potential regulatory mechanisms mechanisms by which SIX1 stimulates cell proliferation and invasion in gastric cancer. Interestingly, the frequent upregulation of SIX1 in gastric

adenocarcinomas suggests its utility as a standalone predictor of prognosis and survival duration in patients with gastric cancer [74]. This highlights the clinical significance of SIX1 in assessing disease severity and patient outcomes.

2.7. SIX1 in cervical cancer

SIX1 expression is strongly associated with lymphangiogenesis and lymph node metastasis in cervical cancer [35]. The RT-PCR results demonstrated that SIX1 mRNA levels were substantially elevated in cervical cancer and cervical intraepithelial neoplasia tissues compared to normal cervical tissue [21]. One key mechanism through which SIX1 impacts cervical cancer is by promoting the EMT in coordination with the TGF-β/Smad pathway. SIX1's collaboration with TGF-β/Smad signaling is crucial for EMT induction and enhancing metastatic capabilities in cervical cancer cells. Together, SIX1, in coordination with TGF-β/Smad, increases cell motility and tumor metastasis [77].

SIX1 is also known to upregulate the levels of several essential genes that participate in the initiation of DNA replication in cervical cancer. These genes, which encode proteins within complexes such as the minichromosome maintenance complex (MCM2, MCM3, MCM6), the DNA polymerase α-primase complex (POLA1, PRIM1, PRIM2), the clamp loader complex (RFC3, RFC4, RFC5), the DNA polymerase δ complex (POLD3), and the DNA polymerase ε complex (POLE2), work in coordination to accelerate the transition from G1 to S phase, thereby enhancing the proliferation of cervical cancer cells and contributing to the overall progression of cervical cancer [30].

Moreover, SIX1 enhances the expression of alpha-5-beta-1 (α5β1) in tumor cells. The upregulation of α5β1 is critical for SIX1's ability to enhance various functions essential for a metastatic characteristic. Specifically, it enhances the attachment of tumor cells to the extracellular matrix (ECM), promotes the infiltration of tumor cells into target organs, and accelerates the formation of metastatic lesions [43].

HPV16-induced tumorigenesis exhibits numerous critical characteristics akin to cervical cancer [78]. During the advancement of HPV16-immortalized human keratinocytes (HKc/HPV16) in laboratory settings, a model system for studying the development of HPV-associated cancers, SIX1 mRNA levels significantly increase. SIX1 mRNA expression surged by 200 to over 600 times in HKc/DR compared to their corresponding HKc/HPV16 lines, while SIX1 protein levels rose by approximately two fold [79]. This upregulation is mainly associated with the transition of these cells toward a phenotype resistant to differentiation (HKc/DR). Importantly, HPV16-transformed cells rely on SIX1 for their survival, as well as for the continued expression of HPV16 E6/E7 genes and the stimulation of EMT.

Elevated expression of SIX1 not only results in differentiation resistance but also promotes EMT during the initial phases of HPV16-induced transformation of human keratinocytes [79]. This suggests that SIX1 is key in propelling these cells towards a malignant condition. Moreover, the overexpression of SIX1 is linked to the development of cancer stem cell (CSC) properties and the malignant conversion of HKc/DR cells. These effects are mediated, at least partially, via the activation of the MAPK signaling pathway [80]. SIX1's involvement in cervical cancer encompasses a range of critical mechanisms that contribute to tumor progression, metastasis, and proliferation. Its potential as both a biomarker and a therapeutic target underscores its significance in understanding and managing cervical cancer [30,43,77].

2.8. SIX1 in pancreatic cancer

Pancreatic cancer is characterized by its aggressive nature, and SIX1's role in this malignancy sheds light on potential mechanisms contributing to its progression. qRT-PCR data confirmed the relative expression of SIX1 was notably elevated in pancreatic cancer cell lines (3 to 5-fold) compared to a normal human pancreatic ductal cell line [81]. Increased level of SIX1 in pancreatic ductal adenocarcinomas (PDAC) is pronounced in the nuclei and cytoplasm of malignant tissues. Elevated SIX1 expression is correlated associated with advanced TNM stages, larger tumor size, lymph node metastasis, and higher grading in patients with PDAC. Furthermore, the increased SIX1 protein levels emerge as a potential biomarker for predicting short-term overall survival in patients with PDAC, with patients' survival being diminished to roughly 23 months compared to about 27 months in SIX1 low-expressing tissue following PDAC surgical treatment [82]. The proportion of strongly positive SIX1 protein was 60.2 % (62/103) in PDAC, significantly exceeding that in normal pancreatic tissue [82]. Functional exploration unveils that SIX1 down-regulation attenuates the mobility of pancreatic cancer cells *in vitro*. This effect coincides with decreased expression of CDH1 and vimentin—markers intricately tied to EMT. Notably, pancreatic tumors featuring diminished SIX1 expression exhibit slowed growth and depletion of the $CD24^{+}/CD44^{+}$ phenotype, both common cancers stem cell-like markers, underlining SIX1's pivotal role in steering the trajectory of pancreatic cancer progression [83].

Derived from the chromatin immunoprecipitation (ChIP) assay, SIX1 is found to directly bind to the regulatory region of lactate dehydrogenase A (LDHA), an enzyme responsible for converting pyruvate to L-lactate. This finding unveils the SIX1/LDHA axis as a critical contributor to lactate accumulation within the TME, resulting in dysfunctional natural killer (NK) cells in pancreatic cancer [81]. This mechanism further highlights the significance of SIX1 in modulating the immune response in the pancreatic TME.

2.9. SIX1 in prostate cancer

Analysis of SIX1 protein in prostate tissues revealed a consistent trend of increased SIX1 expression levels in prostate tumors in comparison to adjacent normal prostate tissues [23,84]. The relative SIX1 mRNA expression was notably elevated in prostate cancer tissues (approximately 5-fold) compared to adjacent non-tumorous tissues [84]. The study found elevated SIX1 expression predicts unfavorable cancer outcomes compared to patients with low expression. This elevation in SIX1 expression underscores its potential

utility as an additional marker for identifying prostate cancer patients at risk of tumor advancement. Numerous studies have demonstrated the reduced expression of miR-30a in several cancers, including prostate cancer. Low levels of miR-30a were correlated with elevated expression of SIX1 in both prostate cancer tissues and cell lines [85]. Liao et al. showed glucose-regulated protein 75 (GRP75), a member of the mitochondrial heat shock protein family of molecular chaperones, directly hinders the degradation of SIX1 by facilitating its deubiquitination through ubiquitin-specific peptidase 1 (USP1) in prostate cancer cells [23]. Consequently, tumor growth was significantly inhibited by blocking the formation of the protein complex involving GRP75, USP1, and SIX1, along the response to androgen receptor-targeted therapy, was enhanced in preclinical models. Additionally, abnormal expression of GRP75 and SIX1 in tumor tissues was linked to unfavorable prognosis in prostate cancer patients. Given its ability to distinguish between cancerous and normal prostate tissues and its link to tumor progression, SIX1 holds promise as a valuable tool in assessing the disease's severity and predicting patient survival. Indeed, the potential role of SIX1 in predicting survival outcomes adds to its clinical relevance in the context of prostate cancer management [84].

