

Personalised therapy for Pancreatic Cancer: Challenges and Opportunities

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Abstract

Pancreatic Ductal Adenocarcinoma (PDAC) has one of the lowest 5-year survival rates of all cancers. Early metastatic spread and failure of standard chemotherapeutics both contribute to this statistic. Over the last decade many other cancer types have benefited from the targeted therapy revolution in which signalling networks found to be altered in cancer cells are specifically targeted. Despite an ever-growing understanding of the mutation profiles in PDAC, the vast majority of these approaches have failed for this form of malignancy. Chemotherapy, which simply targets dividing cells, remains the most effective available treatment option alongside surgery. Although targeting a broad range of tyrosine kinase receptors with erlotinib has been shown to offer a modest improvement in the 5-year survival when combined with the pyrimidine-based chemotherapeutic gemcitabine; this suggests that targeted approaches may be of benefit in at least some patients. PDAC is characterised by a long genomic tail of potentially actionable mutations but each appearing in only a small number of patients, making it difficult to show a survival benefit of a targeted therapy in an unselected group of patients. Identifying specific subpopulations based on molecular characterisation of the tumour is particularly difficult in PDAC because of problems in biospecimen acquisition; only a small proportion of patients undergo surgery and the organ is difficult to biopsy due to its location. Furthermore, PDAC is characterised by cellular heterogeneity within primary tumours and their metastases, meaning that targeted

therapies may have only a transient effect, killing dominant cellular populations but leaving behind potentially even more aggressive forms of cancer cells that have unidentified (and so unexploited) molecular targets. In this review, consideration is given to overcoming the barriers of personalised medicine specific to PDAC with the aim of improving the 5-year survival rates

Key Words: PDAC, Targeted Therapy, Chemotherapy, Circulating Tumour Cells, Next Generation Sequencing

INTRODUCTION

In the USA, death rates are stable or decreasing for most forms of cancer, largely due to advances in treatment. However, for pancreatic ductal adenocarcinoma (PDAC) increasing incidence more than compensates for any tiny improvement in survival and so despite accounting for less than 5% of all newly diagnosed cancers, PDAC has grown to become the 4th largest cancer killer (Siegel et al, 2015). The median survival for patients after a diagnosis with PDAC is less than 5 months (Yuan et al, 2015). Chemotherapy with 5-fluorouracil (5FU) or gemcitabine following surgical resection of primary tumours improves prognosis (Neoptolemos et al, 2010; Neoptolemos et al, 2004), but only less than 20% of patients are suitable for surgical resection of their tumour (Hackert et al, 2015). Gemcitabine became the standard of treatment for advanced PDAC due to slight superiority over 5FU in patients with advanced cancer (Burris et al, 1997). FOLFIRINOX (oxaliplatin, irinotecan, leucovorin, and 5FU) is the only non-gemcitabine regime shown to be superior to gemcitabine in patients with metastatic pancreatic cancer (Conroy et al, 2011) but its toxicity and side effect profile limit its use to patients with a high performance indicator.

Targeting cell division by interfering with microtubule depolymerisation using albumin-bound paclitaxel (nab-paclitaxel) combined with gemcitabine gave modest improvement in overall survival (OS) in advanced PDAC (Von Hoff et al, 2013). The addition of an orally administered precursor of 5FU (capecitabine) to gemcitabine significantly improved response rates and progression free survival (PFS) but only showed a trend towards increasing OS in patients with advanced cancer (Cunningham et al, 2009). A severely limited repertoire of effective chemotherapeutics in a disease characterised by treatment resistance is a major factor contributing to the poor 5-year survival of PDAC.

Targeted therapy in PDAC

Targeted therapies have shown significant survival benefit in many different cancer types. It was hoped that similar outcomes would follow for PDAC but most trials have failed to demonstrate a survival benefit. For example, bevacizumab (a recombinant humanized anti-VEGF monoclonal antibody) gave improved overall survival (OS) in phase III trials in advanced colorectal (Hurwitz et al, 2004), non-small cell lung (Sandler et al, 2006), renal cell (Escudier et al, 2007), and breast cancer (Miller et al, 2007). Despite

strong pancreas specific pre-clinical evidence (Bockhorn et al, 2003) and an encouraging phase II study (Kindler et al, 2005) no improvement in OS was seen in a phase III study in advanced PDAC (Kindler et al, 2010). Lack of response may be due to the pattern of VEGF isoforms present in the PDAC patients on the trial. Bevacizumab inhibits angiogenesis via VEGF-A but the majority of pancreatic cancer patients have high levels of expression of the VEGF-D which can compensate for reduced VEGF-A activity. There is evidence that for a small subset of patients with low levels of VEGF-D bevacizumab does have efficacy (Weickhardt et al, 2015), unfortunately this is too few patients to allow the benefit to be observed in an unselected population.

