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From integrative genomics to therapeutic targets

Rachael Natrajan and **Paul Wilkerson**
Breakthrough Breast Cancer Research Centre, The Institute of Cancer Research, London SW3 6JB, UK

Abstract

Combinatorial approaches that integrate conventional pathology with genomic profiling and functional genomics have begun to enhance our understanding of the genetic basis of breast cancer. These methods have identified key genotypic-phenotypic correlations in different breast cancer subtypes that have led to the discovery of genetic dependencies that drive their behavior. Moreover, this knowledge has been applied to define novel tailored therapies for these groups of cancer patients. With the current emphasis on characterizing the mutational repertoire of breast cancers by next generation sequencing, the question remains as to what constitutes a driver event. By focusing efforts on homogenous subgroups of breast cancer and integrating orthogonal data-types combined with functional approaches, we can begin to unravel the heterogeneity and identify aberrations that can be therapeutically targeted.

Introduction

Traditionally, breast cancers have been characterized into biologically and clinically meaningful subgroups according to histological grade and type (i.e. growth pattern)(1), with the majority of breast cancers being classified by histological exclusion i.e. invasive carcinomas of no special type (IC-NST). On the other hand the remaining tumors can be histologically classified according to their distinctive growth patterns and are termed 'special' histological types. Over the last decade seminal class discovery expression profiling studies have identified a number of molecular subtypes of breast cancer defined at the transcriptomic level that are characterized by distinct histological features, clinical behaviors and responses to therapy(2). Indeed, different breast cancer subtypes (both histological and molecular) harbor distinct patterns of genetic aberrations and are driven by alterations in distinct molecular pathways and networks(3, 4). It is now widely accepted that breast cancer heterogeneity may be underpinned by myriad mechanisms of genetic aberration (e.g. gene amplifications, in-frame fusion genes or mutations and homozygous deletions, disrupting fusions or deleterious mutations causing gene activation or inactivation respectively), and that phenotypic subgroups harbor distinct patterns of genomic aberrations(3, 5). Moreover, targeting these genomic alterations has proven an effective way of developing tailored therapies for subgroups of breast cancers(5-9).

The use of high-throughput technologies has enabled the investigation of biological phenomena and allowed its correlation to specific disease behavior. Research has focused on integrative approaches combining high-throughput genomic data through the use of microarray-based comparative genomic hybridization (aCGH), gene expression profiling and more recently the use of next generation sequencing, to define the genetic underpinning

Corresponding author: Rachael Natrajan PhD, The Breakthrough Breast Cancer Research Centre, The Institute of Cancer Research, 237 Fulham Road, London, UK, SW3 6JB, Tel: +44 2071535546, Fax: +44 2071535533, rachael.natrajan@icr.ac.uk.

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of different subtypes of cancer with the ultimate goal of identifying novel therapeutic targets. However the main challenges for the translation of the genetic alterations identified by massively parallel sequencing into benefit for cancer patients lie in the identification of biologically relevant aberrations among the deluge of sequencing data being produced, which can be used as therapeutic targets or predictive biomarkers.

Exploring genotypic-phenotypic correlations

There is evidence to suggest that at least some subtypes of breast cancer are underpinned by distinct arrays of genomic alterations. In fact some special histological types of breast cancer harbor specific pathognomonic alterations such as the *ETV6–NTRK3* oncogenic fusion gene in secretory carcinomas, the *MYB-NFIB* fusion gene in adenoid cystic carcinomas, and inactivation of E-cadherin through mutation and gene methylation in lobular carcinomas of the breast (for a review on special histological types of breast cancer see(10)). Perhaps the best example in breast cancer is the characterization of *ERBB2* (*HER2*) as the driver of the 17q12 amplification, which has spurred the hunt for additional amplified driver events. We, and others have explored the genotypic–phenotypic correlations of different molecular subgroups of breast cancers through the use of high-throughput genomic analyses using aCGH(3, 5). Through aCGH profiling of a series of 95 high-grade breast cancers, we have shown that distinct patterns of copy number alterations are found in different molecular subtypes(5). These analyses highlighted the genotypic-phenotypic association between specific amplifications and subtypes of breast cancer(3, 4).

