Mouse xenograft models for elucidating drug resistance mechanisms

Divergence of treatment responses to chemotherapy exists across patients (often with unknown underlying mechanisms), with some patients exhibiting worsened outcome upon treatment. Genomic approaches (such as microarray profiling and whole genome sequencing) hold promise for transforming cancer treatment, particularly, in tailoring drug regimen to specific patients. Nevertheless, formulating effective personalized treatment via surveying the mutational landscape remains difficult, due to current deficiencies in predicting drug sensitivity from genotype. Xenografts, both indirect (via cell line) and direct (from primary tumours), are good physiologic models of cancers. Their utility in informing cancer treatment, however, is constrained by high cost of generating and maintaining genetically modified animals, and the paucity of tissue samples from biopsies. Advent of high throughput biomolecular profiling tools finally enable reading out the expansive molecular fingerprints that encode observed phenotypes in xenografts. Using pheochromocytoma (an adrenal medulla cancer) as example, this short essay provides a broad overview of the scientific and clinical possibilities offered by xenograft models for understanding resistance mechanisms to particular chemotherapeutic regimens, and upon identification of the putative mutations, confirms their functional roles as either oncogenes or tumour suppressors. Additionally, workflow involved in generating a predictive platform, based on non-invasive blood biomarkers, for informing drug treatment options is discussed. Known as an integrated genomic classifier, combination of physiological response of direct xenografts to drug treatment and bioinformatics enabled correlation of blood biomarkers to observed phenotype at cellular and animal levels, and provides the biological basis for predicting patients' prognosis without invasive biopsy of solid tumours. Elucidation of drug resistance mechanisms entails: (i) recapitulating *in vivo* tumour behaviour using cell lines derived from primary tumour; (ii) identification of aetiological

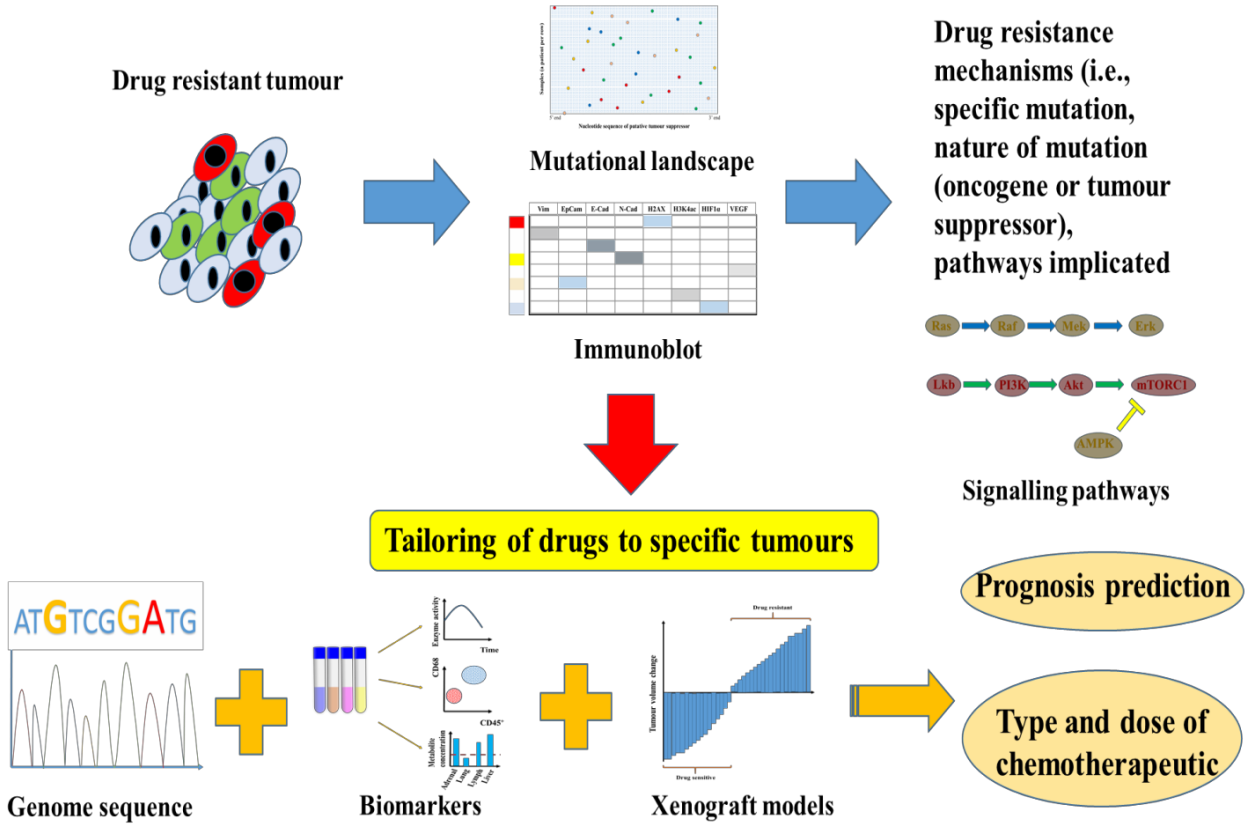
mutations and longitudinal profiling of phenotypic response; and (iii) validation of mutations and phenotype via both knockout mice and direct allogenic xenografts. Biological models seek to recapitulate human physiology at specific levels of abstraction for answering designated questions, but incongruence in phenotype is inevitable. Nevertheless, xenografts (especially those derived from patients, PDTX), are powerful tools for addressing basic science, clinical and treatment related questions using close functional replicas of patient physiology in an animal model. Residual incompatibility between model and patient response would require the expertise and clinical experience of oncologists for fine tuning model suggested drug regimen to particular patients.

Mouse xenograft models for elucidating resistance mechanisms to cancer therapeutics

Wenfa Ng

Novena, Singapore, Email: ngwenfa771@hotmail.com

Graphical Abstract



Short description

What drugs do we prescribe to patients? Would there be drug resistance? What are the markers available for predicting prognosis and tailoring chemotherapy regimen? Grafting of primary tumours onto mouse models (xenografts) affords physiological mimics of humans, and facilitate the elucidation of drug resistance mechanisms and evaluating efficacy of specific drug treatments. Wealth of information captured in mouse genotype and physiology can be readily profiled using whole-genome sequencing, molecular cytogenetics, and a suite of high throughput assays interrogating the proteome, transcriptome, epigenome and metabolome; thereby, allowing the reconstruction of the mutations and molecular effectors important to reactivation of pathways previously sensitive to drugs. Beyond the case-specific formulation of treatment plan, the larger goal of translational cancer research lies in generating a holistic model combining drug efficacy data from xenografts with blood biomarkers-based genotyping and phenotyping for predicting prognosis and formulating chemotherapy regimen.