2.10. SIX1 in breast cancer

Breast cancer is a highly studied malignancy, and SIX1 emerges as a key player in its pathogenesis and progression. Several studies have confirmed the substantial role of SIX1 in the development of breast cancer. SIX1 expression was notably increased in breast cancer tissues (approximately 1.5-fold) compared to paired non-tumor breast tissues [86]. SIX1 overexpression is associated with advanced clinical stage, lymph node metastasis, and adverse overall survival (OS) and disease-free survival (DFS) in breast cancer patients. Moreover, patients with high SIX1 expression experienced a poorer prognosis than those with low SIX1 expression, particularly in late-stage breast cancer cases [87].

SIX1 gene amplification and overrepresentation are observed in multiple breast cancer cell lines, promoting cancer cell proliferation, and alterations in gene dosage are associated with elevated SIX1 mRNA levels [17]. Increased expression of SIX1 in breast cancer has been shown to increase cyclinA1 mRNA levels and activity [31]. Beyond cyclin A1, SIX1 exerts control over numerous pro-tumorigenic genes, including cyclin D1, c-Myc, and Ezrin [50].

Gene amplification serves as one mechanism underpinning the overexpression of SIX1 in breast cancer. This overexpression, in turn, leads to significant downstream effects, including the induction of lymphangiogenesis and distant metastasis through the up-regulation of VEGF-C [50,88].

SIX1, often in collaboration with the co-factor EYA proteins, is a formidable driver of metastasis in breast cancer [89]. It achieves this through a multifaceted approach, including the activation of TGF-β signaling and initiation of EMT [90], expansion of the cancer stem cell (CSC) pool, initiation of lymphangiogenesis, and induction of Hedgehog (Hh) signaling in neighboring cells that may not express SIX1 themselves.

The level of SIX1 expression in breast cancer cells is associated with their resistance to paclitaxel [91]. Furthermore, SIX1's pro-metastatic role in breast cancer extends to its involvement in the lncATB/miR-200s axis within the EMT signaling pathway. This axis represents a critical component of the metastatic cascade, further highlighting SIX1's role As a significant contributor to the progression of breast cancer [86]. The increased expression of SIX1 leads to a decrease in miR-204-5p expression, thereby impacting the migratory and invasive properties of breast cancer cell lines [92].

In light of these findings, SIX1 holds promise as both a diagnostic marker and a candidate for therapeutic intervention against clinically advanced breast cancer. Its multifaceted involvement in the proliferation of tumors and their spread to other parts of the body underscores its significance in understanding and combatting this complex disease [86].

2.11. SIX1 in lung cancer

Lung cancer, a complex and challenging malignancy, presents an intriguing relationship with SIX1, further emphasizing the multifaceted nature of this transcription factor's impact on cancer. Bioinformatic analyses have unveiled a distinct pattern of SIX1 upregulation in non-small cell lung cancer (NSCLC) tissues. SIX1 was overexpressed in NSCLC samples as determined by qRT-PCR. The relative mRNA expression level of SIX1 compared to GAPDH in human NSCLC tissues is about 3-fold higher than in corresponding adjacent tissues [93]. Importantly, this heightened expression of SIX1 in NSCLC tissues is not merely a biomarker but holds clinical significance, as it strongly correlates with poor patient prognosis. This finding positions SIX1 as a potential prognostic marker, offering valuable insights for patient stratification in NSCLC [94]. However, the role of SIX1 in NSCLC extends far beyond prognostic implications.

Experimental investigations have demonstrated that the ectopic expression of SIX1 within NSCLC cells substantially impacts cancer biology. Specifically, SIX1 overexpression triggers a cascade of aggressive behaviors, including enhanced proliferation, migration, invasion, and the induction of EMT in NSCLC cells. Conversely, following the promotion of cell proliferation, migration, invasion, and the initiation of EMT in NSCLC cells, the knockdown of SIX1, the aggressive cancer behaviors are reversed, further highlighting its functional significance in NSCLC [95].

Delving into the underlying mechanisms, it becomes apparent that SIX1 exerts its influence by activating the Notch pathway. This activation serves as a central hub through which SIX1 drives the malignant biological behaviors observed in NSCLC cells. Significantly, therapeutic interventions aimed at inhibiting the Notch pathway, such as the use of a γ-secretase inhibitor, have shown promise in counteracting the oncogenic effects induced by SIX1 in NSCLC [95].

2.12. SIX1 in brain cancer

In the realm of brain cancer, SIX1's role is particularly intriguing, with its expression and dysregulation shedding light on its significance in this challenging disease. Normal brain tissues typically exhibit minimal expression of the SIX1 protein. However, a pivotal transformation occurs in glioma, where SIX1 expression becomes dysregulated, particularly in tumor-initiating cells derived from glial progenitor cells within the normal white matter of the brain. The level of SIX1 protein expression varies among different grades of glioma and correlates closely with the WHO grade classification. Notably, the overexpression of SIX1 is more common in high-grade gliomas, and it independently serves as an indicator for forecasting unfavorable clinical results in affected individuals [96].

Functional investigations with human glioma cell lines provide further insights into SIX1's influence within this context. These studies reveal that SIX1 controls the expression of SOX2, a pivotal regulatory protein implicated in stem cell maintenance, senescence regulation, and self-renewal. This functional connection underscores the complexity of SIX1's role in glioma pathogenesis and progression [28].

Glioblastoma multiforme (GBM), the most lethal and commonly occurring type of malignant primary brain tumors in humans, represents a particularly challenging subset of brain cancer. The expression of SIX1 mRNA in GBM samples, when normalized to GAPDH, exhibited an approximately 2.5-fold increase compared to adjacent non-tumor brain tissues [97]. Within GBM, overexpression of SIX1 stands out as a significant contributor to the disease's aggressiveness. SIX1 exerts this impact by markedly promoting glioblastoma cell proliferation and invasion. A key mechanism underlying this effect is the upregulation of connective tissue growth factor (CTGF), which plays a crucial role in facilitating the malignant behavior of GBM cells [97].

2.13. SIX1 in sarcomas

The influence of SIX1 in cancer goes beyond the confines of carcinomas and extends into sarcomas, a notable example being its involvement in rhabdomyosarcoma (RMS) and osteosarcoma, two challenging malignancies with distinct characteristics.

RMS, a type of muscle sarcoma primarily affecting children, known for its expression of myogenic lineage transcription factors MYOD1 and MYOG, prominently features SIX1 in its molecular landscape. SIX1 assumes a pivotal role within RMS, where it is highly expressed and functions critically in preserving a state resembling muscle progenitor cells. This crucial role positions SIX1 as a master regulator, orchestrating.

