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Pancreatic cancer tumour initiating cells: the molecular regulation and therapeutic values

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Abstract

Pancreatic cancer is an aggressive solid tumour characterized by its local invasion, early metastasis and resistance to standard chemotherapy or radiation therapy. Tumour initiating cells (TICs) are not only capable of self-renewal and differentiation, but also play an important role in multi-drug resistance, and thus become a popular topic in cancer research especially in pancreatic cancer. In this review, we summarize the current progress of TICs in tumourigenesis, various newly identified surface markers of pancreatic TICs, and the signalling pathways such as epithelial-mesenchymal transition, sonic hedgehog and Notch that regulate TICs. We also discuss the role which microRNA plays in TICs as well as its application in TIC-targeted therapy along with other approaches.

Keywords: tumour initiating cells • microRNA • pancreatic cancer

Introduction

Pancreatic cancer is known for its notorious mortality rate and the lowest overall survival among all cancers. In 2011, the incidence of pancreatic cancer has gradually increased with an estimated 44,030 newly diagnosed cases in the United States. Unfortunately, 37,660 of these patients are expected to succumb to this deadly disease, making pancreatic cancer the fourth leading cause of cancer death. Pancreatic cancer is an aggressive solid tumour with local invasion, early metastasis, and resistance to standard chemotherapy and radiation therapy [1,2]. In 1997, gemcitabine was approved by the FDA as the first-line chemotherapy drug for patients with locally advanced or metastatic pancreatic adenocarcinoma [3]. From then on, many studies have been done with the goal of improving clinical efficacy of chemotherapy. Unfortunately, little progress has been made and the overall survival has not improved up to now [4]. Drug resistance usually contributes to treatment failure and plays a significant role in high mortality in patients diagnosed with this cancer. Previous studies have indicated various mechanisms of drug resistance in pancreatic cancer, such as changes in individual genes or signalling pathways, and the influence of the tumour microenvironment [5].

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Current therapeutic strategies for patients with pancreatic cancer involve targeting and killing differentiated cancer cells as well as the quiescent tumour initiating cells (TICs) [6].

Tumour initiating cells are defined as a unique subpopulation of cells that possess the ability to initiate tumour growth and sustain self-renewal as well as metastatic potential from which they were isolated or identified [7]. The definition implies TICs' ability to induce tumourigenesis in xenotransplanted immunodeficient mice. Tumour initiating cells are also referred to as "cancer stem cells (CSCs)" or "tumourigenic cells" in many studies [8]. The first evidence regarding TICs was observed in a lymphoma study, sparkling the debate on the role of TIC in cancer progression [9] while the TICs were first identified by Park *et al.* when they observed extensive proliferation of a subset of cancer cells isolated from myeloma mice in 1971 [10]. These special sets of cells can self-renew and differentiate to develop the cellular and molecular heterogeneity of the originating tumour [11].

The lineage of TICs is still under great debate, however, many investigators have hypothesized that TICs arise from normal stem or progenitor cells after accumulation of genetic mutations [12]. In some cases, TICs may also arise from differentiated cells such as acinar cells that are once committed but re-acquire stem cell characteristics after mutations take place. As the lineage of pancreatic cancer still remains unclear, tracking down the origin of TICs in pancreas has posed great challenges.

The TICs' existence has now been validated in several studies on solid tumours, such as breast cancer [13], glioblastoma [14], colorectal cancer [15] and liver cancer [16]. The concept of TICs provides a distinct view of carcinogenesis and may give rise to novel therapeutic strategies for pancreatic cancer, preventing tumour recurrence. Therefore, elucidating the mechanisms underlying pancreatic tumourigenesis employing TICs is clinically significant to improve the treatment of pancreatic cancer [17].