Integrating data-types to identify therapeutic targets

By using a combination of aCGH and gene expression profiling, we have shown that canonical pathways involved in estrogen receptor (ER) signaling, proliferation and DNA repair are enriched for genes whose expression is driven by copy number in basal-like, HER2 and luminal tumors(3), suggesting that the diversity of breast cancer and the molecular subtypes may stem, to some degree, from the different patterns of genetic aberrations found in these cancers. Moreover, biological phenomena characteristic of each subtype (e.g. proliferation, HER2 and ER signaling) may be driven by specific patterns of copy number aberrations. This approach has also led to the identification of genes that are consistently overexpressed when amplified, which are considered potential ‘amplicon drivers’. However not all genes within an amplicon are overexpressed, and an amplicon may harbor more than one driver(6). The expression of some driver genes is also more pervasive i.e. are overexpressed by other mechanisms in addition to amplification. That said, such approaches have been successful in identifying novel targets for subgroups of breast cancer, by exploiting the concepts of oncogene addiction. For instance, *FGFR1*, one of the genes mapping to the 8p11-p12 amplicon, is amplified in 10-15% of breast cancers and is associated with ER-positive disease and poor survival(11). FGFR1 is consistently overexpressed in tumors harboring *FGFR1* amplification both of which have been shown to constitute a mechanism of resistance to endocrine therapy(9). A phase II clinical trial is currently testing the efficacy of small molecule FGFR inhibitors for these patients. By performing genome wide correlations between amplifications in different subgroups of breast cancer, we have identified a number of subgroup specific amplifications. This approach, coupled with integrating these data with matched gene expression data led to the identification of *PPM1D* as a putative amplicon driver(5). RNA interference-induced silencing and chemical inhibition of *PPM1D* in a panel of phenotypically matched *PPM1D* amplified and non-amplified cells showed that PPM1D expression and phosphatase activity is selectively required for the survival of cells harboring *PPM1D* gene amplification(5, 12). These data suggest that *PPM1D* may prove a viable therapeutic target for the subset of HER2-positive breast cancers harboring amplification at 17q23.2. Through a similar

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approach, we identified 38 genes that were significantly overexpressed when amplified in a series of 56 triple negative breast cancers, including *FGFR2* amplifications in approximately 4%. Our work demonstrated that cancer cells harboring *FGFR2* amplification are exquisitely sensitive to inhibition of FGFR2 *in vitro* and *in vivo* through the use of RNA interference and treatment with FGFR small molecule inhibitors [8], suggesting that FGFR inhibitors may constitute a tailored therapy approach for a subgroup of triple negative tumors. More recently, we have shown that 5% of ER-negative high-grade breast cancers that harbor amplification of *CCNE1* within the 19q12 amplicon are dependent on CCNE1 and CDK2 kinase activity for their survival. Cancer cells with *CCNE1* gene amplification are sensitive to CDK2 inhibitors, providing a rationale for the testing of these chemical inhibitors in a subgroup of patients with ER-negative grade III breast cancers in the context of clinical trials(6).