Global changes in transcription are regulated by MYOD1 and MYOG across the genome. Significantly, SIX1 actively represses RMS differentiation, contributing to the tumor's undifferentiated and aggressive nature [98].

The impact of SIX1 is discernible in its upregulation within Osteosarcoma cell lines in contrast to the human osteoblastic cell line hFOB1.19. The expression of SIX1 mRNA increased ~3-fold in osteosarcoma cell lines compared to normal human osteoblastic cell lines, while protein levels of SIX1 increased by about 3-fold [99]. Functionally, SIX1 exerts a substantial influence on osteosarcoma progression. It promotes cell proliferation and migration while concurrently suppressing apoptosis. Importantly, SIX1's expression levels emerge as a valuable indicator of tumor progression and survival outcomes among osteosarcoma patients. Its overexpression strongly correlates with poor prognosis, establishing SIX1 as a potentially invaluable prognostic biomarker in osteosarcoma patients [100].

The mechanistic underpinnings of SIX1's impact are further unveiled by its influence on specific molecular pathways. It enhances the levels of cyclin D1 and VEGF-C expression while concurrently diminishing the level of caspase-3 expression. These molecular alterations contribute to the aggressive behavior of osteosarcoma cells [99]. SIX1-promoting osteosarcoma progression is mediated by its PTEN/PI3K/AKT signaling cascade regulation. This signaling pathway emerges as a potent target for intervention in osteosarcoma, offering prospects for innovative therapeutic strategies [101].

2.14. SIX1 in blood cancer

In acute myeloid leukemia (AML) patients and murine leukemia stem cells (LSCs), SIX1 is commonly upregulated. SIX1 overexpression in human AML patients is a predictor of a worse overall prognosis [102]. Crucially, the knockdown of SIX1 significantly extends survival by reducing invasion into surrounding tissues and the total tumor load in MLL-AF9 AML mice models. AML cells from mice with suppressed SIX1 expression exhibit a substantial reduction in the number and functional capacity of LSCs. This effect is underpinned by augmented apoptosis of LSCs and a corresponding decrease in the expression of genes involved in glycolysis in mice with suppressed SIX1 expression [102].

In addition, the significance of SIX1 extends to other malignancies, including Hodgkin lymphoma (HL). In HL, SIX1 acts as a newly identified oncogene, disrupting basic developmental transcription factor genes to alter B-cell differentiation. This disturbance in B-cell differentiation underscores the diverse roles of SIX1 in different cancer types and highlights its potential as a therapeutic target in these contexts [103].

3. SIX1 influence on cancer metabolism

SIX1 not only influences pathways promoting tumor progression but also orchestrates a metabolic shift within cells, tailoring their landscape to thrive in the hostile TME. By exerting control over genes that drive the Warburg Effect, SIX1 fosters an environment conducive to cancer growth. The Warburg Effect characterizes a metabolic phenomenon where cancer cells exhibit a preference for aerobic glycolysis over oxidative phosphorylation, even when oxygen is present. This metabolic strategy allows cancer cells to generate

intermediate substrates crucial for building the macromolecules needed for rapid growth and proliferation. SIX1's regulation of numerous glycolytic pathways paves the way for the Warburg effect to take hold.

Upon SIX1 knockdown, the expression of key glycolytic proteins such as GLUT1, HK2, PFKL, ALDOA, GAPDH, PGK1, ENO1, PKM2, and LDHA is downregulated, independently of HIF-1α. Remarkably, the reexpression of SIX1 rescues the suppression of these glycolytic proteins. ChIP-seq analysis indicates that SIX1 interacts with the promoter regions of PFKL, ALDOA, PGK1, ENO1, PKM2, and LDHA, a finding corroborated by increased luciferase activity in promoter assays [104]. These findings highlight the critical importance of SIX1 in shaping the glycolytic landscape essential for the Warburg Effect.

Central to its contribution towards enhancing the Warburg Effect, SIX1 exercises its control over glycolysis through interactions with the Histone Acetyltransferase Binding to ORC1 (HBO1) protein kinase and the Amplified in Breast Cancer 1 (AIB1) coactivator protein, propagating glycolysis-associated gene expression through decondensation of heterochromatin [104].

Furthermore, SIX1 drives aerobic glycolysis, the Warburg Effect, lending insight into the nuanced interplay underpinning tumor metabolism. Intriguingly, microRNA-548a-3p emerges as a pivotal counter-regulatory factor, directly repressing SIX1's glycolytic function. This microRNA's downregulation inversely correlates with SIX1 expression and emerges as a strong predictor of prognosis for breast cancer patients.

This complex interplay between microRNA-548a-3p and SIX1 unravels a significant axis linking aerobic glycolysis to carcinogenesis [104]. This discovery not only advances our understanding of cancer metabolism but also presents an exciting prospect for potential therapeutic intervention. By delving into this intricate landscape, we uncover novel avenues for intervening in cancer's metabolic vulnerabilities and forging innovative approaches for curbing its devastating impact.

4. SIX1-associated natural product therapeutic prospects

The investigation into the role of SIX1 in cancer has unveiled promising avenues for therapeutic intervention, underscoring its potential as a robust drug target. Several candidates have emerged, which will be discussed in this section.

Apigenin (**1**) can diminish the levels of the oncoprotein SIX1, a critical factor in cancer oncogenesis, via the inhibition of CK2α [105]. Furthermore, Apigenin's utility extends to diverse cancer types, exhibiting substantial in *vivo* efficacy against pancreatic, breast, and colorectal cancers [106,107].

Manzamine A (**2**), sourced from an Indo-Pacific sponge, *Acanthostrongylophorra* sp., has garnered attention for its notable antiproliferative effects on cervical cancer cells [108–110]. Impressively, these effects manifest at relatively low and non-harmful

