Cancer remains an outstanding cause of global morbidity and mortality, despite intensive research and unprecedented insights into the basic mechanisms of cancer development. A plethora of clinical and experimental evidence suggests that cancers from individual patients are likely to be molecularly heterogeneous in their use of distinct oncogenic pathways and biological programs. Efforts to significantly impact cancer patient outcomes will almost certainly require the development of robust strategies to subdivide such heterogeneous panels of cancers into biologically and clinically homogenous subgroups, for the purposes of personalizing treatment protocols and identifying optimal drug targets. In this review, I describe recent progress in the development of both targeted and genome-wide approaches for the molecular stratification of cancers, drawing examples from both the haematopoietic and solid tumor malignancies.

Key Words: Molecular stratification, cancer genomics, targeted therapies
Progress in the Molecular Stratification of Cancer

The prognosis of these cancers, often associated with overall 5-year survival rates of < 20%, can be ascribed to several reasons. First, there is currently no effective screening methodology to detect these cancers at early disease stages, leading to most patients presenting with advanced stage disease, which is traditionally associated with poor prognosis. Highlighting the importance of early detection, the five-year survival rate of patients in the US with operable early stage gastric cancer is 50%, but decreases to < 10% in individuals with late stage disease. Second, conventional strategies for clinically treating these conditions, usually involving a combination of surgery, radiotherapy and chemotherapy, are far from optimal. In gastric cancer, chemotherapy response rates can range for 20% for single agent therapies to 40% for combination therapies, and even if short-term responses are observed, most tumors will eventually develop drug resistance and relapse. The shortcomings of existing therapies for these conditions has understandably fueled intense interest in the use of so-called “targeted therapies”, which are antibody-based or small-molecule drugs designed to selectively inhibit particular oncogenes and oncogenic pathways, such as HER2, EGFR, and the pro-angiogenic VEGF pathways. Results from recent clinical trials evaluating these drugs are starting to confirm that they can indeed convey a significant, albeit modest, increase in overall survival and lifespan when administered in combination with conventional therapy. Third, as we now argue in this review, another likely reason for the high mortality rate of these cancers is that these tumors are in reality highly heterogeneous, frequently differing between individual patients in their fundamental biology and reliance on specific cellular pathways. We will present a case for why considering cancer heterogeneity is likely to be important for improving patient outcomes in these diseases, and describe a variety of approaches currently being used to dissect cancer heterogeneity and subclassify cancers at the molecular level. For clarity, it is also important to note that for this Review, the term “cancer heterogeneity” or “tumor heterogeneity” will be used to refer to variations in tumors between individual patients, and not to the populations of different cell types within a given tumor (e.g., cancer cells, stromal cells, infiltrating immune cells, etc).

The Confounding Reality of Cancer Heterogeneity

An ample body of research over the past decade supports the notion that many cancers are likely to display significant variability at the clinical, histopathologic, and molecular level between individuals. For example, in non small cell lung cancer (NSCLC) clinical trials evaluating the EGFR inhibitor gefitinib, clinical responses were observed only in a small proportion of patients. The existence of such ‘high-responder’ patient subpopulations provides compelling evidence of clinical heterogeneity in NSCLC. In terms of histopathology, tumors can also exhibit significant differences in histologic architecture, tumor microenvironment, tumor grade and differentiation status. In stomach cancer, it is well known that tumors can be subdivided into at least two distinct histological types - an intestinal variant where cancer cells develop as tubular structures exhibiting features of well-differentiated glandular tissue, and a diffuse subtype where tumor cells are poorly differentiated and spread along the lining of the stomach, sometimes resulting in the classical ‘linnitis plastica’ phenotype. Such phenotypic variability logically suggests that different biological programs are likely to be acting in individual tumors, even when they are derived from the same target organ. Reinforcing this notion, recent comprehensive genomic analyses of lung, breast, and brain tumors have confirmed that individual tumors can frequently exhibit heterogeneous patterns of somatic mutations, gene amplifications and deletions, epigenetic profiles, and gene expression portraits.

