REVIEW ARTICLE

Future Directions in Pancreatic Cancer Therapy

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ABSTRACT

Pancreatic cancer is a major disease burden that is essentially incurable at present. However significant understanding of the molecular basis of pancreatic cancer has been achieved through sequencing. This is allowing the rational design of therapeutics. The purpose of this review is to introduce the molecular basis of pancreatic cancer, explain the current state of molecular therapy and provide examples of the ongoing developments. These include improvements in chemotherapy, small molecule inhibitors, vaccines, immune checkpoint antibodies, and oncolytics.

Introduction

Pancreatic cancer (85% of which are adenocarcinomas) is a deadly cancer with a very poor survival frequency [1]. It is the eighth leading cause of death from cancer in men and the ninth leading cause of death from cancer in women throughout the world [1]. In Canada as of 2014 the overall five year observed survival proportion (proportion of patients alive five years after diagnosis) was estimated to be 7% [2]. This poor survival rate is in line with world-wide statistics. The best survival rate results from early diagnosis of localised cancer before spread to the lymph nodes. This represents only 9% of cases at diagnosis. In the USA the survival rate for this group of patients is 26% [3]. Much of this poor survival outlook is due to the late stage of diagnosis and the ineffectiveness of current pancreatic cancer therapies. However with the advent of high throughput cancer sequencing the molecular "landscape" of pancreatic cancer is now well understood and this could allow the development of more effective patient specific therapies.

Microarrays and Next Generation Sequencing

Before the advent of next generation sequencing, microarrays were an important method of comparing the expression level of mRNA between tumour samples and normal tissues. The method involves spotting microlitre volumes of probe DNA (typically cDNAs) onto glass slides (this can comprise the total human genome). The array is then hybridised with fluorescently labelled total cDNA from normal and pancreatic cancer tissues. Typically a two colour analysis is performed in which the cDNA from cancer and normal tissue are labelled with different fluorescent chemistries and hybridised to the same microarray. Several such studies have been performed for pancreatic cancer [4–11]. The method indicates relative expression levels of mRNAs between tumour and normal tissue. However the results between studies are rather inconsistent. This was perplexing at the time however with the advent of more sensitive profiling techniques such as next generation sequencing it has become apparent that pancreatic cancer is highly heterogeneous in terms of specific gene alterations. That is it is extremely rare that the tumours of two pancreatic cancer patients have the same molecular profile. However the mutations that occur fall within predicable signalling pathways such as RAS. An important shift in thinking is required: pancreatic cancer is a genetic disease whose sole linking factor is the location of the disease. Successful treatment requires detailed molecular knowledge of the patient's individual tumour. This information can be obtained by next generation sequencing.

Next generation sequencing describes platforms that sequence DNA via whole genome sequencing as opposed to bacterial artificial chromosome (BAC) based approaches commercially introduced in 2004. Adaptors are annealed to blunt ended DNA fragments and these fragments are either amplified before sequencing or directly sequenced. No cloning is required. In addition to sequence information next generation sequencing also preserves information relating to the relative abundance of each DNA fragment in the original test sample. The conversion of total mRNA to cDNA before next generation sequencing allows the generation of data equivalent to microarray or quantitative PCR but with sequence information for each mRNA [12].

Genetic Basis of Pancreatic Cancer

When considering the frequency of mutations in the population of a particular cancer the most common

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Abbreviations EMT epithelial to mesenchymal transition
FOLFIRINOX folinic acid, 5-fluorouacil, irinotecan and oxaliplatin
GEM gemcitabine
GM-CSF granulocyte–macrophage colony-stimulating factor
HSV-1 herpes simplex virus 1
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mutations have come to be termed "mountains" and the majority of the other mutations in a particular tumour sample thought of as "peaks" [13]. In descending order of frequency the mutation "mountains" of pancreatic adenocarcinoma are in KRAS, CDKN2A, TP53, SMAD4 [14]. Like colon adenocarcinoma, the average pancreatic adenocarcinoma develops through a predictable set of genetic changes which are associated with progression to cancer. An important outcome of sequencing pancreatic cancer is an appreciation that there are certain pathways in which the majority of mutations lie such as KRAS and Hedgehog signalling. The major pathways have been summarised by Jones *et al*. Their data is also publically available for independent analysis [15].