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Abstract

Divergence of treatment responses to chemotherapy exists across patients (often with unknown underlying mechanisms), with some patients exhibiting worsened outcome upon treatment. Genomic approaches (such as microarray profiling and whole genome sequencing) hold promise for transforming cancer treatment, particularly, in tailoring drug regimen to specific patients. Nevertheless, formulating effective personalized treatment via surveying the mutational landscape remains difficult, due to current deficiencies in predicting drug sensitivity from genotype. Xenografts, both indirect (via cell line) and direct (from primary tumours), are good physiologic models of cancers. Their utility in informing cancer treatment, however, is constrained by high cost of generating and maintaining genetically modified animals, and the paucity of tissue samples from biopsies. Advent of high throughput biomolecular profiling tools finally enable reading out the expansive molecular fingerprints that encode observed phenotypes in xenografts. Using pheochromocytoma (an adrenal medulla cancer) as example, this short essay provides a broad overview of the scientific and clinical possibilities offered by xenograft models for understanding resistance mechanisms to particular chemotherapeutic regimens, and upon identification of the putative mutations, confirms their functional roles as either oncogenes or tumour suppressors. Additionally, workflow involved in generating a predictive platform, based on non-invasive blood biomarkers, for informing drug treatment options is discussed. Known as an integrated genomic classifier, combination of physiological response of direct xenografts to drug treatment and bioinformatics enabled correlation of blood biomarkers to observed phenotype at cellular and animal levels, and provides the biological basis for predicting patients' prognosis without invasive biopsy of solid tumours. Elucidation of drug resistance mechanisms entails: (i) recapitulating *in vivo* tumour behaviour using cell lines derived from primary tumour; (ii) identification of aetiological mutations and longitudinal profiling of phenotypic response; and (iii) validation of mutations and phenotype via both knockout mice and direct allogenic xenografts. Biological models seek to recapitulate human physiology at specific levels of abstraction for answering designated questions, but incongruence in phenotype is inevitable. Nevertheless, xenografts (especially those derived from patients, PDTX), are powerful tools for addressing basic science, clinical and treatment related questions using close functional replicas of patient physiology in an animal model. Residual incompatibility between model and patient response would require the expertise and clinical experience of oncologists for fine tuning model suggested drug regimen to particular patients.

Keywords: drug resistance; direct xenograft; indirect xenograft; next generation sequencing; transgenic mice; tumour suppressor; loss of function; non-invasive biomarkers; biopsy; solid tumour;

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Introduction

Tumour heterogeneity and differing physiologies of individual patients meant that huge variation exists in patients' response to chemotherapy. Such a wide spectrum of responses suggests cancer treatment should be personalized; in fact, the individualized nature of chemotherapy in both drug combination and dosage meant that personalized medicine is mainstream in cancer therapy. Cognizant of the phenomenon of oncogene addiction, where a specific oncogenic signaling or metabolic pathway is dominant (amongst a variety of other mutations) in a cancer's phenotype, modern chemotherapy is combinatorial in nature, where a main drug is paired with other therapeutics such as DNA damage agents to achieve cytotoxicity and, more important, reduce incidence of drug resistance. Nevertheless, the specific targeting of highly proliferative cells by most chemotherapeutics, and the attendant severe side-effects meant that formulation of chemotherapeutic combination requires a fine balance between treatment response and patient's tolerance to side effects.

Pheochromocytoma (PCC), a neuroendocrine tumour of the adrenal gland medulla [1], is characterized by excessive secretion of catecholamine, norepinephrine, and epinephrine, which results in hypertension. Hypervascularization is a key feature of this cancer; thus, anti-angiogenic agents such as VEGF inhibitors are used in chemotherapy [1]. To date, no suitable human PCC cell line is available, and rat PC12, a well characterized polyclonal cell line, serves as a model system [1]. Although most patients respond well to treatment, a subpopulation of patients exhibit worsened prognosis with treatment and they eventually succumb to the disease. While poor response to specific combination therapy is not uncommon, and significant effort has been expended on understanding the molecular mechanisms underlying exceptionally good responders [2], less understood is the exacerbation of patients' condition upon treatment. Drug resistance could arise, for example, from the reactivation of pathways (previously sensitive to inhibitory effects of therapeutics) through upregulation of specific signaling molecules or acquisition of new mutations in oncogenes [3]. Amplification of causative mutation is also a possibility, and overwhelms maximum tolerable doses of chemotherapeutics. Additionally, intravital imaging using a two fluorophore biosensor also reveals that stroma density in the tumour microenvironment is an important factor potentiating drug resistance [4]. From the treatment perspective, tumour cells are known to develop resistance to both small molecule inhibitors and antibodies. But, human FcγRIIB (CD32B) antibodies have been demonstrated recently to afford both anti-tumour activity and is less prone to inducing resistance [5]; thus, raising the intriguing possibility of developing a new class of antibody-based anti-resistance chemotherapeutic agents. Nevertheless, development of resistance to small molecule inhibitors and antibody remains significant barriers to achieving greater treatment success in cancer therapy.

In essence, the clinical problem described belongs to the broader unsolved problem of tailoring chemotherapeutics to specific tumour types at the patient level. Multiple approaches have been developed to this end, the most prominent of which is the profiling of tumour mutational landscape via next-generation sequencing (NGS) for prognostic indications and formulation of chemotherapeutic regimens; in essence, the matching of oncogenic signature to chemotherapeutic combinations [6].

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Nevertheless, poor correlation between genotype and phenotype, and lack of concordance between mutational profile and drug response, meant that the goal of predicting chemotherapeutic regimen (drugs and dosage) from tumour genotype remains elusive. Cell lines and animal models are workhorses of cancer research and have informed the multifaceted biology of various cancers. Poor correspondence between *in vivo* conditions and physiology, however, significantly constrains their utility. Recent developments on using implantable microdevices for testing drug efficacy *in vivo* [7], if shown to be reproducible and effective across larger cohorts, offers a promising path forward for personalized chemotherapeutic treatment to patients, and holds important implications for increasing survival rates especially in relapsed cancers recalcitrant to first-line treatments. On a parallel front, platform technology is also available for multiplex evaluation of drugs' efficacy in xenograft models of lymphoma [8], and raises the possibility of expediting screening of multiple drug combinations at different doses for tailoring chemotherapeutic treatment to patients.