Conceptually aflibercept offers an advantage over bevacizumab in treatment of PDAC because as well as targeting VEGF-A it also acts against at least two other members of the VEGF family (VEGF-B, and PLGF) (Sun, 2012). However, aflibercept again gave no survival advantage when combined with gemcitabine over gemcitabine alone for advanced PDAC patients (Rougier et al, 2013).

An alternative would be to inhibit the receptors rather than VEGF itself. Axitinib is

a fairly broad range kinase inhibitor but is best characterised in terms of its inhibition of VEGF Receptors 1, 2 and 3 (McTigue et al, 2012), it has also received approval for use in treatment of renal cell carcinoma, nevertheless it proved ineffective when used in combination with gemcitabine in a phase III trial with advanced PDAC (Kindler et al, 2011).

Other targeted therapies with proven effectiveness in several cancer types and strong pre-clinical evidence have also failed to show improved OS. The following are examples, all of which were used in combination with gemcitabine and all of which showed no survival benefit:

Tipifarnib targets RAS by inhibiting farnesyl transferases, as stated above nearly all PDAC tumours have K-Ras mutations, but despite this targeting K-Ras in the clinic has not yet proved successful (Macdonald et al, 2005). Including a clinical trial of tipifarnib with gemcitabine (Van Cutsem et al, 2004). Possibly because K-Ras unlike other forms of Ras protein does not absolutely require farnesyl transferases to become active (using geranyl transferases instead) and perhaps because of the ability of cancer cells to survive having lost K-Ras (Viale et al, 2014).

Marimastat targets matrix metalloproteinases (MMPs). MMPs are important in the metastatic process that is so characteristic of PDAC and so they would appear to be a very suitable target, but in combination with gemcitabine marimastat had little or no effect on survival (Bramhall et al, 2002). This may be because by the time of treatment the cancer had already metastasised and the MMPs were not necessary for cancer cell survival.

Cetuximab targets epidermal growth factor receptors (EGFRs); in other cancers, EGFR inhibitors have been shown to be most effective in patients with EGFR mutations (Pao et al, 2004) and without K-Ras mutations (Allegra et al, 2009). In PDAC EGFR is rarely mutated (less than 5% of cases) and K-Ras is mutated in over 90% of cases (Waddell et al, 2015; Witkiewicz et al, 2015). On this basis, it seems unlikely that Cetuximab would be effective in PDAC, which was confirmed in clinical trials (Philip et al, 2010).

Sorafenib is a broad-spectrum kinase inhibitor, targeting not only tyrosine kinases (e.g. EGFR and VEGF receptors) but also serine/threonine kinases (e.g. RAF kinase), it could therefore be considered as a more likely prospect for PDAC than cetuximab. Nevertheless, it was not successful when

tested in a clinical trial (Goncalves et al, 2012).

Of all the targeted therapy tested to date against PDAC only the addition of the tyrosine kinase inhibitor erlotinib to gemcitabine provided a significant survival advantage (Moore et al, 2007). Like cetuximab and sorafenib, erlotinib targets EGFR. Therefore, it is perhaps surprising that erlotinib should be effective. Sorafenib and erlotinib are both small molecule kinase inhibitors but they have a different spectrum of affinities to specific targets: for example sorafenib is more effective at inhibiting VEGF receptors than erlotinib but less effective at inhibiting EGFR (Uitdehaag et al, 2014). However, erlotinib does not seem to be any more effective in PDAC patients with wild type K-Ras than in patients with mutations (Boeck et al, 2013), in marked contrast to the K-Ras dependence seen in other tumour types (Allegra et al, 2009; Pao et al, 2005), suggesting that the efficacy of erlotinib seen in PDAC may be due to inhibition of targets other than EGFR. It could be that although the kinases inhibited by erlotinib cover a narrower spectrum than those inhibited by Sorafenib this spectrum matches more closely the profile of kinases

driving tumour growth and development in PDAC.

The problem is that all the targeted agents described above might be effective against PDAC in some patients, none of them is effective against the majority of patients and this means it is difficult to establish efficacy in a clinical trial.