It is now known that there are two major distinct precursor conditions with unique genetic profiles that lead to pancreatic adenocarcinoma. Pancreatic intraepithelial neoplasia (PanIN) are microscopic premalignant pancreatic lesions associated with the pancreatic ducts. At least 90% of PanINs contain mutations in KRAS regardless of stage; however the proportion of cells that contain the KRAS mutation increases with stage. This suggests that KRAS mutation is the driver that initiates PanIN [16]. In the 10% of cases that lack KRAS mutation GNAS or rarely BRAF, and CDKN2A mutations may be responsible [16].

The second less frequent precursor to pancreatic adenocarcinoma are cystic lesions the most common of which are intraductal papillary mucinous neoplasms (IPMNs), representing ~20% of surgically resected cystic lesions of the pancreas and occur in the main pancreatic duct or branch ducts [17]. It has been found that between 40-66% of IPMNS contain GNAS mutations [17, 18]. Furthermore greater than 96% of IPMNs have either a GNAS or a KRAS mutation [17]. It may well be worth considering pancreatic adenocarcinomas that arise from cysts a "different disease" to those that arise from PanIN [17].

Standard Cytotoxic Chemotherapy

Only 15-20% of pancreatic cancers are resectable at diagnosis [1]. In 1997 gemcitabine (GEM) became the standard chemotherapeutic of choice in preference to 5-fluorouacil (5-FU) for the treatment of non-resectable pancreatic cancer, when a clinical trial demonstrated an increased survival duration of about a month and a 24% response rate compared with 5% for 5-FU [19]. Gemcitabine is also commonly used following surgery (adjuvant therapy) although it has no survival benefits over 5-FU in this context [20] (see Table 1 for a list of the drugs utilised for pancreatic cancer treatment and their targets discussed in this review).

The recent introduction of two new chemotherapeutic regimes have superseded gemcitabine monotherapy for advanced pancreatic cancer patients with a good performance status. In a 2011 phase III trial, a four drug regimen, which included folinic acid, 5-fluorouacil, irinotecan and oxaliplatin (FOLFIRINOX), was shown to have significantly superior survival outcomes compared to GEM alone [21]. In a recent phase III clinical trial gemcitabine and nanoparticle albumin–bound paclitaxel (Abraxane), as compared with gemcitabine alone, was associated with a prolongation of overall survival [22]. These cytotoxic drugs provide a base for multimodal therapy including pathway targeted small molecule inhibitors of protein, and immunotherapy.

Pathway Targeting Therapeutics

The new understanding of the molecular pathways involved in pancreatic cancer should enable highly effective therapeutics targeting specific pathways to be developed. The use of viral vectors to modulate gene activity in pancreatic cancer cells and tissues is a fast and economic method of preclinical target evaluation. These vectors have the potential to be used as *bona fide* therapeutics (for example the adenovirus based oncolytic vector Oncorine approved in China for head and neck cancers [23]) but can also provide validated targets for the pharmaceutical industry to develop stable therapeutic small molecules.