Herein, an approach is described for determining drug resistance mechanisms via a combination of primary tumour sample, indirect and direct xenografts, as well as stable and conditional knockout mouse, with readout provided by a plethora of genomic, biochemical, and cellular assays. In the approach, surveys of the mutational landscape and chromosomal instability by pyrosequencing and molecular cytogenetics tools, respectively, is followed by detailed characterization of the proteome, transcriptome and metabolome using high throughput molecular approaches. Identification of putative mutations and affected pathways in primary tumour cells and indirect xenografts afford creation of knockout mouse for functional validation of the mutations' roles in tumourigenesis at the organismal level. Concomitantly, direct xenografts derived from primary tumour serve as reference for assessing the clonal nature of tumour cell population, and sensitivity (or lack thereof) to drug treatment it was previously resistant to. Thus, cell lines derived from primary tumour are useful for identifying the signaling and metabolic pathways and, more importantly, mutations and their corresponding molecular effectors mediating drug resistance; knowledge of which would be translated to functional study in transgenic mouse models, with direct xenograft of primary tumour serving as an observational reference. On the other hand, treatment naive tumours from other patients could be used in generating direct xenograft models useful for providing a physiological reference.

Two mechanisms, in general, underpin carcinogenesis: oncogenic gain of function, and tumour suppressors' (TS) loss of function. Gene amplification and elevated expression of receptors are prototypical examples of oncogenic gain of function, which can be detected by direct quantification of gene copy number and protein expression. On the other hand, mutations in TS manifest as loss of function, which are more difficult to discern through phenotyping since homozygous deletion of twin TS alleles would result in embryonic lethality. Hence, detecting TS presence would require either indirect physiological assays or direct gene sequencing. Specifically, in contrast to oncogenes, TS does not exhibit conserved mutational signature at the nucleotide level. Functional expression assays for TS activity typically require the generation of either stable or conditional knockouts, where the latter is particularly useful in cases such as homozygous loss of function of the putative TS, which results in severe physiological stress that hamper experiment readout and reduce assay accuracy.

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Finally, the use of mouse xenografts for obtaining treatment response data in support of predictive models that correlates non-invasive blood biomarkers with prognostic indicators for specific chemotherapy regimen is discussed. Overall, the utility of indirect and direct xenograft models lies in providing a retrospective understanding of mechanisms underlying poor response to specific chemotherapeutic combination, and they are also useful for prospectively validating predictive models of patients' prognosis and likely response to specific drug treatment. These are discussed from the translational perspective.

Indirect and direct xenografts

Much of cancer research revolves around the use of cell lines and animal models (especially mice, given its physiological resemblance to humans). While we have learnt how to treat and cure cancer in mice, poor survival rates in humans, particularly those with regional and distant metastasis, continues to motivate scientists and clinicians in understanding disease mechanisms and formulating innovative and effective treatment modalities. Physiologically, the gap between animal and human physiology cannot be bridged, but certainly could be narrowed. To this end, xenograft models, whether from cell lines or primary tumour samples, are used as human mimics for studying tumour biology and informing cancer treatment; especially from the perspective of predicting prognosis and efficacy of specific drug combination. With the growth of tumours from patient's primary tissue samples, xenograft models exhibit closer physiological resemblance to human cancers compared to cell lines or transgenic animals, especially in recapitulating the full cascade of events in tumour invasion and metastasis [9].

Within xenografts, direct xenografts or patient derived tumour xenografts, PDTX (i.e., from patient's tissue sample without first creating a cell line) [10] would be better models of human cancer relative to indirect xenografts obtained from cell lines, which was derived from primary cancer cells. Specifically, indirect xenografts are known to be poor predictors of efficacy of new therapeutics, probably due to long-term selection for growth in cell culture rather than in host environment, and the lack of peri-tumoural stroma important for inducing tumourigenesis and metastasis [9,11,12]. Additionally, evolutionary divergence after 10-14 passages, and emergence of heterogeneity during long-term culture [13] negates any genotypic and functional similarity that a cell line had with its primary tumour; a problem ameliorated by using primary tumour sample in direct xenografts. Global gene expression patterns of primary tissue derived cell lines are also significantly different from primary tumours [11]. Nevertheless, generation of direct xenografts is limited by the availability of patient's tumour sample, which is divided, for example, between immunohistochemistry staining for specific proliferation, migration and invasion markers, and genome sequencing for identifying global mutational landscape. Additionally, there is the need of biobanking tissue for future analysis. Hence, a combination of logistic and cost factors accounts for indirect xenografts predominance over the more physiologically relevant PDTX in cancer research. Better maintenance of original tissue architecture (with supporting stroma) and histological characteristics, and the close resemblance of gene expression and pathway activity with primary tumour are key advantages of PDTX [11].

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Beyond understanding tumour response to drugs at the patient level, PDTX is also used in establishing and characterizing stem cell compartments in cancer [11].

Besides tumour sample, circulating tumour cells (CTC) and disseminated tumour cells (DTC) are other potential cell sources with intriguing scientific possibilities [14]. Specifically, released from the primary or secondary tumour, CTCs are highly heterogeneous and represents a clinically relevant model for studying self-seeding metastasis [14,15]; a pertinent scientific question given documented metastasis of PCC to lymph nodes and lung, which are the main causes of death for PCC patients [1]. Relevance of CTCs for studying metastasis largely accrue to the high concordance of mutations in CTCs with those in primary and secondary tumours [16]. Additionally, CTCs are also useful prognostic markers of metastatic breast cancer [16,17]. But, clinical utility of CTCs is beset by the difficulty of isolating viable CTCs, since many CTCs are apoptotic or dead [17]. One approach for circumventing the problem involves isolating the proteins secreted by CTC or DTC, combined with negative enrichment (leukocyte depletion) [17].

Xenografts could be implemented in a variety of animal models. Specifically, zebrafish is an emerging and increasingly important animal model for various cancers [18-20]; however, it is better suited for forward genetic studies, which seeks the genotype associated with a phenotype [20]. Mouse, in contrast, are more suited for reverse genetics studies identifying the phenotype of a specific genotype. Given mouse's high genotypic and physiological resemblance to humans, the following discussion will focus on mouse xenograft models for studying many facets of a solid tumour's biology.