Surface markers of pancreatic TICs

Consistent to its role in other solid tumours, TICs are also responsible for tumour recurrence as well as tumour metastasis in pancreatic cancer. Li *et al.* identified a subpopulation of highly tumourigenic cancer cells expressing the cell surface marker $CD44^+$, $CD24^+$ and epithelial-specific antigen (ESA^+), and these $CD44^+CD24^+ESA^+$ cancer cells display stem cell-like characteristics such as self-renewing and the ability to produce differentiated cells as well as drive continuous malignant cell expansion in a invasive and metastatic manner. As few as hundreds of $ESA^+CD44^+CD24^+$ cells were able to generate a tumour in 50% of the animals compared with the cells negative for all three markers ($CD44^-CD24^-ESA^-$) that would require 10^4 or more cells implanted to induce tumour formation [18]. In another study, Hermann *et al.* used $CD133^+$ as a marker to isolate pancreatic cancer cells with a significantly higher tumourigenic potential and found these $CD133^+$ expressing cancer cells are highly resistant to standard chemotherapy. In addition, the authors also found $CD133^+CXCR4^+$ expressing cancers cells to be essential for tumour metastasis [19]. Moreover, Shah *et al.* showed that gemcitabine-resistant pancreatic cancer cells have increased expression of $CD24^+$, $CD44^+$ and ESA^+ while possess the morphological and biochemical properties of epithelial-mesenchymal transition (EMT) [20]. In a more recent study, Rasheed *et al.* identified aldehyde dehydrogenase positive ($ALDH^+$) pancreatic cancer cells with stem cell-like features, clonogenic potential and characteristics of EMT [21]. They also suggested that $ALDH^+$ pancreatic cancer cells can negatively affect the overall survival of cancer patients. A recent study by Kim *et al.* [22] demonstrated cell populations with high $ALDH^+$ activity alone are sufficient for efficient tumour-initiation and recapitulating the phenotype of original tumour in NOD/SCID mice regardless of the level of $CD133^+$ expression, indicating $ALDH^+$ might possibly be a more ideal marker to identify novel therapeutic targets for pancreatic cancer.

Signalling pathways in pancreatic TICs

Conventionally, EMT is recognized as a pathological mechanism during the progression of various diseases including inflammation, fibrosis and cancer [23]. In recent years, emerging evidence has implicated that EMT plays a critical role in the molecular mechanism of TICs in pancreatic cancer. Mani *et al.* [24] found that EMT generates stem cell–like cells when they induced EMT in non-tumourigenic human mammary epithelial cells (HMLEs) and later identified those EMT-induced cells displaying $CD44^{high}/CD24^{low}$ pattern, associated with both human breast CSCs and normal mammary epithelial stem cells. Their findings suggested cells undergo EMT share many markers and properties with tumour-initiating cells, indicating a possible mechanism involved in both EMT and self-renewal. Thus, the inhibition of EMT along with other pluripotency maintaining factors in pancreatic TICs isolated from $Kras^{G12D}$ mice by anticancer drug resveratrol indicated that resveratrol can be used to target TICs and suppress their metastatic potential [25]. Other than inhibiting EMT markers, the authors also found resveratrol's ability to inhibit the self-renewal capacity of pancreatic TICs by preventing the formation of primary and secondary spheroid, implicating its role in pancreatic TICs management.

In addition to EMT signalling, other important signalling pathways associated TICs such as sonic hedgehog (SHH) was elucidated by several studies. It has been shown that down-regulation of SHH by cyclopamine, an inhibitor of SHH, can reduce the growth and viability of pancreatic cancer cells [26]. Li *et al.* assessed the expression levels of SHH by real-time reverse transcriptase polymerase chain reaction (RT-PCR) and found a 46-fold increase in the expression levels of SHH in pancreatic cancer TICs and a four-fold increase in $CD44^-CD24^-ESA^-$ pancreatic cancer cells compared with normal pancreatic epithelial cells [18]. The SHH inhibitor cyclopamine is administered as a part of the dual-compartment therapeutic approach to further reduce tumour growth and

decreased both static and dynamic pancreatic TIC markers such as CD24 and ALDH [27]. These studies suggest that SHH represents a promising target for therapeutic interventions which may facilitate in eliminating pancreatic TICs.