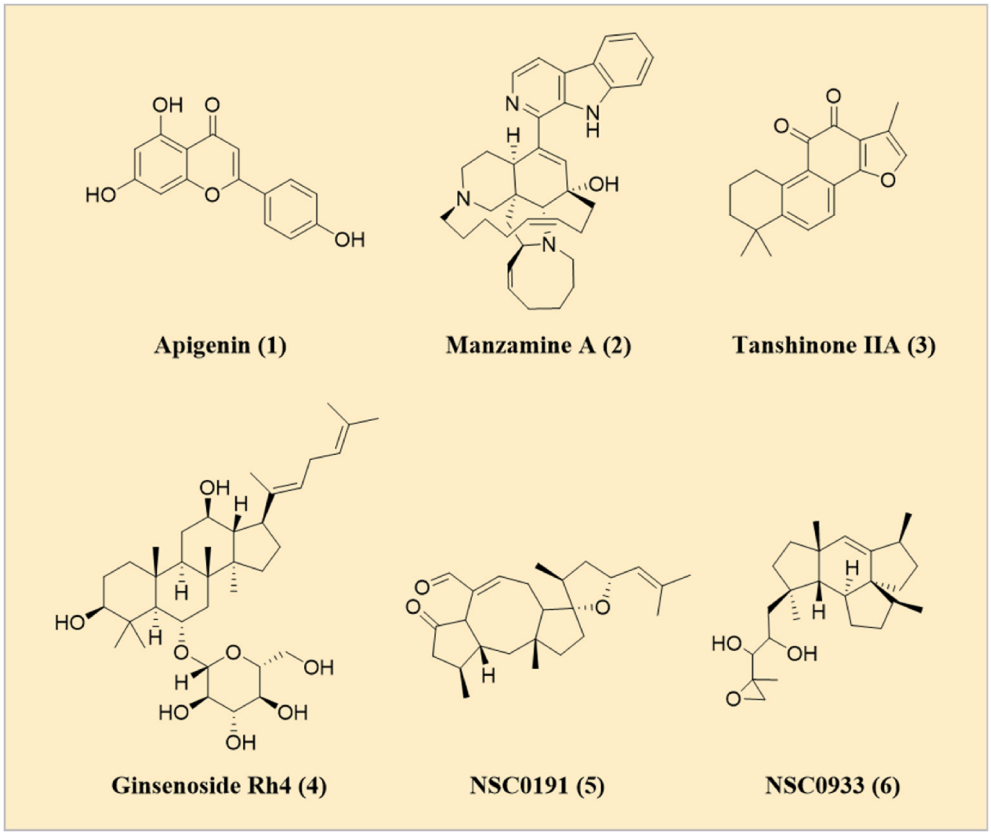

Fig. 3. Natural products with *in vitro* or *in vivo* activity against tumor cells via SIX1 inhibition.

concentrations (up to 4 μM). Mechanistic investigations have elucidated its ability to arrest cell cycle progression at the G1/S phase, concurrently orchestrating the regulation of cell cycle-related genes. Particularly noteworthy is Manzamine A's capacity to reinstate p53, p21, and Rb levels in cervical cancer cells. Moreover, molecular targeting investigations revealed that Manzamine A inhibited CK2α and its downstream target, the SIX1 oncoprotein, which is linked to tumor formation and invasiveness in various cancer types [111,112]. In its mode of action, Manzamine A is thought to target the E2F Transcription Factor 8 (E2F8) gene. The involvement of E2F8 in tumorigenesis due to its overexpression underscores the importance of this finding [113,114].

Tanshinone IIA (**3**), a bioactive component derived from *Salvia miltiorrhiza*, emerges as a potent inhibitor of NSCLC cell proliferation in a manner dependent on the dose. Impressively, the half-maximal inhibitory concentrations (IC_{50}) in A549 and H292 cells are 5.45 μM and 5.78 μM, respectively. Tanshinone IIA operates by suppressing SIX1 expressions. This orchestrated downregulation translates into the inhibition of the expression of pyruvate kinase isozyme M2 (PKM2), hexokinase 2 (HK2), and lactate dehydrogenase A (LDHA) in A549 cells. In essence, Tanshinone IIA (3) induces the downregulation of SIX1, consequently reducing glycolysis in NSCLC cells [115].

Ginsenoside Rh4 (**4**), sourced from Ginseng (Panax spp.), has demonstrated remarkable anti-gastric cancer (GC) efficacy both *in vitro* and *in vivo*. The IC_{50} values for HGC-27 and BGC-823 cells are 83.15 μM and 92.38 μM, respectively. Further substantiation via proteomic analysis, combined treatment with disitertide (a TGF-β inhibitor), and the SIX1 signaling pathway silencing underscore its potency. Specifically, Ginsenoside Rh4 targets SIX1, reducing the expression levels of TGF-β1, P-Smad3, and Snail. Consequently, this inhibition of the TGF-β/Smad2/3 signaling pathway prevents EMT processes in GC cells [116].

The manifestation of CRC pathogenesis through the overexpression of the SIX1/EYA1 complex has prompted the investigation into potential interventions. Two promising small molecules, NSC0191 (**5**) and NSC0933 (**6**), derived from Mangicol G of *Fusarium heterosporum*, have displayed remarkable promise. These compounds significantly impede the SIX1-EYA1 interaction with IC_{50} values of 12.60 ± 1.15 μmol/L and 83.43 ± 7.24 μmol/L, respectively. Their administration culminates in the substantial repression of cyclin A1 (CCNA1) and TGFβ1 expressions, thereby impeding CRC cell growth both *in vitro* and *in vivo* [117].

In conclusion, these natural compounds (**1**–**6**) derived from diverse sources present a promising array of candidates in the pursuit of cancer therapeutics, particularly by targeting SIX1 or related pathways involved in oncogenesis. However, a thorough understanding of their mechanism and efficacy requires extensive research and clinical trials, comprehensively addressing their safety profiles, mechanisms of action, and potential side effects in human cancer therapy. The chemical structures of natural compounds **1–6** derived from natural products are portrayed in Fig. 3, and protein/pathway targets are illustrated in Fig. 4.