As well as using genetic and transcriptomic data to identify potential therapeutic targets in a candidate driven approach, integrating functional profiling data offers an unbiased way of identifying genetic dependencies. This approach has been used to identify additional amplicon drivers in *HER2* amplified tumors by systematically assessing cell viability in a panel of *HER2* amplified cell lines after silencing of all genes that were significantly overexpressed when amplified identified in a cohort of primary *HER2* amplified breast cancers. This approach identified the transcription factor *TFAP2C* as a novel genetic dependency in 5% of *HER2* amplified breast cancer cells(13). Whilst such screening approaches as these can identify novel amplicon drivers, many of the targets identified (e.g. transcription factors) are not directly targetable. By exploiting the concept of synthetic lethality(7), (where loss of either gene is compatible with cell survival, however loss or inhibition of both genes results in cell death), the alterations in the cells' physiology that arise as a consequence of aberrant activation of oncogenes or tumor suppressor gene loss, rather than oncogene/tumor suppressor proteins themselves, are targeted to achieve tumor selectivity. This concept has been successfully applied to identify novel therapeutic targets including PARP inhibitors in *BRCA1/2* mutant patients, further corroborated by the identification of a resistance mechanism to PARP inhibitors(14). High-throughput RNA interference screening of the kinome (i.e. pharmacologically tractable genes), in a panel of commonly used breast cancer cell line models, identified a series of novel genetic dependencies, in basal, luminal and HER2 subgroups(15). This approach also led to the identification of genetic dependencies of cells with specific mutations, e.g. PTEN-null breast tumor cells were found to be dependent on signaling through mitotic checkpoint kinases. Integration of viability data with transcript and protein profiling also identified a correlation between sensitivity to ADCK2 silencing and high ADCK2 mRNA and protein levels in ER-positive cells(15). Such unbiased approaches provide a framework upon which additional dependencies and candidate therapeutic targets may be identified.

The next generation

The advent of next generation sequencing has increased our understanding of the complexity of cancer genomes tremendously and has identified a number of subtype-specific mutations associated with different cancer types. Massively parallel sequencing studies in breast cancer have identified a plethora of novel mutations, including *MAP3K1* mutations in ER-positive cancers(16), and *PARK2* mutations in triple negative disease(17). In addition RNA-sequencing studies have enabled the identification of novel recurrent targetable expressed fusion genes, involving the *MAST* kinase and *NOTCH* gene family members(18). Large-scale sequencing efforts currently being undertaken with consortia such as The International Cancer Genome Consortium (ICGC) and The Cancer Genome Atlas (TCGA), are leading to an unprecedented amount of data. However, the main challenges that lie ahead are for the translation of the genetic alterations identified by next-generation sequencing into benefit for

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cancer patients. These mainly depend on i) the identification of 'driver' mutations and ii) the targeting of 'driver' genetic aberrations. While the identification of 'driver' genetic aberrations so far has been largely based on statistical algorithms(19), the targeting of the 'driver' aberrations has proven difficult. However, the majority of the novel mutations observed in the common types of breast cancer are at relatively low frequency, and the main challenge lies in the distinction of what constitutes a 'driver' mutation event versus a 'passenger' event (i.e., has no biological significance on the cell harboring its mutation at a given point in time)(7).

Traditionally, the identification of driver events stems from the fact that they are recurrent at a significant frequency above the background mutation rate within the tumor cohort studied. We can integrate different sorts of genetic alterations to aid the identification of recurrent activation or tumor suppressive events, such as mutation and homozygous deletions, or gross DNA rearrangements of a tumor suppressor gene, or amplification and activating mutations of an oncogene (Fig. 1). We have used this approach to identify novel candidate cancer genes in *BRCA1* mutant tumors, by integrating a list of mutations identified from whole genome sequencing, with published aCGH data for the presence of homozygous deletions(20). This can also be taken further to look for functional recurrences in the form of genetic alterations in members of the same gene family or members of the same signaling network or pathway. For example mutations in chromatin remodeling genes appear to be a common alteration in many types of solid tumors(19, 21), and identifying ways of targeting these tumors with chromatin remodeling defects is a key challenge that needs to be explored in future studies. There are a number of computational tools that exist to predict the functional effect of a mutation of interest on a protein and to identify pathways that are deregulated in cancer and therefore are likely to contain significant driver genes. Algorithms that identify key transcriptional regulators of oncogenic programs can be used to prioritize mutations for follow-up studies (for a review of these see(22)). Algorithms that predict the pathogenicity of somatic mutations based on the selection pressure and type of mutation have also been developed(19, 23). However, novel predicted 'drivers', still need to be functionally investigated in appropriate model systems before they can be definitively defined as driver events. The recent functional validation of *HER2* mutations in breast carcinomas without *HER2* amplification has highlighted the importance of this step(24). Through the use of functional genomic screens we can begin to identify driver events in a high-throughput manner. This can be achieved a number of ways, from cross-species comparative approaches identifying driver genes as those that are conserved in human and mouse tumors, high-throughput insertional mutagenesis screens through to whole genome shRNA screens(22). Perhaps a more intuitive approach, which aids in the identification of both tumor suppressor genes and oncogenic drivers lies in the generation of cancer genome focused screens by generation of overexpression libraries of mutant open –reading frames (ORF's) and short hairpin RNA's (shRNA's) that target the same set of genes identified by sequencing primary tumor samples. These libraries can then be screened for their ability to transform pre-malignant cells. In addition, wild-type ORF libraries generated from primary tumors without prior knowledge of the mutations may also provide an effective approach for gain of function screens.