Cell preparation and choice of immunodeficient mice

Assuming sufficient tumour sample is available, and execution of a well planned sample extraction, preservation and delivery protocol, the tissue sample is first washed in serum-free antibiotic medium, followed by fragmentation into smaller sample size [9]. Depending on the quantity and quality of tissue available, the subcutaneous heterotopic or orthotopic implantation route could be selected [11]. Typically, the orthotopic route requires more tissue given the inevitable loss of cells during multiple centrifugation and filtration steps in cell preparation; but it would be of higher functional and physiological relevance. Additionally, accessibility of host tissue and organ, and desire of reducing animal pain during surgical procedures are other factors hampering more wide spread adoption of the orthotopic route. Choosing the orthotopic route for PCC would lead to significant tissue morbidity given the externalization of the adrenal gland, to which the prepared cell suspension would be injected. Another important consideration in generating xenograft models lies in judicious selection of types of immune deficient mice (i.e., T-cell deficient athymic nude, T and B-cell deficient non-obese diabetic (NOD)/severe combined immunodeficiency (SCID) mice, and T, B and natural killer cells deficient NOD/SCID IL-$2^{-/-}$ [9]. In addition to the F_0 generation, PDTX could also be maintained in immunodeficient mice by passaging in successive mouse generations [9]. Similar to transplantation, the extent of graft

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"take" is a critical factor underlying xenograft success. Typically, engraftment requires between 2 and 4 months [11], and is dependent on tumour size, squamous histology, and tissue differentiation state [11].

Molecular profiling tools

Growth of tumour in mouse would translate into observable physiological impacts, both at the molecular and tissue/organ level. Although animal care and ethical requirements prevents tumour growth beyond 1.5 cm, important understanding of tumour biology could still be derived from a range of histochemical, proteomic, transcriptomic, metabolomic, cytogenetic, and genomic profiling of both tumour and "within limits" normal tissue samples in a longitudinal study design [2]. All assays have their limitations, but when appropriately sequenced and data analyzed holistically, complementary techniques yield a coherent picture of the underlying aetiology and mechanistic basis of a cancer. To probe the mechanism underlying observed drug resistance, broad survey approaches such as molecular cytogenetics [21-23], whole genome sequencing (WGS), and a haematoxylin and eosin (H&E) stain reveal the mutational landscape and pathology of the cancer. Besides direct analysis using tumour samples, tissue microarrays could also be created for expediting experimentation [11].

Probing mechanisms of drug resistance

Probing drug resistance mechanism(s) would require creation of cell lines from primary tissue (indirect xenografts), even though they do not closely mimic human physiology (Fig. 1). Specifically, indirect xenografts are useful for elucidating dysregulated pathways in tumourigenesis, while avoiding double dosing of drugs on treated tumours. In essence, the approach outlined below uses indirect xenografts for identifying specific pathways and mutations pertinent to emergence of drug resistance. Useful information could be gleaned from cell line and animal models of an indirect xenograft. Specifically, potential non concordance in mutations and pathways could be assessed from genome sequencing and functional assays conducted at the cellular and animal level, which informs how hierarchical regulation across biological organization impacts tumour biology. Subsequently, knowledge gained would be utilized in creating mouse knockout models of the identified mutations, which after mutagenesis and tumour development, would be useful for observing the phenotypic consequences of the altered genotype during drug treatment. PDTX of untreated patients' tumours would serve as allogenic controls, even though they do not exactly match patients' physiology. Patients' bone marrow could be transplanted in mouse knockout models and PDTX for providing the stroma environment known to be important for mediating tumour progression [24], and which potentiates metastatic processes. Recent research also highlights important roles of tumour stroma in potentiating drug tolerance [24]; for example, by providing a "safe haven" against anti-cancer drugs' onslaught [4,25]. High concordance at the phenotypic, genotypic and biochemical levels across primary tumour, indirect xenograft, and mouse knockouts, would lend credence to the putative roles of identified mutations in mediating drug resistance. Genomic, molecular, and biochemical characterization methods (i.e., histochemical, proteomic, transcriptomic, metabolomic, cytogenetic, and genomic profiling) outlined below would be generic across different levels of experimental interrogation.

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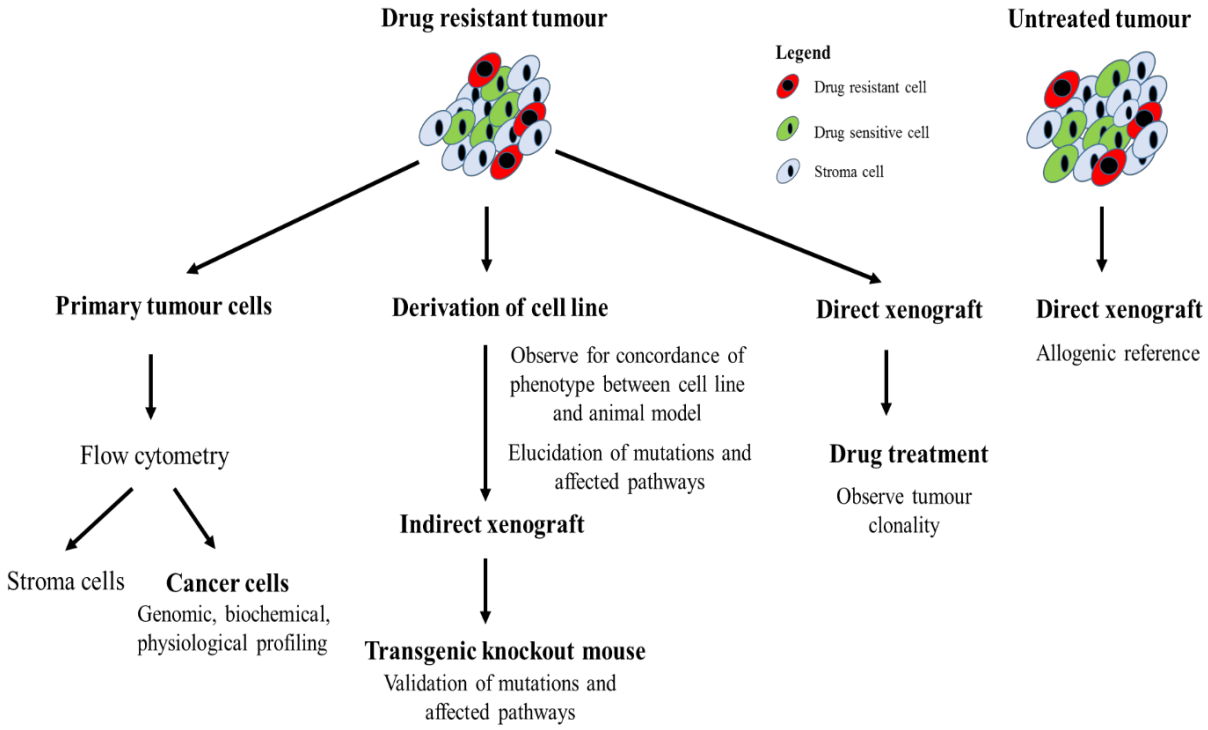

Figure 1. Schematic diagram illustrating the workflow for using a combination of primary tumour samples, indirect and direct xenografts in probing the mechanistic basis (mutations and pathways) of resistance to chemotherapeutic treatment. Molecular profiling tools useful for this endeavour include a variety of genomic and biochemical techniques, while physiological assays afford phenotypic responses at cellular and whole organism levels. The approach should be generic across all solid tumour types.