The Notch signalling pathway is typically involved in cellular development through regulation of cell proliferation and apoptosis [28]. Notch gene is abnormally activated in many human malignancies such as lung cancer [29], prostate cancer [30] and pancreatic cancer [31]. Four Notch proteins have been identified in a variety of stem cells [32]. Down-regulation of Notch-1 using specific small interfering RNA (siRNA) was correlated with decreased proliferative rates, increased apoptosis, reduced migration and decreased invasive properties of pancreatic cancer cells. Notch signalling could induce the activity of its downstream target NF-κB in pancreatic cancer [33], and forced over-expression of Notch-1 could lead to overgrowth, clonogenicity, migration and invasion of AsPC-1 cells. The over-expression of Notch-1 can also lead to the induction of EMT phenotype and up-regulation of TIC markers such as $CD44^+$ and ESA^+, suggesting that targeting Notch-1 pathway may be an effective therapeutic strategy to treat pancreatic TICs [34].

Function of microRNAs in TICs regulation, self-renewal and differentiation

MicroRNAs (miRNAs) are small conserved non-coding RNAs with the length of 18–25 nucleotides that act as translational regulators of genes involved in many cellular processes, such as development, differentiation, cell proliferation, survival and death [35]. These regulatory molecules can directly interfere differentiation in both normal stem cells [36] and TICs [37]. Recently, miRNAs have become a spotlight in cancer genetics because of their ability to regulate gene expression. Previous studies found that the expression of miRNAs is altered in haematologic [38] and solid tumours [39] when compared with their normal counterparts. Meanwhile, emerging evidence has shown that several miRNAs may play an important role in the development and progression of diverse tumours, such as breast [40], ovarian [41], endometrial [42], hepatocellular [43], colon [44], esophageal [45], lung [46] and pancreatic cancer [47]. To date, the associations between miRNAs and TICs are still not completely understood. Ji *et al.* found that miR-34 restoration inhibits the $CD44^+/CD133^+$ MIA PaCa-2 cells, accompanied by significant inhibition of tumour sphere growth *in vitro* and tumour formation *in vivo.* Furthermore, miR-34 is involved in pancreatic TICs' self-renewal, potentially *via* the direct modulation of downstream targets Notch and Bcl-2 [48]. Interestingly, in another study of prostate TICs, Liu *et al.* found that systemically delivered miR-34a inhibited prostate cancer metastasis as well as extended survival of tumour-bearing mice. Moreover, $CD44^+$ was identified as a direct and functional target of miR-34a, and the $CD44^+$ knockdown mimicked the effect of miR-34a over-expression in inhibiting prostate cancer regeneration and metastasis [49]. These studies strongly suggest that miR-34a is a negative regulator of TICs and can serve as a novel therapeutic target in cancer treatment.

Another TIC-regulating miRNA complex in pancreatic cancer is the miR-200 family which is reciprocally linked to zinc-finger enhancer binding (ZEB)/miR-200 feedback loop, whereas ZEB transcription factors are crucial to EMT activators [50]. According to their seed sequences, the members of miR-200 family can be further divided into two subgroups which showed slight difference in their target gene sets. Subgroup A contains miR-141 and miR-200a while subgroup B contains miR-200b, miR-200c and miR-429. The most prominent targets of the miR-200 family are two E box binding transcription factors, ZEB1 and ZEB2, and *C. elegans* Sma and *Drosophila* Mad (SMAD) interacting protein 1 (SIP1) [51–56]. Transient over-expression of miR-200c decreased the sphere-forming capacity of wild-type Panc-1 and MIA PaCa-2 cells. Conversely, specific inhibition of endogenous miR-200c led to an increase in sphere numbers in the differentiated pancreatic cancer cell line BxPC-3 cells [57]. The result is consistent with another study which found that the expression of miR-200b, miR-200c, let-7b, let-7c, let-7d and let-7e was significantly down-regulated in gemcitabine-resistant cells with TICs' characteristics [58].