Clinical Trial design

The randomised controlled trial (RCT) was developed nearly 70 years ago to investigate the treatment of a relatively simple disease (pulmonary tuberculosis) with a single treatment (Streptomycin) (1948). This was a population-based approach; if more people benefit from a drug than are harmed by it then it is a good drug. It was always apparent that a simplistic use of RCTs would miss many beneficial agents if the benefit was restricted to only a subset of the population, similarly there is an ethical issue in licensing an agent for use because the majority will benefit when it is possible that a minority will be seriously harmed. Statistical methods such as multivariate assessment of proportional hazards can be used to mitigate this problem (Cox, 1972), but use of such approaches requires recruitment of large numbers of patients to give each subgroup an adequate representation. This approach also requires

identification of the relevant parameters defining the subgroups, for example, subgroups could be defined by the genotype of the tumour. Unfortunately, the more parameters (e.g. clinical features or mutations) considered the greater the number of subgroups and so the greater the number of patients that need to be recruited. Clinical trials are expensive and so recruitment is restricted, if 1,000 patients are recruited to a trial and only 1% have a genotype that interacts positively with the treatment then the effect is likely to be missed. The solution is to selectively recruit patients who are predicted to benefit from the treatment.

The problem is exemplified by the failure of early trials with the EGFR inhibitor gefitinib in unselected cohorts with non-small cell lung cancer (NSCLC) (Kim et al, 2008; Maruyama et al, 2008; Thatcher et al, 2005). A later phase III study, which restricted trial entry to patients harbouring an EGFR mutation, was required to uncover the survival benefit (Mitsudomi et al, 2010).

In NSCLC EGFR mutations are relatively common and so in the successful trial described above only 337 patients had to be screened in order to recruit 118 patients

(Mitsudomi et al, 2010). Conducting a comparable study restricting entry to patients harbouring a mutation with a prevalence of less than 5% (e.g. EGFR in PDAC) would require over 2,000 patients to be screened: this would be expensive and time consuming. Basket trials recruit patients with different histological cancers but identical actionable mutations to allow completion of trials with statistical power in a feasible timeframe (Berry, 2015; Mehta et al, 2014; Redig & Janne, 2015). PDAC with its long tail of low frequency actionable mutations has the most to gain from such a strategy. The first basket trial including PDAC examined the effect of vemurafenib in non-melanoma cancers with BRAF V600 mutations, it recruited 2 PDAC patients and has now been published (Hyman et al, 2015). The National Cancer Institute's Molecular Analysis for Therapy Choice (MATCH) study is a large basket trial open to all patients with solid tumours who have progressed on first line chemotherapy. The trial includes 10 separate targeted therapies matched to actionable mutations. The likelihood of PDAC recruitment is significantly diminished by the inclusion criteria which requires four core biopsies of primary tumour with >70% tumour content (Do et al, 2015).

Basket trials offer an elegant solution to overcome some statistical challenges with standard RCTs. However, in reality acquired resistance to monotherapy typically seen in PDAC demands a methodology that can evaluate multi-drug therapy according to a plethora of actionable mutations, which evolve throughout a patient's treatment (see later section). Neither RCTs nor basket trials can accommodate these vast permutations. Single-patient or n-of-1 trials use multiple time points and crossover two or more treatments using individual patients as a control. They may be the method of choice to re-evaluate previously dismissed treatments (Duan et al, 2013; Schork, 2015).

Genomics of PDAC

Obviously targeted therapy requires the target to be present. The key to success therefore lies in proper characterisation of tumours, to this end the International Cancer Genome Consortium (ICGC) has set up large-scale cancer genome studies to generate a comprehensive catalogue of somatic mutations from a variety of cancer types including PDAC (International Cancer Genome et al, 2010). Such studies offer new insights into the genomic landscape of PDAC (Biankin et al, 2012; Nones et al, 2014;

Waddell et al, 2015) and provide the platform from which new approaches to PDAC treatment can be developed. A classification of the PDAC based on genomic structural variation has been proposed (Waddell et al, 2015). Two of these groups have direct therapeutic relevance and will be considered below.

Locally rearranged

This subgroup accounts for 30% of PDAC patients, one third harbour a focal amplification in an oncogene (*ERBB2*, *CDK6*, *PIK3CA*, *MET* and *FGFR1*) which can be targeted therapeutically.