As many mutations and fusion genes identified may not be directly targetable, synthetic lethality approaches constitute an alternative for the identification of novel targets. These can be achieved through screening of isogenic cell line models with and without the genomic alteration of interest and/or a panel of heterogeneous cell lines with and without the alteration, with si/shRNA screens of druggable genes and high-throughput small molecule drug screens. Through the use of drug screens using small molecule inhibitors that are already FDA approved, the time needed from target identification to phase II clinical trials is much shorter. In fact there are a plethora of small molecule inhibitors available that have no

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useful predictive biomarker. Identifying these biomarkers through these integrated approaches would ultimately lead to patient benefit more quickly. Concerted efforts within the scientific community are being aimed at addressing these issues, and interrogation of systematic pharmacogenomic screening data for an aberration of interest is becoming a reality(25, 26). In parallel, the growing field of metabolomics is yielding interesting possibilities for classifying tumors based on their metabolic signatures, and in identifying pathways related to drug resistance or toxicity through metabolomic profiling. Furthermore, metabolic dependencies resultant from specific genomic alterations, offer novel therapeutic opportunities. Readers are directed to excellent reviews on the subject(27). In addition, other factors such as the importance of epigenetic mechanisms (including methylation and acetylation)(28) and non-coding RNAs upon gene regulation(29), and the role of the tumor micro-environment need also to be considered(30, 31).

However, not all recurrent mutations and fusion genes are represented by the available breast cancer cell line models, and pathognomonic events underlying some types of breast cancer can only be studied in the context of forced expression models, making the use of synthetic lethal approaches limited. Such models may not recapitulate the network state space of primary tumors harboring the genetic aberration of interest. These caveats must be born in mind when interpreting pre-clinical functional validation data. That said, there are a number of common genetic aberrations which are not directly targetable (e.g. *TP53* and *KRAS* mutations, and PTEN loss of function), where adequate models are in abundance. This provides an opportunity to leverage the power of synthetic lethal screens in multiple isogenic models, thereby providing some control for the context-dependent nature of many genetic dependencies. Furthermore, by subjecting samples with and without the mutation of interest to deep sequencing one might identify a pattern of co-mutation (e.g. are there a set of genes frequently mutated in *TP53*-mutant triple negative breast cancers but not in *TP53* wild type cancers), which could be modeled *in vitro* through synthetic lethal screens to interrogate potential cooperative interactions. Systems biology approaches would likely prove invaluable in these strategies.

Finally, next generation sequencing studies have highlighted the scope(32) and important role of intra-tumor genetic heterogeneity in cancer evolution and emergence of drug resistance(33). High depth multi-region sequencing and single cell sequencing can be used to characterise the repertoire somatic variants or patterns of copy number changes in non-modal clones within a tumor. Of course, not all these mutations will be biologically relevant. Integration of these data with pathway analysis tools and on-line resources such as the Connectivity Map(34) which identifies connections between drugs, disease and genes, aids prioritization of mutations and subsequent compound library screening, using chemical libraries of drugs currently in clinical trials. This approach would identify which mutations confer resistance to which drug; the ideal scenario would then be to analyze pre- and post-treatment samples from neoadjuvant trials to confirm the role of these non-modal clones in the evolution of drug resistance. Focused high depth sequencing could be effectively employed as a screening strategy to exclude patients from treatment with agents they are likely to develop resistance to, or early relapse after.