Similar to the miR-34 and miR-200 families, most miRNAs were down-regulated in many types of tumours [39, 59] except for miR-21, which was dramatically up-regulated in the analysed tumours of breast, colon, lung, stomach, prostate and pancreas [59]. The human miR-21 gene is well characterized and mapped to chromosome 17q23.2, where it overlaps with the protein-coding gene VMP1, a human homologue of rat vacuole membrane protein [60,61]. Meng *et al.* found that miR-21 is a downstream effecter of PI3-kinase signalling pathway in human cholangiocarcinoma cells [62]. It has been shown that aberrant expression of miR-21 may contribute to the growth of hepatocellular carcinoma and regulate the expression of the tumour suppressor phosphatase and tensin (PTEN) homologue [63]. Similar results were found in another study of pancreatic cancer cell lines by Bao *et al.* [64]. In addition, expression of miR-21 is found to be up-regulated in gemcitabine-resistant pancreatic cancer cells with TIC markers ($CD44^+$ and ESA^+). Furthermore, suppressing the expression of miR-21 could lead to re-expression of PTEN and miR-200, reversing the EMT phenotype in the cells. Those studies indicate that aberrant expression of miRNAs certainly contribute to the molecular regulation of pancreatic TICs and may play important roles in TIC-targeted cancer therapy.

New therapies targeting the deregulated pathways and miRNAs to treat TICs in pancreatic cancer

Because of its self-renewal capability and high resistance to chemotherapy, targeted killing of pancreatic TICs may become an