ERBB2 (*HER2*) has been found to be over expressed in 2% of PDACs, it is suggested that this is associated with absence of liver metastasis and propensity for lung and brain metastasis (Chou et al, 2013). *ERBB2* is well known to be over expressed in up to 30% of breast cancers (Slamon et al, 1987) and has long been effectively targeted with the monoclonal antibody trastuzumab (Slamon et al, 2001), which has also been shown to have antitumor effects in PDAC (Kimura et al, 2006). As well as directly targeting cells dependent on *ERBB2*, trastuzumab can also be used to target cytotoxins to *ERBB2* positive tumour cells by conjugating the cytotoxin to the antibody. For example, a

derivative of the tubulin inhibitor maitansine has been conjugated to trastuzumab to give ado-trastuzumab emtansine (Kadcyla) (Lewis Phillips et al, 2008). Kadcyla is one of the drugs that is being assessed in the NCI MATCH trial in patients with proven *ERBB2* over expression (Do et al, 2015).

One of the most frequently mutated genes in PDAC is *CDKN2A* gene which encodes the p16ink4a protein, an inhibitor of Cyclin Dependent Kinases 4 and 6 (*CDK4/6*) (Schutte et al, 1997), taken together with the frequency of focal amplifications involving *CDK6* (Waddell et al, 2015), this suggests an exquisitely significant role for *CDK4/6* in pancreatic cancer. Palbociclib is an oral and selective inhibitor of *CDK4/6* and studies in PDAC animal models suggest palbociclib may be effective in PDAC treatment (Franco et al, 2014).

The *PIK3CA* gene encodes for the p110 subunit of phosphatidylinositol 3-kinase (*PI3K*), which activates the mammalian target of rapamycin (*mTOR*) pathway (Bjornsti & Houghton, 2004). *mTOR* inhibitors such as afinitor (everolimus) have improved PFS in pancreatic neuro endocrine tumours (Yao et al, 2011) advanced breast (Baselga et al, 2012) and renal cell carcinoma (Motzer et al, 2008). In pre-clinical studies, inhibition of the

mTOR pathway has shown anti-tumour effect in PDAC models (Asano et al, 2005; Bondar et al, 2002; Bruns et al, 2004; Ito et al, 2006). Although treatment with everolimus in an unselected group of patients with metastatic PDAC who had progressed on gemcitabine showed minimal clinical advantage (Wolpin et al, 2009), sub-groups defined by mutations in the PIK3CA gene may benefit.

MET can be targeted using tivantinib (Perez-Ramirez et al, 2015), early phase I trials have found tivantinib to be safe and tolerable in patients with advanced PDAC (Pant et al, 2014).

Ponatinib is an inhibitor of FGFR1 (amongst other receptors) and may be effective in FGFR1 mutated cancers (Gozgit et al, 2012) although this has not yet been tested in a clinical trial with PDAC patients.

Unstable

This subgroup accounts for 14% of PDAC (in the Wadell *et al* study (Waddell et al, 2015)) and is defined by a large number of structural variations, implicating defects in DNA maintenance and association with both germline and somatic mutations in the *BRCA2* and/or the *PALB2* genes. Though numbers were small combined patient and patient-

derived xenograft data indicated that these patients respond better to platinum based chemotherapy than other groups ($P=0.007$) (Waddell et al, 2015). *In vitro* (van der Heijden et al, 2005) and phase II studies (Kaufman et al, 2015) suggest that patients with recombination repair defects (e.g. with *BRCA2* mutations) respond better to Poly (ADP-ribose) polymerase (PARP) inhibitors, it is therefore reasonable to assume that PARP inhibitors would be more effective in the Unstable subgroup than in others. In a prospective phase II non-randomised study Kaufman *et al* investigated the PARP inhibitor olaparib in 23 patients with advanced PDAC and a known deleterious germline mutation in *BRCA1/2* who had already progressed on gemcitabine and platinum based chemotherapy (Kaufman et al, 2015). The response rate of 22% in this population is encouraging.

The emerging picture from whole genome sequencing studies is that PDAC tumours are very heterogeneous. Positively, many of the driver mutations are dominant focal mutation in oncogenes which have matching targeted therapies which in many cases are tried and tested in more homogeneous cancers types; where the prevalence of mutation is over 5%. The challenge of extending this strategy to

low prevalence mutations is multi-faceted. New methods of evaluating treatments in very small sub-groups have been discussed above. The next section will consider how such subgroups can be identified.

PDAC tumour sample

The availability of high quality bio-specimens is a prerequisite for entry of patients into clinical trials of personalized medicine and ultimately will be required to apply personalized medicine in clinical practice. An ideal bio-specimen accurately reflects the contemporaneous molecular composition of the tumour; is adequate for the analysis (e.g. offers enough DNA of good enough quality for sequencing); and allows minimally invasive acquisition to permit serial sampling to track clonal evolution.