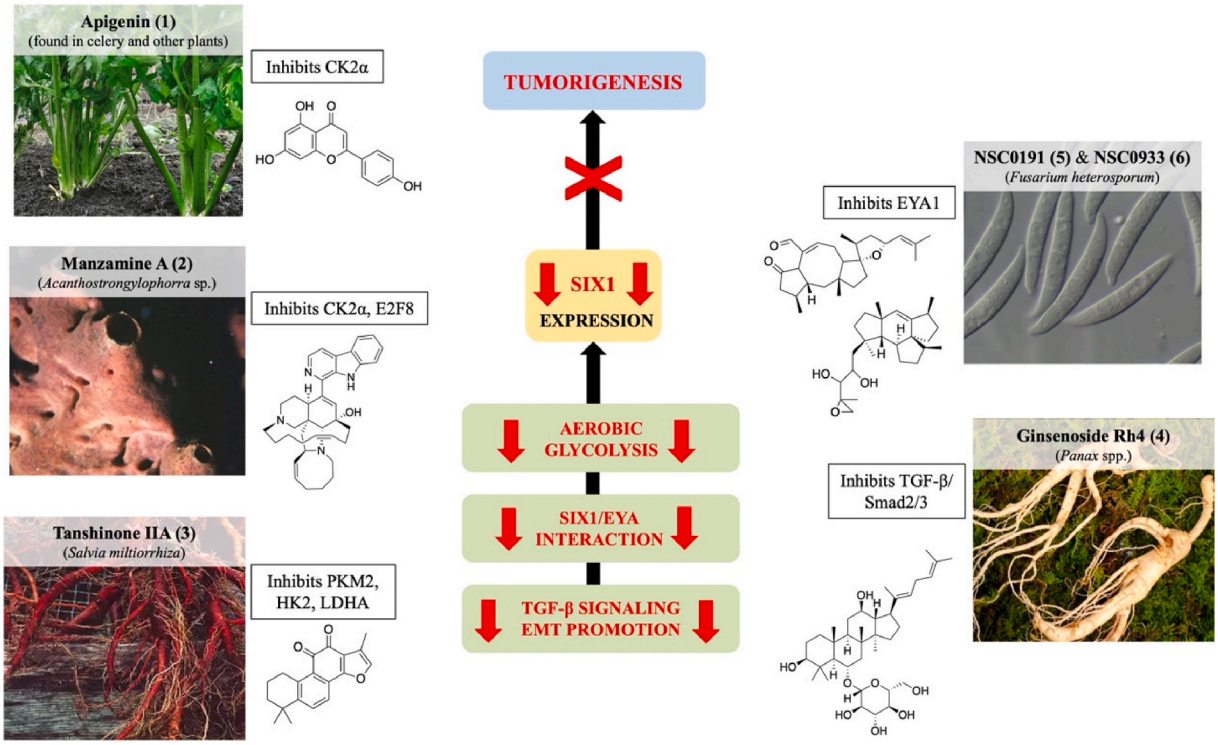

Fig. 4. Natural SIX1 inhibitors. Apigenin (**1**) - isolated from celery, Manzamine A (**2**) - isolated from an Indo-Pacific sponge, Tanshinone IIA (**3**) - isolated from the Red Sage root, Ginenoside Rh4 (**4**) - isolated from Ginseng, and NSC0191 (**5**) and NSC0933 (**6**) - isolated from a hyphomycetes fungus. Images of specimens generated with the assistance of artificial intelligence (AI) via OpenAI's DALL·E image modeling program- https://openai.com/dall-e-2. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

5. Strategies for targeting SIX1 in cancer therapy

The overwhelming role of SIX1 in cancer emphasizes potential targets for drug therapy. For example, previous research has shown that phosphorylation is a crucial step for the activation and subsequent downstream activity of SIX1, regardless of overexpression. Another feasible approach could be seeking out SIX1-related pathways in cancer. Potential SIX1 pathways that can be selected for drug therapy include those involved in cell cycle regulation, promotion of the transition from epithelial to mesenchymal phenotype (EMT) involves the activation of the transforming growth factor (TGF) pathway, and stimulation of aerobic glycolysis (Fig. 5) [50]. Considering the various mechanisms underlying SIX1's role, potential methods for inhibiting SIX1 could include: 1) targeting the phosphorylation of SIX1, 2) inhibiting the phosphatase activity of EYA2, and 3) disrupting the interactions between SIX1 and its co-factors or promoters [2].

In the realm of SIX1 precision targeting, attention is drawn to selected protein kinases such as protein kinase C (PKC), casein kinase II (CK2), Cdc2 kinase (CDK1), and CDK2 (Fig. 6a–d). These kinases are attractive candidates as they orchestrate SIX1 phosphorylation at distinct stages of the cell cycle. Previous research has suggested that CK2 primarily contributes to the phosphorylation of SIX1 during the quiescent phase of the cell cycle, while Cdc2 (CDK1) and PKC play a more significant role during the dividing phase, known as mitosis [118]. The influence of SIX1 on the cell cycle involves a complex mechanism. An example of a drug candidate that can block the activation of SIX1 via kinase inhibition is Apigenin (**1**). This compound (**1**) selectively inhibits SIX1 by targeting CK2α, one of the upstream kinases, and has thus been shown to reduce the phosphorylation of SIX1 during the resting phase of the G1/S phase. Additionally, CDK2 has been recognized as the kinase accountable for phosphorylating SIX1 during both the resting phase and mitosis. CDK2 controls the advancement of the cell cycle beyond the boundary of G2/M [2,119]. Disruptions in the phosphorylation process mediated by these protein kinases lead to the deactivation of SIX1, ultimately resulting in cell arrest and apoptosis [105]. Furthermore, natural products, including Manzamine A (**2**), have shown promising potential as inhibitors of protein kinases [112,120–122], demonstrating their promising ability to inhibit the activity of SIX1.

Another approach to blocking SIX1's effects in cancer is by targeting its cofactors. For example, the upregulation of SIX1 and EYA2 has been detected in different types of tumors, suggesting that targeting the SIX1-EYA complex could be a promising approach to impede tumor advancement. SIX1, lacking inherent activation domains, relies on EYA cofactors to facilitate transcriptional activation. The connection between SIX1 and EYA2 is crucial for SIX1-mediated TGF-β signaling, which triggers processes like the transition from epithelial to mesenchymal phenotype (EMT) and the acquisition of cancer stem cell properties, and metastasis. Examination of the crystal structure indicates that SIX1 primarily employs a lone amphipathic α-helix to bind to a hydrophobic groove in EYA (Fig. 7). This mechanism of binding shares similarities with successful small molecule-targeted protein-protein interactions seen in potential cancer therapeutics like nutlin (an inhibitor of p53-MDM2 interaction) [123] and navitoclax, a compound that inhibits interactions among Bcl-2 family proteins [124]. The utilization of natural products, such as NSC0191 (**5**) and NSC0933 (**6**), demonstrates their potential to disrupt the SIX1-EYA1 transcriptional complex. However, the specific mechanism by which these natural products achieve this disruption is yet to be determined [45,117,125].

Cellular proliferation in various cell types relies on the presence of both SIX1 and EYA2 proteins [126]. Previous research has demonstrated that reducing the levels of EYA2 in breast cancer cells with elevated SIX1 expression inhibits SIX1's ability to activate