An example of the approach of using viral vectors to validate potential pancreatic cancer targets are investigations of therapeutics targeting G1-S initiation. CDKN2A encodes two proteins $p16^{INK4a}$ and $p14^{ARF}$ which inhibit the cyclin D-CDK4/6 complex responsible for G1-S initiation by phosphorylating retinoblastoma thereby releasing E2F to initiate transcription. Loss of CDKN2A in pancreatic cancer therefore promotes cell proliferation. Kobayashi *et al*. demonstrated that an adenovirus encoding $p16^{INK4a}$ could significantly reduce the proliferation of the pancreatic cancer cell line MIAPaCa-2 [24]. Pfizer (palbociclib) [25], Novartis/ Astex Pharmaceuticals (LEE011) and Eli Lilly (abemaciclib) [26], have developed orally administered potent small molecule inhibitors of CDK4/6. In theory these compounds are highly suitable for advanced pancreatic cancer with loss of CDKN2A, however studies involving pancreatic cancer cell lines have shown that not all CDKN2A negative cell lines are responsive to CDK4/6 inhibition alone [19, 27]. Combination with chemotherapeutic agents must also be carefully considered as CDK4/6 inhibition reduces the effectiveness of gemcitabine but perhaps not 5-FU [19, 27]. In addition CDK4/6 inhibition may induce epithelial to mesenchymal transition (EMT) in pancreatic cancer cell lines responsive to TGF-β and with a wild-type SMAD4 protein [28]. There is a clear need for further investigation in 3D preclinical models. Nonetheless, Novartis are currently investigating LEE011 in a phase II clinical trial of solid tumours with activation of the CDK4/6 complex either by mutation or amplification of the complex or by deletion of CDKN2A [ClinicalTrials.gov identifier: NCT02187783].

The Hedgehog signalling pathway is almost always activated by mutations in pancreatic cancer [15]. All current small molecules in clinical development inhibit the SMO receptor [29]. Unfortunately this leaves a large number of downstream mutations that can nullify any inhibition of

Table 1. Pancreatic cancer drugs and their targets mentioned in this review

Drug	Target
5-fluorouacil	Thymidylate synthase
Gemcitabine	DNA
FOLFIRINOX	Thymidylate synthase, Topoisomerase 1, DNA
Abraxane	Tubulin
Palbociclib	CDK4/6
LEE011	CDK4/6
Abemaciclib	CDK4/6
Tipifarnib	Farnesyltransferase
L-778.123	Farnesyltransferase
Tanomastat	MMP-2, MMP-3, MMP-9, and MMP-13
Marimastat	All MMPs
Erlotinib	EGFR
Algenpantucel-L	Pancreatic cancer cells (vaccine)
GVAX pancreas	Pancreatic cancer cells (vaccine)
TG01	Mutant RAS (vaccine)
Ipilimumab	CTLA4
Talimogene laherparepvec	Interferon pathway negative cancer cells
CG0070	E2F1 over-expressing cancer cells

SMO. All clinical trials targeting SMO in pancreatic cancer patients have failed, most likely for the aforementioned reason [29]. None of the trial participants were screened for mutations in the hedgehog pathway. These findings in no way rule out the Hedgehog pathway as a valid target for single agents. These drugs may be effective for rare pancreatic cancer patient populations and as discussed in more detail below these non-significant trials should be re-examined for outlier patients who showed regression. Some of these SMO inhibitors may be orphan drugs (of benefit in rare patient populations) and could potentially be brought to market as such. Especially if a regulatory framework were put in place that recognises that cancer including pancreatic cancer is essentially a vast collection of rare diseases [30].

Farnesyltransferase inhibitors originally envisaged to target the RAS signalling pathway have proven disappointing in clinical trials (no statistically significant improvement in patient outcomes). This may be due to alternative prenylation of K-RAS [31]. Two farnesyltransferase inhibitors have been tested in clinical trials of pancreatic cancer, Tipifarnib (Johnson & Johnson), and L-778.123 (Merck & Co.). However L-778.123 is no longer available due to cardiac safety concerns [32].

In preclinical testing of Tipifarnib it became clear that farnesyltransferase inhibitors could inhibit cancer cell proliferation by mechanisms other than through RAS signalling. The majority of wild-type RAS cell lines tested were in fact more sensitive to farnesyltransferase inhibition than cell lines with RAS activating mutations [33]. Roughly fifty percent of K-*RAS* active cell lines were resistant to farnesyltransferase inhibition by Tipifarnib. However importantly in mouse xenographs of the sensitive K-*RAS* pancreatic cancer cell lines, such as CAPAN-2, inhibition significantly reduced tumour volume.