Incisional biopsies

In breast, colorectal and ovarian cancer the majority of patients undergo surgical resection, but in prostate, lung and pancreas cancer patients undergoing resection are in the minority: for example, resection rates for colon cancer is 85.5% compared to 16.6% for PDAC (Speelman et al, 2015). As a result, molecular profiling of primary PDAC is more dependent on incisional rather than excisional biopsies, which is also true of other cancer types such as prostate. The anatomical

position of the prostate allows easy access to transrectal core biopsies which are widely used for molecular profiling in prostate cancer (Yadav et al, 2015). The pancreas however, occupies a retroperitoneal position, in close proximity to major vascular structures such that only an endoscopic approach to biopsy is possible.

Endoscopic ultrasound-guided fine-needle aspiration (EUS-FNA) is the most common modality for obtaining a tissue diagnosis of PDAC but the aspirate is often of limited or no cellularity and allows at most very limited histological analysis (Varadarajulu et al, 2012). EUS-FNA will often be inadequate for diagnosis let alone Next Generation Sequencing (NGS). Whilst successful NGS on pancreas FNA has been reported and in fact shown good concordance with paired formalin fixed paraffin embedded (FFPE) samples from the primary tumour, this approach has not been widely replicated (Young et al, 2013). A core tissue biopsy is the gold standard incisional biopsy and often is a minimum requirement for clinical trials enrolment. EUS-guided Tru-Cut biopsies (EUS-TCB) of the pancreas was first reported in 2002 (Wiersema et al, 2002), however, this is a technically difficult procedure and as a consequence improvement in diagnostic

accuracy (over FNA) proved marginal in early studies (Shah et al, 2008) and EUS-TCB has not yet been adopted into routine clinical practice (Fuccio & Larghi, 2014). Without core biopsies entry into basket trials, such as the MATCH trial which stipulates four core biopsies each with minimum tumour content >70%, will be limited to the small percentage of PDAC patients undergoing resection.

Excisional biopsies

Less than 20% of PDAC patients undergo surgical resection of their tumours (Speelman et al, 2015). FFPE sections from tumour excision biopsy are by far the most commonly used material in routine diagnostic laboratories due to difficulties in collection and storage of fresh or fresh-frozen samples. The formalin fixation process however, damages DNA through a number of mechanisms including fragmentation and cross-linking to proteins (Auerbach et al, 1977). It is fortunate that the fragmented nucleic acids typically extracted from FFPE specimens are ideally suited, in length at least, to NGS platforms which are restricted to reading short length nucleic acids sequences also of around 200-225 base pairs (Shaw et al, 2016). Despite the DNA damage accrued during the fixation process, studies have

shown comparable sequencing quality with FFPE derived DNA compared to the gold standard of fresh or fresh-frozen samples (Shaw et al, 2016; Spencer et al, 2013). A more significant problem of using FFPE for NGS is the tumour cellularity of the sample. Large scale sequencing studies using conventional approaches requires at least 80% tumour cellularity (Yadav et al, 2015). Dense desmoplastic stroma is a universal feature in PDAC (Mahadevan & Von Hoff, 2007) which dilutes the mean tumour cellularity to between 38-44% (Biankin et al, 2012). To some extent this can be overcome by coring out areas of high tumour cell content (Weng et al, 2010), either on the basis of gross histology (Wagle et al, 2012) or using histological guided laser capture microscopy (Shen et al, 2014), but this is operator dependant and adds time to the workflow which may threaten clinical utility. These difficulties are illustrated in the first trial investigating personalised therapy in PDAC, the IMPACT study (Chantrill et al, 2015). The plan was that patients would be randomised between standard chemotherapy or personalised chemotherapy based on 4 sub-groups of actionable mutations. However, a pilot study although identifying some patients, only served to emphasise the difficulties: indicating that poor quality,

inaccessible, untimely, heterogeneous bio-specimens would make molecular characterisation of the primary tumour to guide therapy impractical in an adequately powered trial.

Intra-tumour heterogeneity

During oncogenesis, genomic instability contributes to the formation of multiple clonal subpopulations with distinct molecular profiles, which can be demonstrated experimentally by NGS from multiple topographical sites within the same primary (Gerlinger et al, 2012). This intra tumour heterogeneity is particularly high in PDAC which, combined with low tumour cellularity, results in the potential for considerable sampling bias undermining personalised therapy efforts (Marusyk et al, 2012). The problem is compounded by the apparent tendency for low frequency sub-clones in the primary PDAC to be enriched in metastatic lesions (Campbell et al, 2010; Yachida et al, 2010), perhaps reflecting the greater metastatic potential of relatively slow growing cancer stem cells (Niess et al, 2014). Clonal diversity is driven by branched tumour evolution, responding to selective pressure from the local microenvironment and potentially by chemotherapy (Burrell et al, 2013; Greaves & Maley, 2012). Studies in

breast cancer have revealed that metastatic sites can acquire HER2 mutations even when the primary tumour is HER2 negative, which has obvious treatment implications (Curtit et al, 2013; Rossi et al, 2015; Santinelli et al, 2008). These findings highlight the inadequacy of directing therapy based on a single primary or even metastatic tumour sample: serial sampling, which can track clonal diversity as it develops is required. Liquid biopsy has recently emerged as a potential successor to the standard tumour biopsy and has the potential to overcome many of the issues described above.