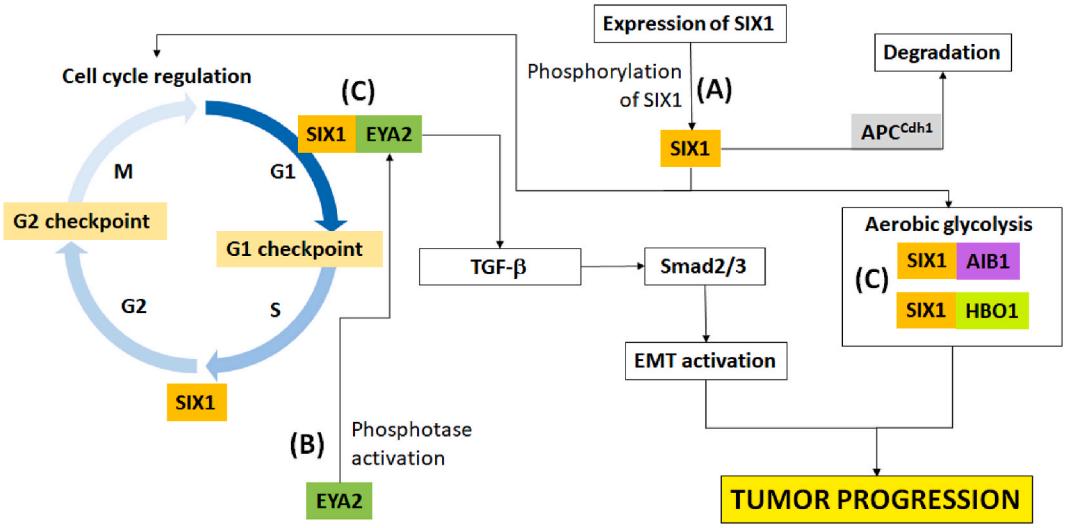

Fig. 5. In the G1 phase, activated SIX1 and EYA2 interacts to form the SIX1/EYA2 complex, which subsequently promotes EMT and tumor progression via the TGF-β dependent Smad2/3 pathway. Activated SIX1 also contributes to aerobic glycolysis through the formation of the SIX1/AIB1 and SIX1/HBO1 complexes, leading to tumorigenesis. Additionally, SIX1 is degraded by APC^{Cdh1}. Mechanisms for inhibiting SIX1 activity include: (A) inhibition of its activation through phosphorylation by upstream targets such as CK2α, Cdc2, PKC, and CDK2; (B) inhibition of EYA2 phosphatase activity; and (C) disruption of SIX1 interactions with co-factors or promoters (e.g., EYA2, HBO1, AIB1).

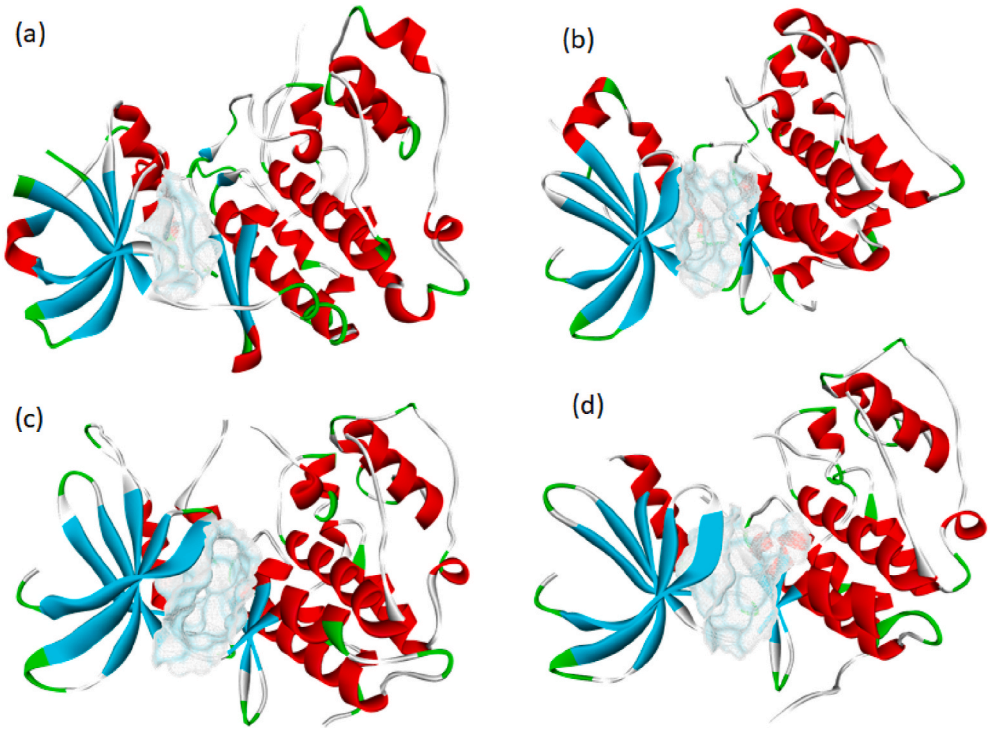

Fig. 6. The protein kinases involved in the phosphorylation of SIX1, along with their corresponding binding domains (shown in pale blue color) in the following representations: (a) CK2α (PDB ID: 3amy), (b) PKC (PDB ID: 1xjd), (c) CDK1 (PDB ID: 6gu6), and (d) CDK2 (PDB ID: 1aq1). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

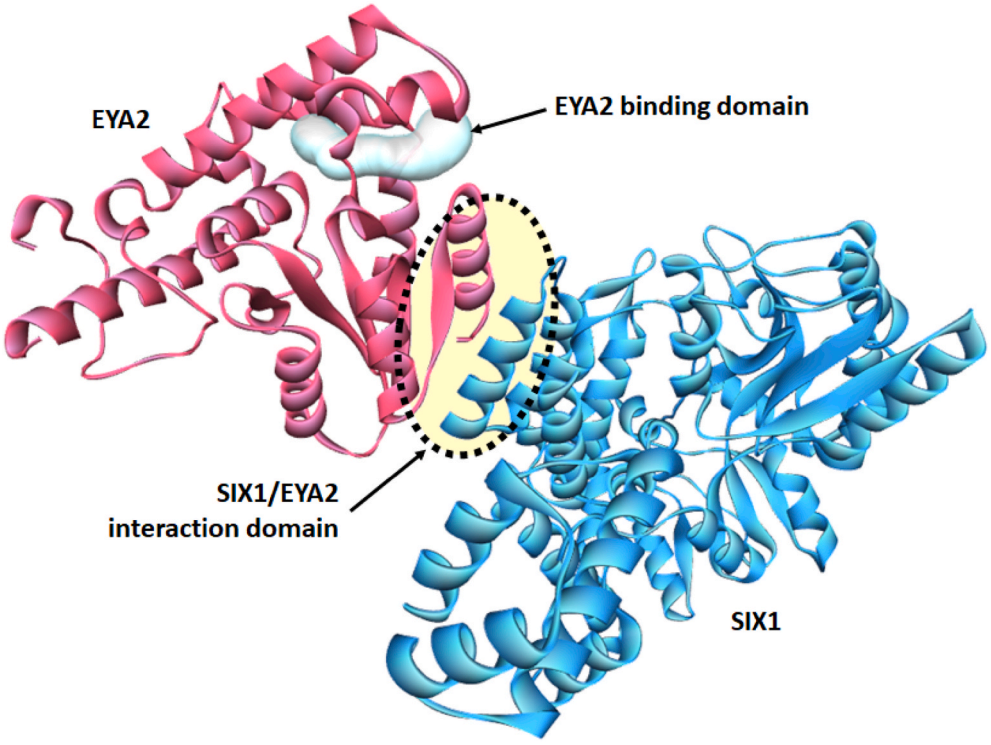

Fig. 7. The domain responsible for SIX/EYA2 interaction and the EYA2 allosteric binding domain (PDB ID: 4egc and 7f8g, respectively).

Table 3
Binding affinity and interacting amino acid residues of compounds **1**–**6** with SIX1 targets.