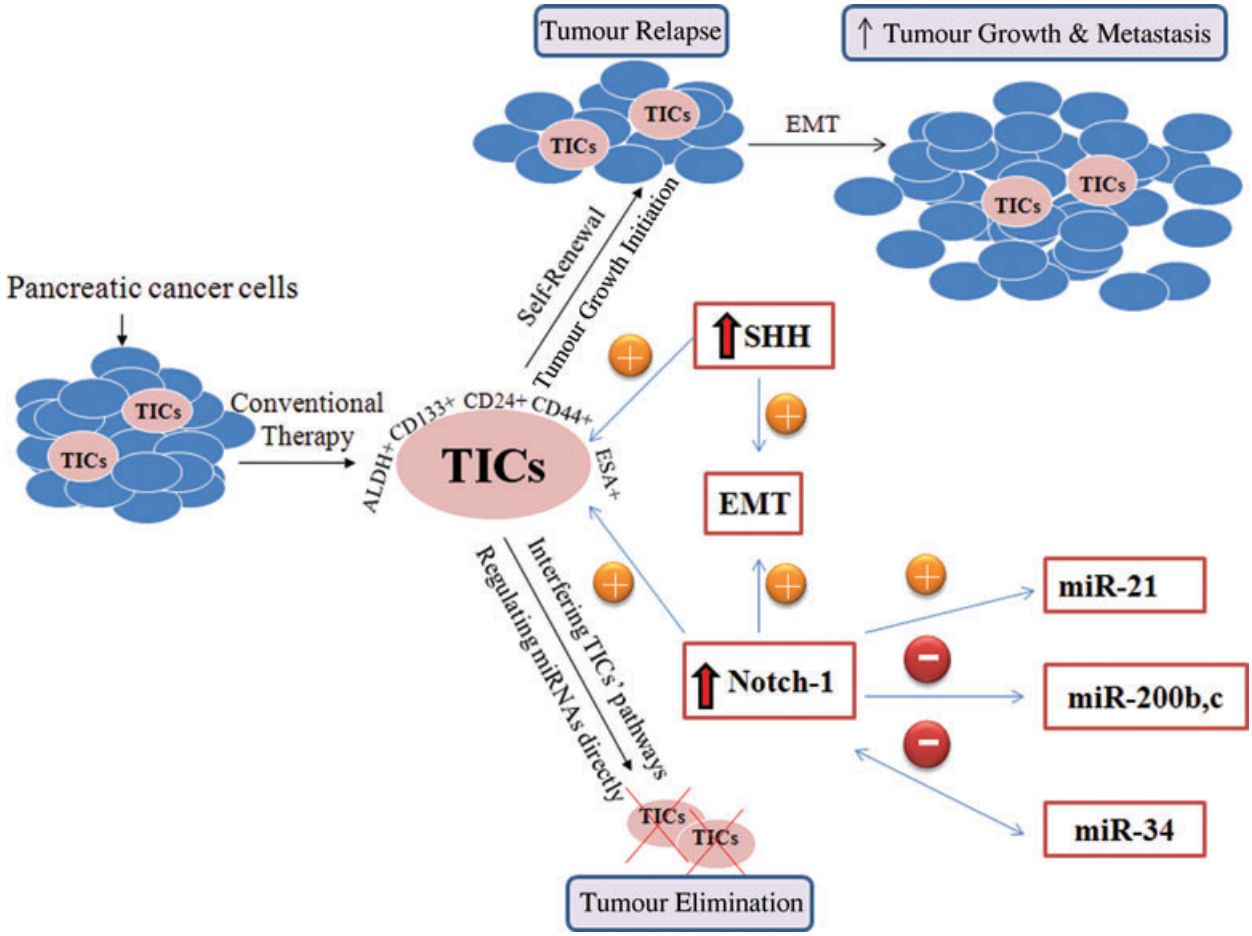

Fig. 1 New therapies targeting the deregulated pathways to treat TICs in pancreatic cancer. Surface markers $CD24^+$, $CD44^+$, $CD133^+$, ESA^+ and $ALDH^+$ have been identified to help isolate TICs. Conventional therapy can reduce the tumour size by exerting effect on cancer cells; however, it does not eradicate TICs. The remaining TICs possess self-renewal ability and can re-initiate tumour growth, increasing the risk of tumour recurrence as well as promoting tumour metastasis. With the use of TIC-targeting therapy that eliminates TICs by either interfering with the signalling pathway or regulating the activity of miRNAs, complete tumour eradication is possible. The signalling pathways associated with TICs include the SHH pathway and the Notch-1 pathway that both up-regulate the activity of EMT and TIC itself. The Notch-1 pathway can also influence the expression of miRNA such as miR21, miR200b, miR200c and miR34 that all play a role in TIC regulation.

effective strategy for preventing pancreatic cancer recurrence as well as overcoming drug resistance. Therefore, one of the most promising approaches to target TICs is to inhibit TIC-associated pathways (Fig. 1). An *in vitro* and *in vivo* model of pancreatic cancer has been used to examine the effects of cyclopamine SHH inhibition and mammalian target of rapamycin (mTOR) blockade on the TICs population. It was found that the eradication of pancreatic TICs only occurred when combined blockade of SHH and mTOR signalling were used together with standard chemotherapy [65]. Curcumin, a well-used dietary polyphenol [22] was demonstrated to down-regulate Notch-1 mRNA level in pancreatic cancer cells. This study has shown that curcumin-induced inactivation of NF-κB DNA-binding activity was potentially mediated by Notch-1 signalling pathway [66]. Recently, difluorinated-curcumin (CDF), a novel synthetic analogue of curcumin, had been proved to be more potent. Difluorinated-curcumin can inhibit the sphere-forming ability of drug-resistant pancreatic cancer cells, which is consistent with inactivation of TIC biomarkers such as CD44 and ESA. The anti-tumour activity was showed in both CDF treatment alone and in combinational therapy of CDF with gemcitabine. Difluorinated-curcumin treatment significantly inhibited tumour growth in a xenograft mouse model of MIA PaCa-2 by decreasing the DNA binding activity of NF-κB, down-regulating the expression of COX-2 and miR-21 as well as increasing PTEN and miR-200 expression in tumour remnants [64]. Interestingly, sulforaphane (SF), another plant compound isothiocyanate enriched in broccoli and other cruciferous vegetables, also demonstrated the ability to eliminate MIA PaCa-2 pancreatic cancer cells with TIC characteristics such as clonogenicity, spheroidal growth and ALDH1 activity. Nevertheless, co-treatment with SF and gemcitabine had