Liquid biopsy

Circulating free DNA (cfDNA) and circulating tumour cells (CTCs) obtained from blood have the potential to molecularly characterise the tumour to meet the aims described in the previous section.

Circulating free DNA

A number of studies have described the use of cfDNA to screen for cancer (Bianchi et al, 2015; Heitzer et al, 2015; Szpechcinski et al, 2015), recurrence (Bratman et al, 2015; Olsson et al, 2015; Stremitzer et al, 2015) and response (Cappelletti et al, 2015; Gong et al, 2015; Mok et al, 2015; Neal et al, 2015; Schwarzenbach, 2015; Tabernero et al, 2015). Plasma contains approximately 1µg/ml of free

DNA (Bagul et al, 2006), most comes from leukocytes and endothelial cells, but in cancer patients the levels can rise by as much as 10 fold; even more during chemo and radio therapy (Holdenrieder et al, 2001). Some of this increase may be due to release of DNA from lysed apoptotic or necrotic tumour cells but the largest proportion results from active secretion from macrophages; work in mouse models suggests that this cancer induced increase includes nucleosomes that have not come from cancer cells (Pisetsky, 2004). This means that in order to detect a specific mutation in circulating DNA a highly robust technique is required that can cope with vast excess of wild type sequences. For example, Garcia-Murillas *et al* used mutation specific digital PCR to identify what they referred to as minimal residual disease in patients with breast cancer (Garcia-Murillas et al, 2015). This could only work if a specific mutant allele was known. In this work the specific mutations were first identified in primary tumours using conventional NGS. Douillard *et al* demonstrated that cfDNA is as effective a biospecimen as primary tumour in determining the presence of a specific EGFR mutation when used in the setting of selecting patients for a targeted therapy trial (Douillard et al, 2014). In both instances *a priori* knowledge of the specific mutation is

required, and thus cfDNA cannot be used with current methodology to uncover the molecular heterogeneity of PDAC in real time.

Circulating tumour cells

CTCs are cells shed from the primary tumour and found circulating in the vasculature, a sub-population of which may be capable of seeding distant metastasis (Gupta & Massague, 2006). Because of the methods used for detection, CTCs have come to be defined as cells isolated from blood with an intact nucleus, which stain positive for cytokeratin, epithelial cell adhesion molecule (EpCAM) and are negative for CD45 (Andreopoulou et al, 2012). The most widely used and only FDA approved technology for identification of CTCs is CellSearch™ (Veridex). Using the definition above and Cellsearch technology, CTCs are very rare (1-10 CTCs/ml blood). It has been over 10 years since this system first demonstrated that enumeration of CTCs, despite their reported scarcity, has prognostic significance in metastatic breast cancer (Cristofanilli et al, 2004) and this has since been confirmed in many other cancer types. More recently, CTCs have been evaluated as a means of directing personalised chemotherapy by

overcoming the issue of genetic discordance between primary and secondary molecular profile (Rossi et al, 2015; Santinelli et al, 2008). The DETECT study for example, aimed to determine whether treatment intervention guided by the HER2 status of CTCs in HER negative metastatic breast cancer patients (determined by primary tumour assessment) is superior to physician assessment (Schramm et al, 2016)

The benefits of CTC analysis in targeting PDAC seem intuitive given all the obstacles precluding solid tumour tissue as a bio-specimen. With its propensity for early haematogenous metastasis CTCs should be more abundant and identification easier in PDAC than in other cancer types. Unfortunately, it has proven more difficult to identify CTCs in PDAC than in other cancer types, indeed there seems to be an inverse relationship between five-year survival and EpCAM-based CTC recovery rates is seen (Figure 1). A low number of CTCs identified in patients even with locally advanced or metastatic disease has been consistently explained by the assumption that CTCs must be exceptionally rare in PDAC (Cristofanilli et al, 2005; Nagrath et al, 2007). A more plausible explanation is that they are exceptionally abundant, just not identifiable

with current Ep-CAM based methods (Gorges et al, 2012).