Useful for direct observation of chromosomal alterations in cancer in an era where genome sequencing was not available or in its infancy, karyotyping and its modern incarnation, molecular cytogenetics, affords a holistic view of chromosomal and genetic instability at the cellular level [21-23]. Specifically, by staining different chromosomal regions with probes conjugated to different fluorophores, multi-colour fluorescent *in situ* hybridization (mFISH) reveals the location and identity of chromosome rearrangements such as inversions, insertions, deletions, and translocations (symmetric and asymmetric). Regions of the chromosome such as centromere and telomere, with high density of repetitive sequence (not readily amenable to readout by pyrosequencing) can be examined by different FISH techniques (quantitative or flow FISH) using centromeric and telomeric probes, respectively. Comparative genome hybridization (CGH) that implements FISH in a microarray format is another important molecular cytogenetics approach [11,26-28]. In the case of telomere, a terminal restriction fragment length analysis via

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pulse-field gel electrophoresis (i.e., teloblot) would also help answer simple but important questions on the role of telomere length in tumourigenesis. Altogether, aneuploidy as a hallmark of cancer can be readily assessed via a variety of molecular cytogenetic tools, which visualizes diverse complicated (and enigmatic) chromosome alterations through hybridization of fluorophore tagged probes to specific motifs of interest [27].

At the opposite length scale, whole genome (WGS) or exome sequencing help reveal mutations important in a patient's aetiology, and, in aggregate, enables the correlation of cancer phenotypes with mutational profile [2]. In particular, next-generation sequencing affords the reliable detection of mutational frequency down to 0.01% [29]. Trend in cancer genomics is certainly towards WGS (predominantly next generation sequencing but single molecule real-time sequencing (SMRT) is expected to become increasingly important). But high cost [30] meant that more restricted sequencing of protein coding genes remains relevant. Sequencing provides two crucial pieces of information: what are the genes mutated and where (i.e., promoter, gene body, untranslated region etc.). Knowledge of the mutated genes (and their frequency) affords a broad understanding of the pathways implicated. In contrast, identification of the specific nucleotide altered provides a granular understanding of the disease mechanism; for example, mutations in the ATP binding pocket of a receptor tyrosine kinase would alter enzyme function. WGS, in particular, offers an unbiased view of the swathe of mutations, at both the gene and epigenetic level, in a specific tumour sample, and vast information on potential molecular targets for downstream functional analysis. The same information, at the global level, could also inform interactions (such as oncogenic collaborations) between different mutations in cancer progression. Mutational landscape [6] profiled also illuminates driver mutations [31] (occurring at high frequency across a cohort) thought to potentiate tumourigenesis [32]; although the relative importance of driver mutations in cancer initiation remains controversial. In contrast, passenger mutations either do not participate in tumourigenesis or only serve as ancillary factors in cancer progression. Nevertheless, recent studies have raised interesting possibilities on the therapeutic potential of targeting passenger mutations given the existence of gene duplication (and partial redundancy), where the remaining allele (or protein isoform) facilitates increased sensitivity of cells to specific drugs [33]. From a clinical standpoint, knowing the source of the point mutation could better match chemotherapeutics to tumour characteristics since drug resistance are mediated by specific mutations; for example, by inducing structural changes in oncoproteins that prevent binding of inhibitors [3]. Conversely, if a particular mutation targeted by a drug is altered [34], the tumour would also be resistant to treatment. Being a technology driven field, advent of SMRT third generation sequencing [35,36] could transform cancer research. Specifically, SMRT overcomes many technical challenges such as the difficulty of sequencing repetitive regions (a significant bugbear of pyrosequencing). More important, SMRT is able to profile the histone modifications and DNA methylation states known to repress or activate transcription. Given the importance of transcriptional dysregulation in tumourigenesis, and how epigenetics help connects environmental factors to carcinogenesis, epigenome sequencing is likely to become increasingly important in understanding how inflammation and diet affect tumour initiation and progression. Besides SMRT and next generation sequencing, RNA-seq is another important tool in the cancer biologist's armamentarium for understanding how epigenetic changes affect tumour behaviour and, by extension, resistance to chemotherapeutic treatment.

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Though WGS appears to be more informatics than experiment, careful sample preparation is critical for obtaining reproducible results. Specifically, given the propensity of DNA and RNA to degrade upon exposure to air, nucleic acids extraction from tumour samples need to be performed expeditiously. Additionally, important caveats exist in interpreting sequence results from short-read sequencing. For example, inability of detecting low frequency mutations is a significant problem of WGS, but improvement in sensitivity and read annotation should ameliorate the problem. Another concern is WGS's higher probability of generating false positives due to a variety of sample preparation issues [37]; which necessitate the validation of mutational data through functional studies. Finally, caution is required in deciphering signals from baseline noise in sequence data, where myriad mutations (with no phenotypic consequences) constitute background heterogeneity. In particular, a P-value of 10^{-7} denotes convincing evidence, and 10^{-8} is associated with genome-wide significance, respectively. [38]. Surprisingly, noise levels could be as high as P-value of 10^{-4}, which in other fields would be associated with high significance; but not in genome science, where the burden of proof is much higher.