Molecular Approaches for Cancer Stratification

Given this remarkable heterogeneity however it is perhaps surprising that with some notable exceptions (discussed below) such patterns of variability are usually not considered in the specific management of cancer patients. Even today, most clinical trials involving many of the major solid cancers are still performed on unselected patient populations without prior stratification. However, the ongoing identification of specific genes and proteins playing important functional roles in cancer development has recently led to advancements in the use of molecularly-targeted assays to stratify and classify tumors. One good example of a protein currently used for molecular stratification in breast cancer is the estrogen receptor (ER). Commonly measured by immunohistochemistry, overexpression of ER in breast tumors has been associated with improved patient prognosis and less aggressive cancers. Perhaps more importantly, ER expression is also used as a predictive biomarker for treatment of breast cancer patients with anti-hormonal therapies such as tamoxifen or aromatase inhibitors. At the genetic level, several assays are now available to test for the presence of specific oncogenic mutations in cancers. For example, in the afore-mentioned lung cancer gefitinib trials, subsequent genetic analysis
revealed that patients exhibiting good responses to gefitinib all exhibited tumor somatic mutations in the tyrosine kinase domain of the EGFR gene.\textsuperscript{32,33} Besides gene mutation, oncogene dysregulation can also occur through the processes of genomic amplification and deletion. Such aberrations, commonly detected either by techniques such as comparative genomic hybridization (CGH or alternatively by higher resolution array-CGH) or fluorescence in-situ hybridization (FISH), can involve a wide range of sizes ranging from whole chromosomes and chromosomal arms, to specific genes. Examples of chromosomal aberrations with clinical significance include losses of chromosome 18q in colon cancer, commonly associated with poor prognosis,\textsuperscript{30} and gain of chromosome 8q in pancreatic cancer.\textsuperscript{31} At the single-gene level, high-level amplifications of the HER2 gene have been reported in 30% of breast tumors, identifying a subtype of patients with particularly poor prognosis.\textsuperscript{32,33} Furthermore, with the development of anti-HER2 therapies such as trastuzamab (Herceptin), HER2 gene amplification is also currently used as a predictive marker for anti-HER therapy.\textsuperscript{34,35}

Besides conventional molecular platforms that typically target only one or two genes/proteins at a time, newer genomic approaches are also being evaluated with the capability of stratifying cancers at the level of multiple biomarkers. For example, gene expression profiles, where the expression level of every single gene is measured simultaneously, have been used to identify subclasses of breast cancer.\textsuperscript{34} An important finding enabled by this technology has been the discovery that the expression profiles of distinct breast cancer “molecular subtypes” (e.g. ER positive and ER negative) differ not simply in their expression of single genes like ER, but are in fact strikingly different from one another at the level of hundreds if not thousands of differentially expressed mRNA transcripts.\textsuperscript{36} The molecular distinctiveness of these subtypes is consistent with proposals that these subtypes are indeed distinct biological and clinical entities. This finding, which has been replicated in several centers across the world including ours, is just one example of how using genomewide information may provide greater accuracy and insights than relying on single biomarkers alone.\textsuperscript{35,36} More recently, several groups have published reports describing how global gene expression profiles can be computationally deconvoluted to provide information regarding the activation levels of different oncogenic pathways in tumors.\textsuperscript{37,38} For example, by identifying gene expression signatures associated with the activation of various oncogenic pathways \textit{in vitro}, Bild et al., were able to predict the oncogenic status of these pathways in a variety of solid tumors such as breast, lung and ovarian cancers.\textsuperscript{38} Excitingly, interpreting gene expression profiles in terms of pathway activation patterns allows such information to be readily linked to the selection of specific targeted therapies designed to inhibit these pathways. Another advantage of genomic assays is that they allow multiple pathways to be interrogated without requiring multiple tests to be performed, as would be the case in immunohistochemistry. Such “high-throughput” pathway profiling could reveal previously unanticipated interactions between different pathways, and even suggest possible strategies for combining different pathway inhibitors. Besides gene expression profiling, the introduction of newer deep-sequencing technologies may also permit individual tumors to be characterized at single-nucleotide resolution resolution, which may provide further insights into the repertoire of genetic aberrations and structural variations in solid cancers.\textsuperscript{39,40}