Unfortunately in a phase III clinical trial of Tipifarnib for pancreatic cancer there was no benefit from farnesyltransferase treatment in combination with gemcitabine compared to gemcitabine alone [34]. In cell lines with activating RAS mutations prenyltransferase inhibitor treatment sensitised the cells to radiation [35]. However clinical trials testing L-778.123 and Tipifarnib in combination with radiation therapy have either not produced results consistent with clinical benefit [32] or the results have not been made available and no further studies initiated [ClinicalTrials.gov identifiers: NCT00077519, NCT00026104].

However farnesyltransferase inhibitors may still have a role in the treatment of a rare subset of pancreatic cancer patients. Importantly rare cases of complete response to farnesyltransferase inhibitors are known in pancreatic cancer patients [36]. It is an open question as to whether this is due to spontaneous regression. This could be addressed however by sequencing the patient's tumour biopsy material (if available). Determining the underlying mechanism is important as these rare instances could be due to a genetic combination in the tumour that could be exploited to both screen for rare patients who would benefit from farnesyltransferase inhibitors and reveal potential targets for a combination therapy involving farnesyltransferase inhibitors. Cell cultures from responding patients (if they exist) and farnesyltransferase inhibitor responsive pancreatic cancer cell lines such as PSN-1 could be employed to experimentally validate the mechanism hypothesises generated from sequencing. This approach has revealed markers that predict durable response to the "failed" bladder cancer drug Everolimus [37]. Farnesyltransferase inhibitors may be appropriate as orphan drugs for specific pancreatic cancer patient subpopulations.

In humans matrix metalloproteinases (MMPs) are a 23 member family of membrane bound and extracellular proteases that bind zinc at their active site [38]. They degrade extracellular matrix components and have been demonstrated to be important for cancer cell line *in vitro* invasion. In pancreatic cancer MMP-2 and MMP-9 are the best characterised MMPs however others are involved and it is an ongoing area of investigation. There is strong evidence for the expression and activation of MMP-2 in pancreatic cancer [39]. Furthermore in pancreatic cancer cell lines MMP-2 expression is correlated with the capacity for *in vitro* invasion and inhibition of its expression by RNAi can lead to reduced cell line *in vitro* invasion [40]. The expression of MMP-9 has been associated with worse prognosis and increased likelihood of metastasis [41]. Initially preclinical data suggested that MMP activation promoted cancer cell invasion of the basement membrane and promoted metastasis in animal models. This lead to the development of broad spectrum MMP inhibitors. MMP inhibitors are not cytotoxic but aim to inhibit metastasis the leading cause of cancer death. Bayer developed tanomastat which inhibits MMP-2, MMP-3, MMP-9, and MMP-13. Unfortunately the development was dropped after a

phase III clinical trial that compared tanomastat alone with gemcitabine alone demonstrated that tanomastat performed worse than gemcitabine [42]. This was however a slightly odd study in that the more appropriate test would have been gemcitabine plus tanomastat especially given that tanomstat is not cytotoxic. Tanomastat may still have utility. Marimastat was developed by British Biotech as a non-selective MMP inhibitor. Unfortunately although marimastat inhibited tumour growth and metastasis in animal models, when it was tested in a rigorous phase III trial in combination with gemcitabine verses gemcitabine alone survival was not enhanced [43]. The reasons for the failure of these trials are two-fold. At the time of development it was not appreciated that some MMPs are in fact tumour suppressors [38]. With this in mind it is much more appropriate to develop specific MMP inhibitors. In addition treating late stage (already metastasised) cancer with a metastasis inhibitor is unlikely to improve survival. It is therefore not so much that MMP inhibitors have failed but that further research was required and the clinical trials initiated were not the most appropriate. The rush to clinical trial was understandable given the lack of treatment modalities for pancreatic cancer. Although the failure was disappointing it does not signify that MMPs are not a good target. Inhibitors that target single MMPs may be appropriate as a maintenance treatment for pre-metastatic pancreatic cancer [44].