Protein name	Binding domain	ATP	1	2	3	4	5	6
CK2a	ATP	−6.6	−9.8	−8.7	−10	−7.4	−8.1	−6.6
PKC	ATP	−7.7	−8.9	−7.8	−9.9	−7.8	−7.6	−7.2
CDK1	ATP	−7.6	−8.1	−7.6	−9.7	−6	−5.9	−7.8
CDK2	ATP	−8.3	−9.3	−8.5	−11.3	−9.1	−9.3	−9
EYA2	Allosteric site	n.a	−9.1	7.3	−7.9	−3.9	−5.5	−5.3
SIX1	SIX1/EYA2 interface	n.a	−7.8	−8.8	−7.5	−7.8	−8.2	−7.2
EYA2	SIX1/EYA2 interface	n.a	−5.6	−8	−6.7	−7.5	−6.3	−6.1
Interacting amino acid residues in SIX1			Leu374, Pro375, Phe379, Arg399, Arg402, Phe403	Ala,370, Leu374, Pro375, Phe379, Ser406, Leu407, Pro408	Ala370, Leu374, Arg399, Arg402	Ala370, Met371, Leu374	Leu374, Phe379, Phe403	Val387, Arg402, Phe403
Interacting amino acid residues in EYA2			Pro516, Arg519	Lys511, Phe517, Trp518	Lys511, Pro516, Phe517	Lys511, Pro516, Trp518, Ala532, Ley538	Lys511, Met515	Phe517, Trp518, Arf519, Tyr537
Interacting amino acid residues in SIX1/EYA2 complex			SIX1: Phe377, Phe379, Glu382, Cys386, Val387, Val390, Glu393 EYA2: Asn514, Pro516, Trp518, Arg519, Ala528, Ala532, Leu538,					

n.a. – not applicable.

TGFβ signaling, initiate EMT and foster tumor-initiating cell properties. Furthermore, a naturally-occurring mutation in SIX1 (V17E), which retains DNA-binding activity but lacks the ability to bind to EYA2, does not promote metastasis in a breast cancer xenograft model [127]. The phosphatase function of EYA2 is pivotal in transcribing a specific subset of genes targeted by SIX1. It also guides cells towards DNA repair rather than apoptosis after experiencing DNA damage. EYA2 is part of the distinct HAD (haloacid dehalogenase) family protein tyrosine phosphatases, characterized by the utilization of aspartic acid as the active site residue instead of the more typical cysteine, which is prevalent in most cellular phosphatases (Fig. 7). While synthetic small molecule inhibitors that target EYA2's activity have been identified [126,128], the discovery of a natural product inhibitor for EYA2 has yet to be reported. However, considering the structural diversity found in natural products, it is highly plausible for such inhibitors to exist within natural product libraries.

A previous investigation has suggested the participation of SIX1 in regulating glycolytic genes' expression via the Warburg Effect [104]. SIX1 exerts this regulatory role by interacting with the promoters of these genes, like AIB1 and HBO1 [118]. However, the precise details of the interaction between SIX1 and these promoters remain to be fully understood, as this area of research is still in its early stages. Advancement in research of SIX1's entwinement with glycolysis holds the promise of broadening the array of targets to curtail its influence on the favored metabolic phenotype in cancer.

Prior investigations have suggested that compounds **1**–**4** could potentially modulate the activity of SIX1 through its upstream regulator. Additionally, there exists a prospect that these compounds may obstruct SIX1 activity by interfering with its interaction with EYA2, resulting in a mechanism reminiscent of compounds **5** and **6**. In an endeavor to elucidate these interactions, a computational modeling approach utilizing previously established methods [111] was used to dock compounds **1**–**6** to the above-mentioned targets. The findings are summarized in Table 3.

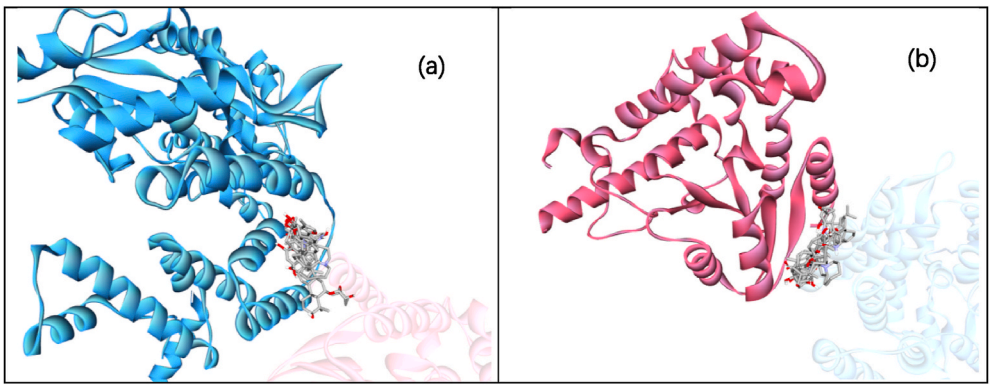

Fig. 8. Compounds **1**–**6** are shown docked to (a) SIX1 (blue, PDB ID: 4egc) at the SIX1/EYA2 interacting interface, and (b) EYA2 (pink, PDB ID: 4egc) at the SIX1/EYA2 interacting interface. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Apigenin (**1**), functioning as a CK2α inhibitor [105], exhibited high affinity towards CK2α, PKC, and CDK2, binding competitively to the ATP-binding domain. While the majority of the compounds demonstrated superior binding affinity compared to ATP, empirical bioassays are necessary to authenticate their activity. The compound's solubility, structural integrity, and binding stability are crucial factors influencing its actual efficacy. Additionally, the findings indicated that only compound **1**, relatively smaller in size, accommodated well in the narrow allosteric binding pocket of EYA2.

Compounds **5** and **6** demonstrated inhibition of the binding interactions between SIX1 and EYA1 [117]. However, owing to the availability of only the SIX1/EYA2 protein structure, it was utilized to assess these interactions, despite the specific binding site for a small molecule inhibitor to SIX1 and EYA2 remaining unidentified. Docking of compounds **1**–**6** independently at the SIX1/EYA2 interaction domain, despite the unknown binding site, provided insights into their potential as disruptors of SIX1/EYA activation and downstream activity (Fig. 8a and b). Given the structural diversity among compounds **1**–**6** in terms of scaffold, shape, and size, this assessment offered an estimation of a prospective SIX1/EYA2 inhibitor's characteristics. The outcomes indicated a superior binding affinity of **1**–**6** to SIX1 compared to EYA2, suggesting a more viable approach targeting SIX1 rather than EYA2. Notably, compounds **5** and **6**, which disrupted SIX1/EYA1 interactions and curbed downstream activity, exhibited enhanced binding affinity to SIX1 akin to other compounds.

6. Conclusion

While SIX1 is a key regulator in early development, its implications clearly extend to intricate pathways and phenotypes in tumorigenesis and progression. SIX1 exerts tumorigenic effects through its attrition of tumor-suppressive genes while augmenting and cross-talking with cellular pathways implicated in cell proliferation, survival, and growth. Furthermore, SIX1 expression also corresponds to decreased immune responses toward tumor eradication, mainly through its facilitation of TGF-β signaling. Apart from its immunosuppressive actions, SIX1 also elicits metabolic reprogramming that favors tumorigenesis through the upregulation of glycolysis. As such, SIX1 overexpression has been noted in a broad spectrum of cancers, prompting the need for therapeutic intervention. Greater efforts and focus are needed to restrain SIX1 and its activity in cancer successfully. However, careful considerations must also be made for SIX1 targeting in cancer, considering its wide-ranging biological roles in development. The main roles of SIX1 in each type of cancer discussed in this review are summarized in Table 4.