A CLINICAL ARGUMENT FOR TUMOR STRATIFICATION

While the availability of these molecular platforms renders the ability to classify cancers technically feasible (i.e. “we can do it”), it is perhaps worthwhile at this juncture to formally consider why the practice of tumor stratification, whereby heterogenous cancers are subdivided into clinically and biologically homogenous groups, might be useful for the improvement of cancer patient outcomes (i.e. “we should do it”). We believe that there are several reasons why this might positively impact the clinical management of cancer patients. First, the ability to stratify patients, at the point of diagnosis, into either good or poor prognosis categories might allow clinicians to personalize standard therapeutic regimens for individual patients through dose-escalation or dose-reduction. In the former (dose-escalation), additional risks associated with the poor prognosis status of the patient might justify the enhanced possibility of adverse side effects caused by intensified therapy, while in the latter (dose-reduction), a priori knowledge that the patient may not require additional therapy might allow clinicians to scale down treatments, thereby minimizing toxic side effects, yet ultimately preserving the same level of clinical benefit. A good example of how such prognostic biomarkers might prove useful can be seen in the management of patients with early stage breast cancer. Retrospective analyses has established that a certain proportion of women with early stage breast cancer are likely to be sufficiently treated with surgery only, requiring no further adjuvant therapy.\textsuperscript{34,41} However, without an assay capable of pre-identifying these good prognosis patients, most early stage breast cancer patients are treated with default with adjuvant chemotherapy, and it has been estimated that at
least 70 - 80% of women with early stage breast cancer are likely overtreated. To address this problem, researchers from the US and Netherlands described in 2002 a 70-gene expression signature capable of subclassify such early stage breast cancer patients into good and bad prognosis categories respectively. Since then, the testing of this 70-gene signature has undergone extensive independent validation and is now the subject of a major European clinical trial (MINDACT, see below).

A second reason for the importance of tumor stratification involves the prediction of response to specific therapies (predictive biomarkers). While we have already cited examples where tumors with specific genetic features have been shown to respond to particular therapies, such as EGFR mutations and EGFR inhibitors, or HER2 gene amplifications and trastuzumab, it should be noted that these cases likely represent only the fortuitous minority where the predictive biomarker corresponds to the drug target itself (e.g. trastuzumab and HER2). In many other cases, identification of the relevant predictive biomarker may not be so clear cut. For example, it has been reported that tumors with mutations in the B-RAF gene may impart sensitivity to MEK inhibitors, or that mutations that constitutively activate the RAS oncogene may impart sensitivity to histone deactylating agents. Besides biomarkers that predict drug sensitivity, there also exists a separate class of mutations in particular oncogenes that impart drug resistance. Examples of such “anti-predictive” factors include the lack of efficacy of EGFR inhibitors in colon cancers with k-ras mutations, and resistance to trastuzumab in HER2-amplified breast cancers with concomitant activation of the PI3K pathway. Beyond targeted therapies, several predictive biomarkers for conventional cytotoxic agents have also been proposed, such as mutated β-tubulin for taxanes and TS for 5-FU.

A third important justification for the implementation of tumor stratification is that it maximizes the opportunity for therapeutic benefits to be observed, particularly in the conduct of clinical trials evaluating novel therapies. Analyses by the pharmaceutical industry of current drug trials have revealed that most anti-cancer drugs typically fail during late stage evaluation (i.e. Phase II or III) typically due to lack of detectable efficacy or toxicity. However, because such trials are typically performed in unselected patient populations, it is quite possible that certain drugs may elicit dramatic responses in a small population of patients, but that this beneficial effect may be diluted when such patients are intermixed with the larger population. Indeed, drugs such as hereceptin and gefitinib would likely have not been approved if tested in an unselected set of cancer patients. Thus, examples such as these provide a strong motivation for the practice of tumor stratification in the management and evaluation of cancer patients.