An example of a modestly successful targeted therapy is that of the tyrosine kinase inhibitor erlotinib which inhibits the epidermal growth factor receptor. Over-expression of EGFR is associated with progression of pancreatic tumour aggressiveness [45]. In a phase III clinical trial erlotinib plus gemcitabine verses gemcitabine alone in patients with advanced pancreatic cancer increased survival from 5.9 months to 6.2 months. The one year survival rate was improved from 17% to 23% [46].

The identification of genetic markers which are associated with targeted therapy responders is very important and is the basis of the success of the targeted therapy trastuzumab for breast cancer. It can be argued that all targeted small molecule inhibitors should be thought of as orphan drugs as pancreatic cancer is not one disease but a group of many rare diseases (differing in their genotype) unified by their origin in the pancreas [30]. In contrast immunotherapy holds the promise of being effective against a much wider range of pancreatic cancer tumours than targeted small molecule inhibitors

Immunotherapy: Vaccines

A promising approach which has been successfully implemented for melanoma treatment and is likely to become a therapeutic reality for pancreatic cancer in the coming years is immunotherapy or boosting the immune response against cancer. One such strategy that has entered phase III clinical trials for both resectable and non-resectable pancreatic cancer [ClinicalTrials.gov identifiers: NCT01072981, NCT01836432] is based on inducing a hyperacute immune response to pancreatic cancer cells as if they were a xenograft. The rejection of xenografts is largely mediated by abundant human antibodies against the specifically non-human disaccharide epitope α-gal present in glycoproteins and lipids of non-human mammalian cells [47]. The pancreatic cancer vaccine Algenpantucel-L consists of two pancreatic cancer cell lines that express murine α-1,3-galactosyl transferase (αGT), which directs the synthesis of α-gal epitopes on their surface proteins and glycolipids. A Phase II trial demonstrated that the vaccine is safe in combination with gemcitabine and showed promising signs that it increased patient survival relative to gemcitabine alone, however this is inferred from previous studies as an Algenpantucel-L negative control group was not included [48]. This is being directly addressed in the aforementioned ongoing phase III clinical trials. Alternative methods of priming the immune system to pancreatic cancer such as cell lines that express granulocyte–macrophage colony-stimulating factor (GM-CSF) or mutant cancer peptides plus GM-CSF require further trials.

In addition to whole cell vaccines small cancer peptide vaccines are also in development. Vaccines based on mutated KRAS peptides in combination with GM-CSF are in the early stages of clinical trials [49]. An interesting approach that demonstrated safety and efficacy in improving patient survival in phase II clinical trial involved the use of two GM-CSF expressing pancreatic cancer cell lines as a primary vaccine followed by booster vaccinations consisting of attenuated *Listeria monocytogenes* expressing the tumour differentiation antigen mesothelin [50]. These strategies boost T cell immune activation through presenting tumour antigens; however it may also be necessary to relieve the immune checkpoint block that tumour cells exert.

Immunotherapy: Antibodies

A well established immune checkpoint protein is CTLA4. This T cell surface receptor counteracts the co-stimulatory signal generated by the interaction of CD28 (also present on the T cell surface) and the antigen presenting cell surface proteins CD80 and CD86 [51]. A phase III trial of fully human antibody (ipilimumab) against CTLA4 was shown to increase survival of patients with metastatic melanoma [52]. It has been approved in the USA, Canada and the EU for treatment of metastatic melanoma. Unfortunately as a single agent at a dose of 3.0 mg/kg ipilimumab was found to be ineffective as a pancreatic cancer treatment [53]. However, in a mouse model of metastatic melanoma a combination of a vaccine consisting of irradiated melanoma cells secreting GM-CSF plus anti-CTLA4 antibodies was shown to be more effective than either agent individually [54]. Similarly for pancreatic cancer a combination of a vaccine of GM-CSF secreting irradiated pancreatic cancer cell lines plus ipilimumab was more effective than ipilimumab alone and increased patient survival rate warranting further clinical trials [55]. Pancreatic cancer is poorly immunogenic compared with melanoma; however the immunogenicity can be increased through oncolytic viral therapy.