Histochemical staining is commonly used to visualize the presence of specific markers important in cancer progression or metastatic initiation. For example, staining for the proliferation panel (e.g., proliferating cell nuclear antigen, PCNA, and Ki-67) [39] informs cell proliferation capacity even though the biomarkers' prognostic relevance is questionable since most cancers are not hyperproliferating. Other panels, however, may offer a better view of cellular- and pathway-dependent characteristics of specific tumours; in particular, the migration and invasion panel featuring molecular markers such as EpCAM, vimentim (Vim), β-catenin, matrix metalloprotease (MMP), claudin-7, α-catenin, fibronectin, and E- and N-cadherin informs the metastatic potential of cells via measuring the extent of epithelial to mesenchymal transition (EMT) with an EMT score. Staining for angiogenesis markers such as VEGFa, integrin β1, cathepsin B, proteinase-activated receptor 1, MMP1, FGF and HIF-1α, on the other hand, provides a broad overview of microvessel density and hypoxia within the tumour microenvironment [11]. Probing the tumour differentiation state via staining for Sox2, Nanog, and Klf-4 is also important since more differentiated tumours are associated with better prognosis. Finally, semi-quantitation of levels of specific cell surface receptors (e.g., epithelial growth factor receptor, EGFR) provides correlative clues implicating specific pathways to tumour progression.

Tissue staining probes cellular phenotype through biomolecule-specific labelling. Biochemical assays, in contrast, quantify the relative abundance of different biomolecules at both global and pathway levels. For example, transcriptome profiling via RNA-seq determines key molecular players involved and their relative abundance [40]. In particular, RNA-seq in combination with WGS identifies microRNA (miRNA) implicated in tumourigenesis, where a wealth of reports have conclusively demonstrated the importance of miRNA in mediating various processes important to carcinogenesis such as initial oncogenesis, induction of migration and invasion etc. as either oncogenes or TS [41-44]. Similarly, various proteomics and metabolomics approaches quantify proteins/peptides and metabolites, respectively, at the global level, leading to better understanding of the role of oncoproteins and onco-metabolites in tumourigenesis.

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Meta-analysis of large omics datasets reveals interactions between different classes of biomolecules, and help identifies nodes where signaling and metabolic pathways are modulated, and thus, suitable for serving as druggable targets. Specifically, observations that mutations frequently cluster into one or more pathways led to the notion that cancer is a disease of pathways; however, network patterns and pathway logic are not complete biochemical explanations of outlier response to therapy, but serve as indicators of the exploratory path forward [2]. Pathway function and roles of post-translational modifications in mediating enzyme function can only be revealed through conventional assays such as Northern and Western blots that examine specific RNA or protein-of-interest in specific pathways. In particular, many chemotherapeutic drugs exert their effects via inhibiting cellular processes that, collectively, trigger apoptosis. To this end, cleaved caspase-3 and Annexin V are critical molecular markers indicating apoptosis induction [1]. Additionally, a variety of cell viability and migration assays (e.g., CellTiter Go, transwell migration, cell cycle progression, and monolayer gap closing) could be used in assessing cellular physiology at different stages of tumour progression. *In vivo* functional short hairpin RNA genomic screens [11], and profiling of circulating tumour DNA (ctDNA), released as fragments from necrotic and apoptotic tumour cells, are emerging tools for monitoring therapy resistance [16]. All aforementioned assays could be carried out for both primary and secondary tumours at the cellular, tissue and organismal levels; thus, enabling the reconstruction of a more comprehensive picture of PCC tumour biology and metastatic potential. Collectively, moving from broad surveys to fine-grained analysis, a variety of molecular and genomic tools enables the progressive elucidation of the molecular determinants of drug resistance or poor response in specific patients. Overall, xenograft models have earned their place as complements to transgenic mouse in studying tumour biology and patients' response to drug treatment [11].

Detecting and confirming the role of tumour suppressors

Observed variation in patient response to chemotherapeutic treatment is, in essence, a natural experiment (i.e., molecular epidemiology), that when examined with precision tools such as deep sequencing, helps illuminate the underlying molecular mechanisms. Mutations in tumour suppressor (TS) result in loss of function, which can arise through nonsense mutations, out of frame insertions or deletions, and splice-site changes [45]. Single or few base pair resolution, common in next generation sequencing (NGS), affords its use in determining aforementioned specific nucleotide alteration in tumours. Specifically, the statistical nature of short read NGS data informs the relative abundance of specific nucleotide altered; thereby, allowing the discrimination of oncogenes and TS mutations. Oncogenic mutations are typified by one or two high frequency nucleotide changes in specific positions (e.g., G12V in HRAS or V600E in BRAF). The mutational landscape of TS, however, is non-specific and random; thus, preventing the association of an altered nucleotide to a mutated gene. According to the allelic two hit hypothesis, presence of homozygous deletion within a mutated region suggests possible presence

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of a TS, whose identity can be confirmed by site-specific mutagenesis and expression analyses (Fig. 2) [45]. Another scenario of a two hit mutation would involve the deletion of 1 allele, followed by mutation of the remaining allele [46]. More specifically, annotation of a TS requires understanding: (i) the impact of deleting a segment of the coding region; (ii) effect of a non-silent sequence alteration; and (iii) expression analysis probing for functional effects of observed mutations [45]. Nevertheless, almost all tumours are heterogeneous; thus, sequencing samples from different regions of the same tumour or, metastatic tumours at different body sites introduces an additional caveat where clonal evolution and mutation capture may potentiate different subpopulations of cells [47], some of which without the putative mutation. Identification of a mutation is not sufficient for confirming TS activity of the wild-type allele without functional analysis. While cell lines do provide phenotypic readouts (such as morphology and changes in metabolite levels) suggestive of a putative TS, PDTX models offer extra informational layers such as organismal level physiology. Transfection of a plasmid with the wild-type TS gene under the control of an inducible expression system (such as TET-On or TET-Off) into tumour cells in a “rescue” experiment would reveal the phenotypic consequences of TS loss of function – and, in turn, biological roles of wild-type TS. Cross-talks between endogenous regulons and heterologous genes, however, meant that most rescue experiments only partially recapitulate the wild-type phenotype [41]. Hence, knockout of the wild-type TS gene in mouse, and phenotypic comparison with PDTX xenografts would provide more confidence in functional assignment of TS activity. Considering the difficulty, time and effort of generating stable knockout mouse models, the alternative of using RNA interference (RNAi), antagomir (anti-sense miRNA binding agent) or morpholino antisense RNA for inducing conditional knockout would be a viable approach. Nevertheless, advent and progressive maturation of CRISPR-Cas precision genome editing technology [48-50] should, in the near future, lower the entry barrier and expense associated with creating stable knockout (and knock-in) mouse models. Cre-loxP, on the other hand, is a more mature technology for effecting stable knockout of specific genes in cells and animals [51,52].