**IMPACT OF CANCER HETEROGENEITY - THE EXAMPLE OF CHILDHOOD ALL**

One example of a major cancer type where patient outcomes have been significantly impacted by molecular stratification has been childhood acute lymphoblastic leukemia (cALL). Prior to 1972, cALL was largely regarded and treated as a “single disease”, with a dismal cure rate of only 5%. Since that time, however, progress in cytogenetic and molecular technologies has revealed that cALL is in fact a heterogeneous collection of distinct diseases. Through detailed comparisons of individual cALLs with their normal counterparts in the hierarchy of hematopoietic development, we now know that distinct subtypes of cALL are likely to originate from blood progenitor cells originally committed to differentiate via a T or B-cell lineage, but in cALL these progenitor cells have acquired mutations causing both deregulated cell proliferation and stage-specific developmental arrest. Currently, cALLs are classified into at least seven distinct subgroups, with each subgroup being either associated with a particular stage of hematopoietic differentiation or a specific cytogenetic aberration such as hyperdiploidy or signature chromosomal translocations including TEL-AML1 (otherwise known at ETV6-RUNX1) or BCR-ABL. Importantly, supporting the notion that these cALL subtypes are likely to represent distinct biological entities, in a large series of hundreds of cALL patients followed up over 15 years, it has been established that each of these subtypes is associated with a distinct course of disease progression. For example, patients having TEL-AML1 or E2A-PBX1 fusions are more likely to exhibit relatively favorable clinical outcomes compared to patients with BCR-ABL or MLL-AF4 fusions. The biological distinctiveness of these subgroups has also been further supported by genome-wide gene expression profiling studies, confirming that the different cALL subtypes are each associated with characteristic patterns of gene expression.

Knowledge regarding the existence of these cALL subtypes has revolutionized treatment for cALL in several ways. First, it permitted clinicians to adapt and modify standard therapy regimens, once used to treat all cALLs, specifically to the different subtypes. For example, while most cALL cases are treated with a three phase regimen of remission-induction, intensification/consolidation, and continuation therapy, patients with mature B-cell ALL are further treated with short-term intensive chemotherapy, while patients with T-Cell ALLs commonly are additionally treated with cyclophosphamide and asparagine during the remission induction phase. Second, knowledge of the
specific molecular aberrations in each subtype allowed drug discovery efforts to focus on these aberrations as potential pharmacologic targets. This is best seen in BCR-ABL positive cALL, a subtype that has traditionally been associated with very poor prognosis.\(^{66}\) However, with the development of imatinib mesilate, a small molecule inhibitor of the ABL kinase, there is now an effective treatment for BCR-ABL positive cALL, with initial response rates being close to 100% in this specific subgroup of cALL.\(^{67}\) Through the tailoring of generic regiments and the availability of subtype specific targeted therapies, the cure rate today for childhood ALL exceeds 80%.

While cALL remains a powerful testament to the utility of tumor stratification, the impressive advances in our ability to manage cALL, however, should not be taken as a sign that this disease has been truly conquered. There is still a significant fraction of cALL patients (20%) who are not cured with existing therapies, and our ability to manage ALL in older patients is still far from optimal.\(^{66}\) One possible reason is that additional genetic and molecular heterogeneity may exist in this disease that has previously been undetected, and that further genetic subdivisions will be required to further differentiate between these various seven subclasses. Fortunately, more comprehensive and high resolution technologies are now available that are capable of identifying genetic and epigenetic aberrations on a genome wide scale. For example, a recent genome wide SNP analysis of ALLs associated with B-cell phenotypes identified several mutations, deletions, and structural rearrangements in various regulators of B-cell development, such as PAX5 and TCF3.\(^{64}\) Future work will then allow ALL treatments to be individualized to the specific genetic makeup of each tumor cell.