Immunotherapy: Oncolyic Viruses

Oncolytic virotherapy for pancreatic cancer is an active area of research. That is the use of viruses specifically targeted to pancreatic cancer that both directly lyse the tumour cells and trigger an immune response against the tumour. It is now appreciated that the immune function may be more important than the lytic function, especially in combination with the new generation of immune checkpoint blocking antibodies entering cancer therapy.

Talimogene laherparepvec is a herpes simplex virus 1 (HSV-1) based oncolytic vector delivered via injection that has undergone a phase III clinical trial for metastatic melanoma with results that strongly suggest it could be useful as a single agent or more likely in combination with immune checkpoint blocking antibodies [56, 57]. It was generated from a fresh isolation of HSV-1 virus (JS1) and has a GM-CSF replacement of the two copies of the ICP34.5 gene which normally reverses the interferon induced phosphorylation of the α subunit of the eukaryotic initiation factor 2 (EIF2S1) [33, 58]. The interferon pathway is usually disrupted in cancer thus lending the vector specificity to cancer cells. In addition the ICP47 gene was deleted which enhances oncolysis [58]. A phase I clinical trial has demonstrated that this vector is safe when injected into primary metastatic pancreatic cancer lesions and tumour size reductions were observed at both the primary site and metastatic lesions that were not injected thus suggesting that the therapy induced a systemic immune response against the cancer [59]. This should be followed up with further clinical trials and perhaps in combination with ipilimumab and other immune checkpoint blocking antibodies as is being pursued for melanoma [ClinicalTrials.gov identifier: NCT01740297].

Adenoviruses are another well established oncolytic agent that could prove useful for the treatment of pancreatic cancer. Their major drawbacks are tropism for the liver and established immunity which make systemic delivery unrealistic. However, as the clinical trial with talimogene laherparepvec has shown, that direct injection to the pancreas is feasible [59]. Furthermore the paradigm shift in viral therapy away from the importance of oncolysis and towards generating an immune response suggests that established immunity to adenovirus may actually be beneficial when the virus is localised to the pancreas. The major benefits of adenoviruses are their safety in terms of non-integration into the genome and the extremely strong understanding of the receptor usage of the many species of adenovirus. This has allowed adenoviruses to be engineered that have improved tropism to cancer including pancreatic cancer [60]. A promising adenovirus candidate for cancer therapy that has entered phase III clinical trials for bladder cancer is CG0070 [36, 61] [ClinicalTrials.gov identifier: NCT01438112]. The virus expresses GM-CSF thus priming the immune system and its replication is driven by the E2F1 promoter. CDKN2A loss is one of the "mountains" of the pancreatic cancer genetic landscape and therefore this adenovirus should also be highly applicable to pancreatic cancer. It is however relatively straightforward to engineer adenovirus replication driven under any desired promoter. Many other types of oncolytic virus have been developed and there are many excellent reviews available. All oncolytic viruses have their advantages and disadvantages. They should be seen as components of immunotherapy involving various agents.

Conclusion

Specific small molecule inhibitors should not be expected to treat all pancreatic cancers. They will only ever treat a subset of patients. The future of pancreatic cancer therapy lies in sequencing. A patient's tumour could be sequenced and appropriate small molecule therapeutics (or viral vectors) selected based on the specific mutations found in their tumour. Immune checkpoint blockade, vaccination and oncolytic agents could be employed to ensure the long term clearance of the disease. A larger repertoire of specific small molecules inhibitors needs to be developed based on objective interpretation of sequencing data for each individual patient. Innovativethe new knowledge of pathways involved in pancreatic cancer to make this feasible.

Conflicting Interest

The authors had no conflicts of interest

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