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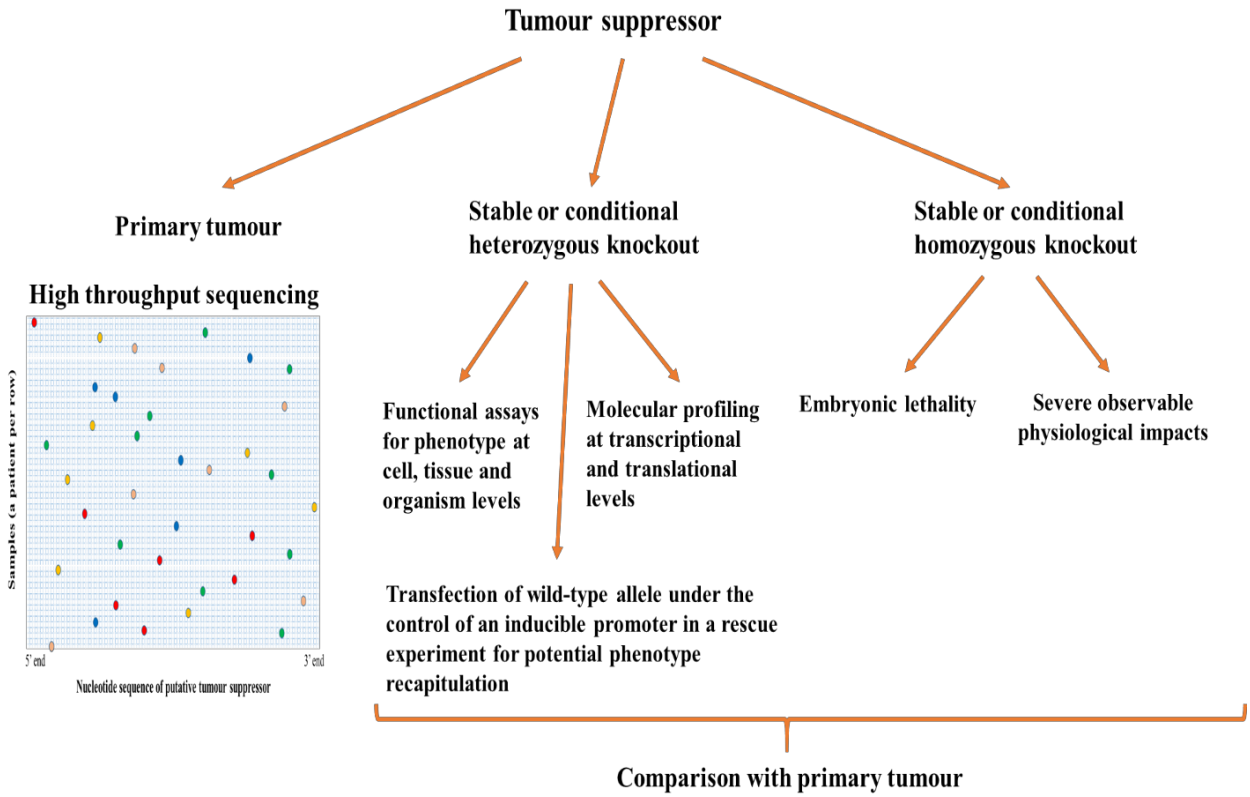

Figure 2. Intrinsic loss of function of tumour suppressor (TS) mutations complicates its identification and assignment of functional roles relative to oncogenic gain of function. Although lack of conserved signature in mutational profiles across patients is suggestive of a putative TS, functional characterization at the molecular and phenotypic levels are *de rigueur* for TS confirmation. Crucially, embryonic lethality of homozygous deletion of many TS (and lack of phenotypic data), meant that creation of stable or conditional heterozygous animal knockouts is a commonly used approach for phenotypic validation of a TS gene.

Moving towards predictive models correlating blood biomarkers to cancer prognosis and treatment

While the multitude of molecular assays available provide ready means for detailed characterization of various aspects of a cancer, we must not lose sight that developing a human mimetic model for informing cancer treatment is the primary driver of xenograft research. Xenografts serve as individualized functional replicas of human subjects in testing the efficacy of particular chemotherapeutic regimen. Although xenograft models could be created for each patient, the overall goal would be to generate a predictive model of PCC for assigning appropriate combination therapy based on minimally invasive assessment (e.g., blood biomarkers) of patients. Other efforts in this direction include using the artificial intelligence-based supercomputer, Watson, for informing cancer treatment decisions given genome sequence data. Hence, moving from biomarker measurement to prescription of targeted therapy

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necessitates the creation of a holistic model that integrates several types of information that spans different levels of biological complexity. In essence, model building (Fig. 3) requires: (i) experimental data of PDTX mouse's sensitivity to a drug combination; (ii) discovery of biomarkers via bioinformatics, with drug sensitivity and resistance data as training and validation tools; (iii) validation of putative biomarkers via preclinical models; and (iv) testing the utility and relevance of identified biomarkers in Phase I and II clinical trials. Specifically, a couple of mouse xenografts (usually 5) would be used as training set in examining, *in vivo*, the efficacy of a specific drug combination relative to control mice. Tumour volume would be an important measurable outcome enabling the assessment of therapeutic efficacy. Nevertheless, clustering of T cells and attendant tumour expansion in immunotherapy has shown that tumour volume alone is not a definitive prognostic yardstick. A "waterfall" plot of tumour volume change of individual PDTX mouse would help segregate the drug resistant and drug sensitive subjects. In parallel, bioinformatics analysis could identify possible biomarkers that correlates observed phenotype with mutational landscape obtained from deep sequencing, which when combined with experiment data from PDTX models (in validation tests) would allow the generation of an integrated genomic classifier [11]. Identifying biomarkers with high correlation to specific disease state is the current trend in clinical diagnostics and translational medicine; however, poor correspondence of biomarkers (validated in a subset of patients) with observed clinical presentation at the population level, meant that individual biomarker is only useful for particular circumstances [30]. While the integrated genomic model serves to facilitate the prescription of suitable drug regimens through analytical readout of a few non-invasive biomarkers, the inability of using a biomarker set for fully capturing the wide spectrum of physiology in a pleiotropic disease such as cancer, meant that the experience and expertise of the clinical oncologist remains important to patient treatment and care.