The regulation of SIX1 involves multiple pathways, and inhibition of SIX1 activities can be achieved by controlling the phosphorylation of protein kinases implicated in SIX1 pathways, inhibiting EYA2 phosphatase activity, or disrupting the interactions

Table 4
The role of SIX1 in blood, brain, breast, carcinoma, cervical, colorectal, endometrial, esophageal, gastric, liver, ovarian, pancreatic, prostate, sarcoma, skin, and thyroid cancers.

Cancer Type	Role of SIX1
Blood	Decreases apoptosis, upregulates glycolysis, and increases stem cell population in leukemia
Brain	Increases cell proliferation and invasion through upregulation of connective tissue growth factor
Breast	Induces metastasis through TGF-β signaling and EMT; Downregulates p53 through RPL26 and microRNA-27a-3p; Induces lymphangiogenesis and metastasis via upregulation of VEGF-C; Mediates EMT, metastasis, and cancer stem cell properties through TGF-β signaling in the presence of EYA2; Confers resistance to Paclitaxel by preventing apoptosis; Potentiates metastasis and EMT through transcription of lncATB, which binds to miR-200c; Promotes glycolytic gene expression through interaction with HBO1 and AIB1; Attenuates DNA-damage induced G2 cell cycle checkpoint
Carcinoma	Involves in O-GlcNAcylation; Heightened MMP-9 expression; Increase SOX2 expression; Activation of ABCC2 expression; Mitigates diethylstilbestrol (DES)-induced endometrial carcinogenesis by promoting basal differentiation within CK14+/18+ cell populations; Increase cyclin D1 expression
Cervical	Promotes DNA replication and proliferation; Promotes expression of α5β1 integrin, improving cell adhesion and resistance to apoptosis; Enhances TGF-β signaling via Smad to promote EMT; Drives cancer cells into a stem cell-like state as indicated by CD24/44 markers and ALDH1 expression; Induces lymphangiogenesis via TGF-β driven VEGF-C
Colorectal	Regulates migration and invasion through miR-30b; Promotes EMT through ZEB1 activation; Promotes proliferation and migration through activation of Wnt/β-catenin signaling pathway
Endometrial	Upregulates ERK, AKT signaling, and Cyclin D1
Esophageal	Reduces sensitivity to radiotherapy through upregulation of AKT and ERK signaling; Induces overexpression of transforming growth factors and their receptor to accelerate self-renewal of tumor basal cells
Gastric	Regulates apoptosis by increasing Bcl-2 and inhibiting caspase-7; Increases the expression of cyclin D1, MMP2, *p*-ERK, and EMT-related proteins
Liver	Inhibits DACH1 from upregulating p53; Enhances glucose metabolism; Improves invasive capabilities and migration by upregulating STAT3 signaling and MMP-9 expression; Promotes chemoresistance through enhancing stemness via SOX2 expression
Ovarian	Drives proliferation in parallel with cyclin A1 induction and reduces TNF-driven apoptosis
Pancreatic	Upregulates cyclin D1; Upregulates CDH1 and vimentin, increases migration, and promotes tumor growth in mice; Increases lactate production through enhanced glycolytic capacity, inhibiting NK cells
Prostate	Drives cell proliferation and cell cycle progression; Increases cell growth and invasion
Sarcoma	Promotes expression of cyclin D1, c-Myc, and Ezrin; Maintains undifferentiated state through repression of MYOD1 and MYOG-mediated transcription; Increases expression of cyclin D1 and VEGF-C, decreases expression of caspase-3 to promote proliferation and suppress apoptosis; Increases activation of the PI3K/AKT pathway and reduces expression of PTEN
Skin	Increases keratinocyte resistance to calcium and serum-stimulated differentiation; Downregulates epithelial-related genes, upregulates mesenchymal-related genes, and activates TGF-β receptor type II and MAPK
Thyroid	Induces EMT via upregulation of TGF-β signaling through Smad2/3; Drives proliferation by elevating STAT3 signaling through EYA1; Promotes expression of GLUT3, MMP2, and Snail, increasing glucose metabolism

between SIX1 and its co-factors/promoters. Consequently, a wide range of structurally diverse natural compounds could serve as SIX1 inhibitors, considering the variety of target proteins involved in regulating SIX1 activities. For example, a more planar structure such as compounds **1** and **2** (i.e., the β-carboline moiety) is likely to fit well in the narrow binding domain of protein kinases such as CK2α. Conversely, for interrupting SIX1 interactions with co-factors/promoters, such as between SIX1 and EYA2, natural compounds capable of forming hydrogen-bonding interactions with SIX1 may be preferred to emulate the protein-protein interactions dominated by hydrogen bonding between SIX1 and EYA2.

The SIX1 oncogene is an underappreciated yet significant target for cancer therapy due to its multifaceted roles in tumorigenesis, ranging from cancer proliferation to metastasis, as well as a biomarker for poor prognosis in patients. Compounds **1**–**6** are natural products that hold the promising potential to perturb pro-tumorigenic actions of SIX1, whether as a single agent or in combination. The predicted binding affinities shown in Table 3 indicate computational selectivity towards protein kinases and transcription factors involved in oncogenic pathways. Further *in silico* studies and the rapid advancements in artificial intelligence (AI) will open doors to discovering structure-activity relationships (SARs) and mechanisms of action (MoA) for inhibiting SIX1.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

CRediT authorship contribution statement

Zhiwei Bian: Writing – review & editing, Writing – original draft, Data curation, Conceptualization. **Menny M. Benjamin:** Writing – review & editing, Writing – original draft, Data curation, Conceptualization. **Lucas Bialousow:** Writing – review & editing, Writing – original draft, Data curation, Conceptualization. **Yintai Tian:** Writing – review & editing, Writing – original draft, Data curation, Conceptualization. **G. Aaron Hobbs:** Writing – review & editing, Writing – original draft, Data curation, Conceptualization. **Dev Karan:** Writing – review & editing, Writing – original draft, Data curation, Conceptualization. **Yeun-Mun Choo:** Writing – review & editing, Writing – original draft, Data curation, Conceptualization. **Mark T. Hamann:** Writing – review & editing, Writing – original draft, Data curation, Conceptualization. **Xiaojuan Wang:** Writing – review & editing, Writing – original draft, Data curation, Conceptualization.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work, the authors used Open AI's DALL·E to assist in the generation of images used in Figs. 2 and 4. After using this tool/service, the authors reviewed and edited the content as needed and took full responsibility for the content of the publication.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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