Summary

Integration of multiple data-types is becoming increasingly useful for the identification of therapeutic targets, within different subtypes of breast cancer. With the advent of next generation sequencing technologies and the vast amounts of data being generated, it is possible to identify recurrent mutational patterns within breast cancer. However, given the relatively low frequency of novel mutations and fusion genes in breast cancer and to fully understand the biology and therapeutic responses of some patients, the clonal genotypes of

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the individual tumors will need to be determined. It is evident that these large-scale sequencing projects need to be integrated with functional screens to achieve the goal of developing novel therapeutic strategies. For functional screening to be useful in identifying key driver events, researchers need to account for the fact that many gene alterations will be context dependent; either through epistatic interactions, or dependence on a particular developmental stage of the tumor. It will be necessary to develop more complex models to assess interactions in a more network-driven approach. The goal of individualized patient management will be a step closer with the inception of clinical trials designed to perform genome-wide or targeted sequencing of cancers to identify targetable aberrations and to determine the mechanisms of resistance to specific therapeutic agents.

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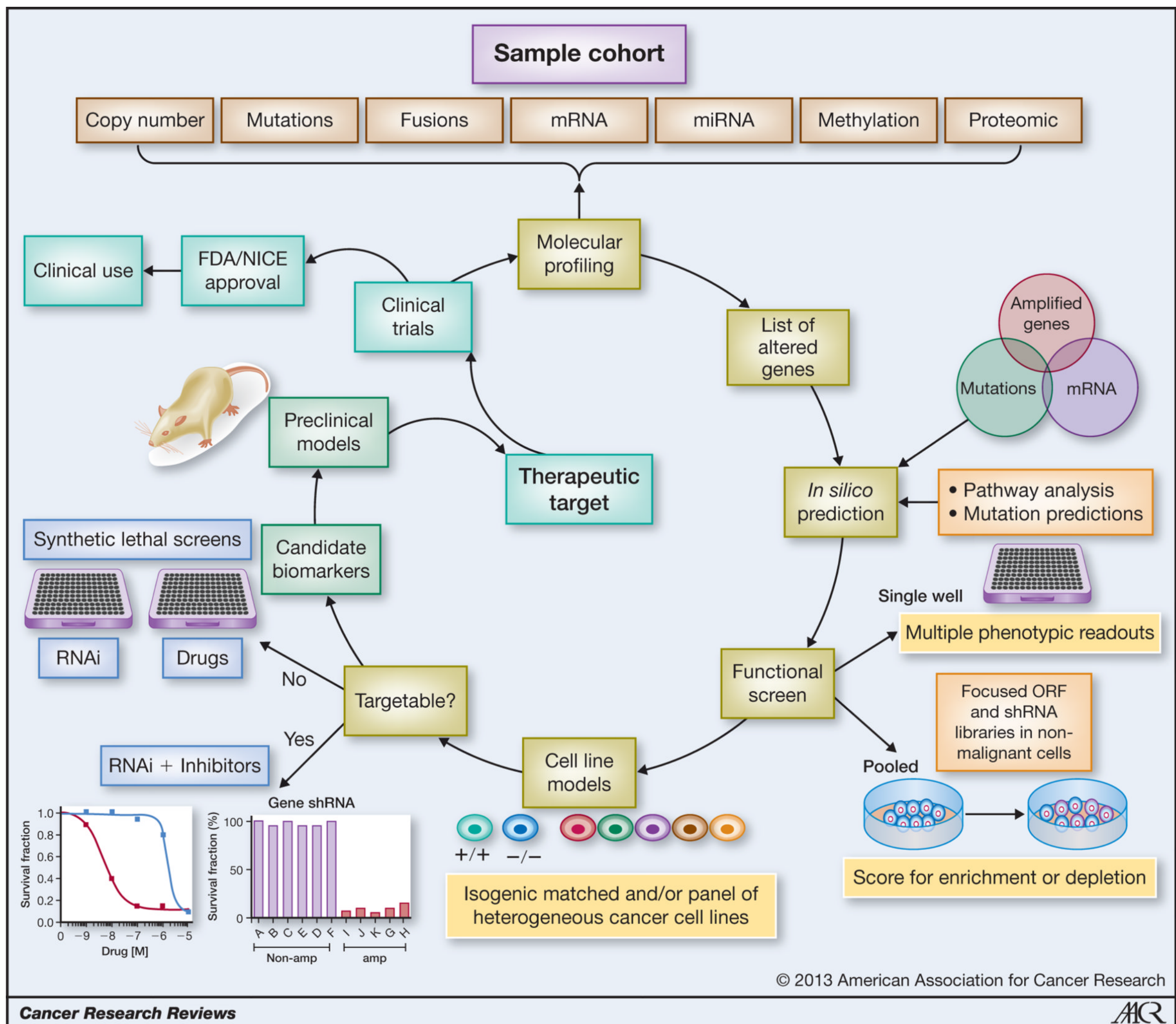