This paradox that less CTCs are observed in a more metastatic tumour type may be explained by the process of epithelial mesenchymal transition (EMT): a process that could make tumour cells more likely to metastasise while simultaneously making them less likely to be detected. EMT plays an essential role in physiological processes such as embryology and tissue repair but also pathological ones such as fibrosis (Kalluri & Neilson, 2003) and cancer progression (Thiery et al, 2009). In cancer, polarised epithelial cells adhered to the basement membrane and must transit through a number of biological changes to assume a mesenchymal phenotype before they can invade the vasculature (Kalluri & Weinberg, 2009). In the process cells shed their epithelial antigens including EpCAM and cytokeratin (CK) and acquire mesenchymal markers such as COL5A2, EGFR, MSN, PDGFRB and Twist. A degree of phenotypic plasticity has been observed whereby cells may transition between epithelial and mesenchymal state with CTCs existing in both forms (Armstrong et al, 2011). The process has been implicated in the rapid formation of primary tumours (Castellanos et

al, 2013; Mani et al, 2008), metastasis (Tsuji et al, 2009; von Burstin et al, 2009), acquisition of therapeutic resistance (Arumugam et al, 2009) and poor survival (Yamada et al, 2013) associated with PDAC. The most widely used CTC enrichment systems including CellSearch™, Adna Test (Demel et al, 2004), Magnetic Activated Cell Sorting System (MACS®) (Griwatz et al, 1995) and microfluidic technologies (Nagrath et al, 2007) all require cell surface expression of EpCAM for CTC capture and will therefore miss mesenchymal sub-populations of CTCs which are known to be responsible for the aggressive characteristics of the tumour. This is supported by evidence suggesting a purely mesenchymal phenotype predominate in the metastatic stages of cancer (Wu et al, 2015).

Protein and DNA based methods have been proposed to extend the utility of CTC analysis to mesenchymal tumour cells. Gorges *et al*, have described the use of new mesenchymal cell surface markers which select for different mesenchymal sub-populations of CTCs (Gorges et al, 2012). This approach is currently being pursued by Adna (Kasimir-Bauer et al, 2012), Cellsearch (Kasimir-Bauer et al, 2012) and CanPatrol CTC (Wu et al, 2015). The use of surface markers requires a

priori selection of the markers or markers and will therefore be vulnerable to missing CTC sub-populations in a heterogeneous population: significant genetic disparity between CTCs, so called ‘micro heterogeneity’, has been demonstrated in cancer patients (Polzer et al, 2014). Another approach is to use the genomic signature of the CTCs, independent of cell surface markings, to deliver an unbiased analysis of all CTC sub-populations. This approach offers the possibility to detect any type of cell in a lineage based on founder mutations. However, it is limited by the contamination of vast numbers of wild type leucocytes (~7 x10⁶/mL blood) (SB, 1996) which drown out the mutant signal from CTC derived DNA. Several negative depletion strategies, which remove leucocytes, thereby enriching the CTC populations are available to overcome this (Lustberg et al, 2012). Developments in NGS now permit sequencing to a much greater depth and PCR errors have been reduced to a level that now permit identification of mutant signal amongst considerable contaminating wildtype DNA.

Next generation sequencing

As discussed previously, multiple actionable mutations have been identified at low frequency in the PDAC genome (Biankin et

al, 2012; Nones et al, 2014; Waddell et al, 2015). Sequencing approaches therefore will need to interrogate multiple candidate genes to provide a meaningful attempt at personalised therapy encompassing all of the targetable oncogenes and genetic biomarkers. Oncology consortiums have recently developed custom gene panels using multiplex PCR combined with amplicon-based NGS from as little as 10ng of DNA derived from FFPE (Tops et al, 2015). The composition of the gene panels reflects both the frequency of mutated genes and oncogenes with potentially actionable mutations. Emphasis remains on validating diagnostic tests across multiple clinical laboratories (Dijkstra et al, 2015; Tops et al, 2015), allowing in-house downstream bioinformatic analysis within the budget and turnaround time required by clinical oncologists. For trials such as the NCI MATCH trial, larger custom panels including up to 200 genes are used on easily accessible platforms (such as the Ion Torrent PGM). Though these panels are designed for sequencing of the primary tumour, application to alternative biospecimens such as enriched CTCs should be considered in PDAC where access to primary tumour is limited and may not properly reflect the clinically important metastatic deposits. In this way treatment can

be adapted to the changing cancer burden as shown in Figure 2: The initial treatment based on the primary tumour, possibly requiring a combination of therapies targeting dominant cancer cell populations; a modified treatment on relapse with further modification as resistant cancer cell populations are selected.