a synergistic effect on the inhibition of tumour growth compared to the treatment with gemcitabine alone [67]. Another study also demonstrated that combining quercetin may increase the efficacy of SF to eliminate pancreatic TICs [68]. Moreover, resveratrol, a polyphenol derived from a wide variety of plants such as grapes, berries, plums and peanuts was also found to be a member of natural dietary compounds which can exert effect on pancreatic TICs by inhibiting the EMT markers [25]. As induction of EMT can lead to invasion of surrounding stroma and colonization of distant sites, inhibiting EMT in TICs will cause decreased metastasis. Meanwhile, Genistein, one of the most active soy isoflavones, was found to display similar effect [34]. Blocking the hypoxia, angiogenesis and inflammation pathways in TICs is also a promising therapeutic strategy to inhibit the interaction of the TICs with the surrounding stromal and vascular endothelial cells to prevent tumour metastasis.

As miRNAs are critical regulators for TICs in different cancers, many miRNA-based therapies have been developed lately to treat pancreatic TICs [69]. Knockdown of doublecortin and Ca^{2+}/calmodulin-dependent kinase-like-1 (DCAMKL-1) in human pancreatic cancer cells by siRNA induces miR-200a but down-regulates ZEB1, ZEB2, Snail, Slug and Twist. Furthermore, DCAMKL-1 knockdown resulted in down-regulation of c-Myc and Kras through a let-7a miRNA-dependent mechanism as well as down-regulation of Notch-1 through a miR-144 miRNA-dependent mechanism. These findings suggest the direct regulatory links between DCAMKL-1, miRNAs and EMT in pancreatic cancer. These results also justify that DCAMKL-1 can serve as a potential therapeutic target for eradicating pancreatic TICs [70]. In a recent study, Pramanik *et al.* synthesized a lipid-based nanoparticle for systemic delivery of miRNA expression vectors with a diameter of 100 nm. Systemic intravenous delivery with either miR-34a or miR-143/145 nanovectors inhibited the tumour growth in both MIA PaCa-2 subcutaneous and orthotopic xenograft mice. MicroRNA restitution was confirmed in treated xenografts by significant up-regulation of the corresponding miRNA while dramatically decreases in miR-34a targets such as SIRT1, CD44 and ALDH and miR-143/145 targets such as KRAS2 and RREB1 [71]. Different from other TICs, pancreatic TICs have unique miRNA expression patterns. Recent data demonstrated that drug resistance is regulated not only by genetic and epigenetic changes, but also by miRNAs [72]. At present, several important tumour suppressor miRNAs, oncogenic miRNAs and their molecular targets have been identified in pancreatic cancer. Thus, utilizing miRNAs as one of the therapeutic targets offers a promising treatment strategy to eradicate pancreatic TICs. However, more in-depth studies are needed to better understand the molecular mechanism and design more effective therapies [73].

Conclusion

Although there have been some improvements in the diagnostic and surgical modalities as well as chemotherapeutic efficacy, lack of early diagnosis and successfully curative resection still poses great challenges for treating pancreatic cancers over the last decades [74]. Tumour recurrence and chemoresistance due to TICs also hinder the survival rate of pancreatic cancer patients. Emerging evidence has gradually revealed an intertwined connection between TICs, signalling pathways and miRNAs, shedding new lights in tailoring successful therapy against pancreatic TICs that can potentially benefit the survival of this deadly disease. In addition to their therapeutic value, circulating TICs can be utilized in assessing the risk of recurrence and prognosis in pancreatic cancer as it has been used in patients who suffer from melanoma [75] or colon cancers [76]. Various surface markers have helped isolate TICs from solid tumour; however, a more refined technique is needed for precise isolation of these tumourigenic cells in upcoming investigation on TICs both *in vitro* and *in vivo*. The validity of these critical TIC markers in TIC identification also has to be justified with larger patient sample size. There is no doubt that pancreatic TICs, their associated signalling pathways as well as miRNAs have come to light as promising targets for cancer therapy, revolutionizing the way of traditional cancer therapy is performed, however, further studies are needed to unravel the detailed mechanism of TICs in pancreatic cancer.

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Conflict of interest

The authors declare no conflict of interest.

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