FIGURE 1. Schematic of data integration to identify therapeutic targets

Molecular profiling of a cohort of primary breast cancers allows the identification of genomic alterations. Integration of this data is useful to identify potential oncogenic and tumor suppressive events *in silico*; e.g. genes that are overexpressed when amplified, in-frame fusion genes where the 3′ partner is overexpressed, mutations that are expressed at the mRNA level; genes that are under-expressed when deleted or methylated, disrupting fusions/structural rearrangements or mutations resulting in under-expression respectively. Additional *in silico* analyses can be performed to identify potential candidate driver genes by using prediction algorithms that ascribe biological meaning to genomic data. For example searching for significantly altered pathways that are more likely to contain driver genes, prediction of key transcriptional regulators of oncogenic programs and prediction of which missense mutations are likely to have a biological effect on the protein. Construction of cancer-focused screens can be a useful tool to investigate which candidate driver genes confer tumor specific dependencies. Oncogenic drivers and tumor suppressors are simultaneously assessed by constructing parallel libraries of cDNA ORF's and shRNA's, which are expressed in pre-malignant cells, and subsequently assayed for tumorigenicity either *in vitro* through the use of 3D models or *in vivo*. In addition, other measures of phenotypic alterations can also be assayed in tandem e.g. invasion, migration and resistance to anoikis. Both pooled screening using next generation sequencing for deconvolution and

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identification of biologically active ORF's and shRNA's, or single well screening can be used. To take forward hits from these screens, the use of appropriate cancer cell line models constitutes a more translationally relevant platform for drug discovery and development. Either a panel of phenotypically matched breast cancer cell lines (ER, PR, HER2, TP53) with and without the aberration of interest are used, or an isogenic cell pair to investigate the selectivity of the genomic alteration of interest. Genes that are directly targetable with validated inhibitors (e.g. kinases) can then be taken forward for further evaluation in the cell line panel e.g. for oncogenic events assessment with RNAi and available inhibitors, can be used to assess tumor dependency (cells with the aberration will be sensitive to gene inhibition, whereas those without will not). Aberrations that are not directly targetable with available inhibitors can be assessed through synthetic lethal screens using siRNA druggable libraries and drug screens. Candidate dependencies can be subsequently validated in pre-clinical models before evaluation in clinical trials. By identifying the genetic alteration and then identifying ways of targeting it, allows the genomic biomarker to be established *a priori*, cutting down the time to identify biomarkers of sensitivity during the drug development process. As is sometimes the case, promising preclinical data do not readily transfer to positive outcomes from early clinical trials. While inter- and intra-tumor genetic heterogeneity almost certainly play a role in resistance to targeted therapies, other molecular mechanisms can be teased out using the same approach described above. We can change the cohort of samples interrogated with molecular profiling to identify biomarkers for resistance or sensitivity to the targeted agent in question (i.e. before or after treatment, responders or non-responders). Identification of targets that are selective to inhibitors already in clinical trials will enhance the time to routine clinical use.