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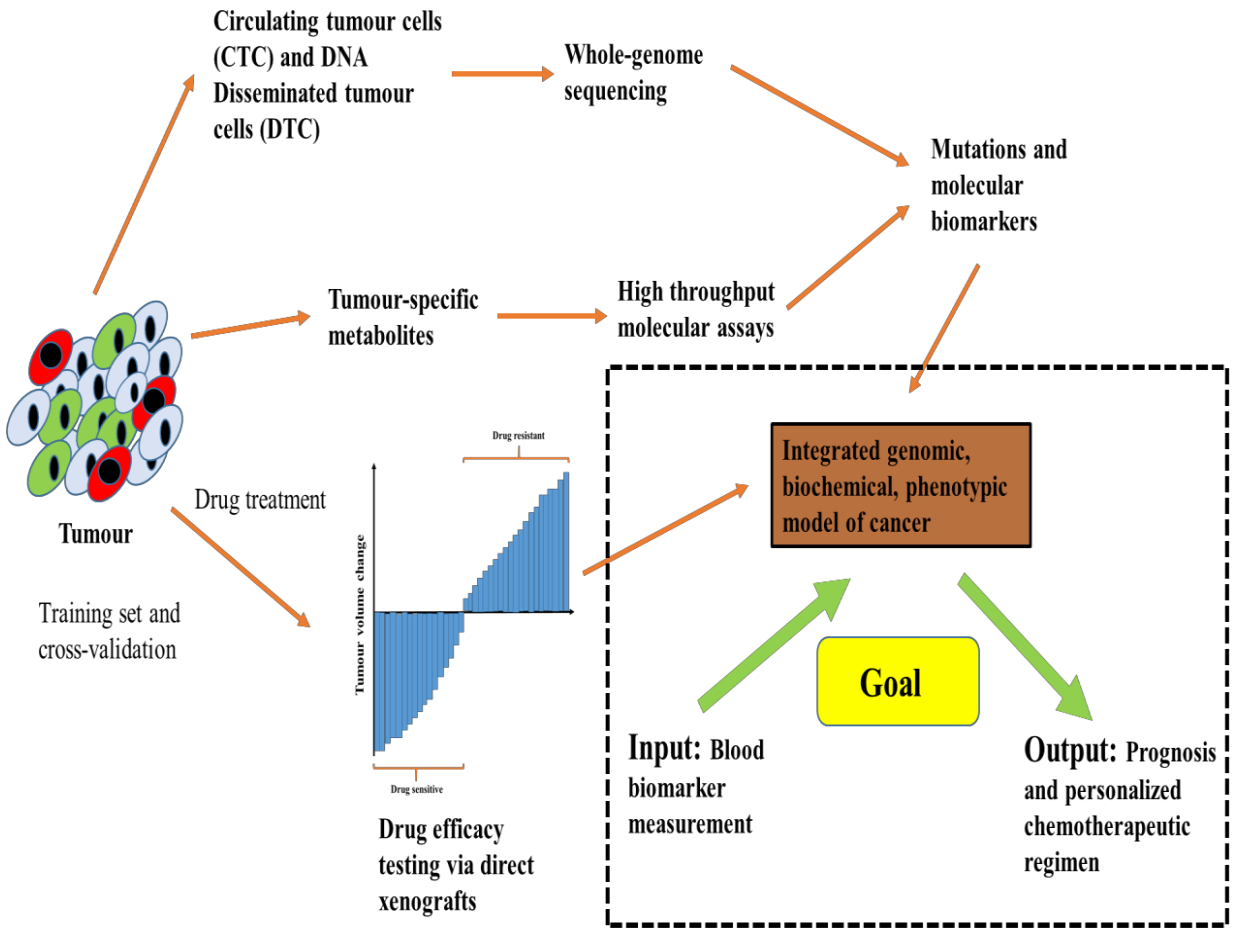

Figure 3. Tumours release a variety of cells such as circulating and disseminated tumour cells (CTC and DTC), circulating tumour DNA (ctDNA), and tumour specific metabolites. Profiling the genetic changes in CTCs and DTCs, and molecular characterization of proteome and metabolome, together with drug sensitivity and phenotype data from direct xenografts enable the creation of a predictive model integrated across genomic, biochemical and physiological space. Such a model would, with progressive refinement via better biomarkers and measurement techniques, move cancer clinical diagnostics and treatment a step closer to the holy grail of predicting patient prognosis and drug treatment strategies from measurement of blood biomarkers (i.e., cells and metabolites). Nevertheless, inevitable inadequacies of all models meant that the art of patient care and treatment rests firmly in the hands of the physician, whose clinical experience and expertise informs judgement necessary for tackling outlier cases.

Prospects

For a long time, tumour xenografts is at the periphery of cancer research. Specifically, high cost presents significant barriers to adoption, even though PDTX models afford close functional resemblance to patient tumour physiology. Recent advent of a suite of high throughput precision molecular assays spanning the genome, proteome, transcriptome, metabolome and

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even whole cell phenome finally provide the means for interrogating the wealth of physiological data encapsulated in PDTX (and to a lesser extent, indirect xenograft) models. High functional and physiological relevance together with availability of assays for probing treatment response led to increasing use of xenografts for understanding mechanisms responsible for emergence and evolution of drug resistance, and tailoring chemotherapeutic options for individual patients. Problems with double dosing of treated tumours and low incidence of chemotherapy induced deterioration and death meant that PDTX are not suitable autologous experimental models. Nevertheless, primary tumour tissue derived cell line circumvents the above problems and help identify possible mutations and pathways involved in tumourigenesis. Subsequent generation of indirect xenografts from primary tissue derived cell lines provide additional biological informational layers, especially concerning hierarchical regulation of gene expression in cancer. Functional validation of the hypothesized roles of mutations and implicated pathways via stable or conditional knockout transgenic mouse models provide the intellectual *tour de force* necessary for understanding resistance mechanisms, which help suggests potential druggable targets or alternative pathways useful from the therapeutic development and personalization perspectives. Possible tumour suppressor function of identified mutations could also be probed with xenograft models serving as positive controls for comparing phenotypic response from knockout mice. Desire of predicting both prognosis and specific chemotherapeutic combinations at the patient level motivates use of xenograft models for elucidating efficacy of specific drug regimens, which when combined with bioinformatics discovery of non-invasive biomarkers (e.g., from blood), enable the development of an integrated tool for guiding clinical evaluation and treatment decisions. Inevitable incongruence between model and physiological response, however, highlights the continued importance of the clinician in the decision loop.

Conflict of interest

The author declares no conflict of interest.

Author's contributions

Wenfa Ng performed the literature review and wrote the paper. Manuscript was expanded from an assignment question for MDG 5102: Techniques for Biomedical Research.

Funding

No funding was used in this work.

Acknowledgement

The author would like to thank Seah Kwi Shan for help in reviewing and editing the manuscript.

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