Conclusion

Improved 5-year survival rates in many cancer types has resulted from a deeper understanding of cancer genomics, pharmaceutical development of targeted therapies and a personalised approach to therapy; these improvements have not been realised in PDAC. Recent insights into the PDAC genome not only explain these failures but point to new approaches to tackle them. Advances in treatment are limited by the weakest link in the chain. The genetic revolution is driving a paradigm shift from histological classification of PDAC according to organ, to considering PDAC as a disparate groups of rare diseases, and finally to truly personalized medicine on an individual basis. The pharmaceutical industry has duly kept pace manufacturing targeted treatments soon after molecular targets are identified. NGS developments have also kept pace and are now capable of identifying all molecular targets on a population level. Improvements

in PDAC survival will come from addressing the weakest links in the chain: treatment evaluation and biospecimen acquisition.

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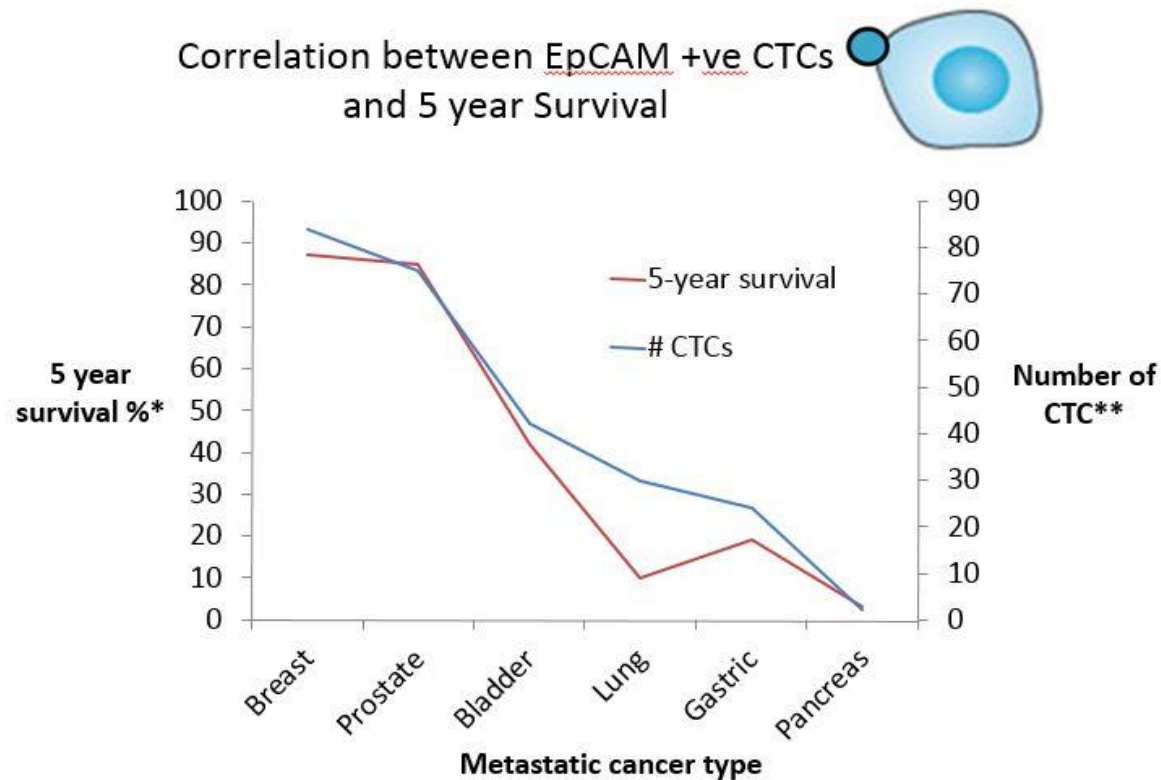
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Figure Legends

Figure 1: More aggressive cancer types (pancreatic, gastric and lung) have fewer EpCAM positive CTCs than less aggressive forms of the disease (prostate and breast).



*Quaresma M et al. *Lancet* 2014

**Allard et al. *Clinic Can Res* 2009 – *CellSearch EpCAM*

Figure 2: Next generation sequencing (NGS) of tumour DNA allows combinations of chemotherapy to be selected which target the dominant forms of cancer cell. During treatment resistant populations of cancer cells will be selected and so will become dominant:

NGS will have to be repeated to identify the optimum therapy at each stage of the disease.