### Challenges for the Molecular Stratification of Solid Tumors

In contrast to the hematopoietic malignancies, similar attempts to stratify many of the major solid cancers such as lung, gastric, and liver cancer have been hampered by many challenges. One major difficulty is that solid tumors are often typically characterized by highly aberrant chromosomal karyotypes that are considerably more complex than those observed in the hematopoietic malignancies.\(^{69}\) With few exceptions, the heightened complexity of solid tumor karyotypes has thwarted attempts by conventional cytogenetics to identify signature aberrations associated with different solid tumor subtypes; for example, recurrent chromosomal translocations that may produce important fusion genes and transcripts. Indeed, it is only in the past few years that recurrent fusion genes such as TMPRSS2-ERG and EML4-ALK in solid tumors have been identified in prostate and lung cancer, respectively.\(^{63,71}\) In both these cases, these fusion genes were not identified through conventional chromosomal cytogenetics, but through innovative high-resolution genomic approaches. Nevertheless, these examples provide important proof that recurrent fusion transcripts do indeed exist in solid tumors, and it is expected that identification of novel fusion genes will continue, particularly with the use of next-generation deep sequencing methods.\(^{72}\)

Besides increased chromosomal complexity, a second challenge faced in the molecular stratification of solid tumors lies in the traditional reliance of diagnostic platforms such as immunohistochemistry or gene sequencing that typically measure single biomarkers (either genes or proteins). While such techniques are amenable to the detection of highly recurrent aberrations, recent large scale comprehensive sequencing analysis of various solid cancers, including pancreatic, brain, breast and colon cancers, has demonstrated that many solid tumors are likely to have a preponderance of mutations in multiple genes with low rates of recurrence.\(^{22,73,74}\) However, when such genes are grouped together by their respective pathways of activation, analysis has shown that such mutations tend to target a series of common core signaling pathways.\(^{73,75}\) These findings suggest that these oncogenic pathway, rather than the gene itself, is likely to represent the unit of mutational selection in solid cancers. Thus, the molecular stratification of these cancers will require analytical platforms that can measure multiple genes simultaneously, rather than one gene or protein at a time. Unfortunately, the expense and complexity of such genomic platforms are still prohibitively expensive and insufficiently robust to validate their regular use in clinical practice.

A third issue of complexity facing the molecular stratification of solid tumors is that unlike blood disorders, solid tumors comprise a diverse mix of many different cell types including cancer cells, stromal cells, infiltrating immune cells, and endothelial vasculature.\(^{76}\) Recent evidence has shown that in many cases, disease progression and treatment response for many solid tumors likely involve a combination of interactions between these different cell types. For example, studies in experimental models have shown that tumor-associated fibroblasts, while non-malignant in their genetic makeup, can play a profound role in the overall rate of tumor growth,\(^{77}\) and studies have shown that colon cancer disease prognosis can be affected either by the rate of immune cells,\(^{78}\) or by the presence of tumor stroma.\(^{79}\) The necessity to consider interactions between such entities raises another level of complexity that is overtly less of an issue in the blood disorders, which are commonly thought of as clonal diseases.
Besides the biological challenges of increased genomic complexity, pathway directed mutational profiles, and interactions with various cell types, it is also worth noting that a number of practical and logistic issues have also challenged research into the molecular stratification of solid tumors. Most solid tumors are collected through some invasive means such as surgery or biopsy, and so analysis of these tumors is limited only in clinical scenarios where such invasive procedures are an option. In the case of NSCLC, since late stage lung tumors are not treated with surgery, our knowledge of the molecular factors at play in late stage NSCLC is still relatively poor, despite the majority of NSCLC patients being diagnosed with advanced inoperable disease. In such cases, samples are typically limited to material through diagnostic methods such as fine-needle aspiration (FNAs), which has led some investigators to devise novel ways to analyze such so-called ‘low-volume’ samples. The challenge of obtaining large numbers of surgical samples has also impeded the establishment of large-scale molecular databases of these cancers to definitely establish overall levels of molecular diversity for many tumor types. Addressing this problem has required the formation of large multi-center consortia, or the development of novel statistical meta-analysis protocols to combine genomic data from multiple data sets to identify conserved molecular patterns.

**PROGRESS IN THE MOLECULAR STRATIFICATION OF SOLID CANCERS**

Despite these significant challenges, progress is indeed being made towards the molecular stratification of solid cancers. One obvious example of a major solid cancer where attempts at molecular stratification are showing progress is carcinoma of the breast. As mentioned above, breast cancers are already routinely subdivided into ER-positive and ER-negative subtypes, which are associated with distinct prognosis and responses to anti-hormonal treatment. Another important subtype of breast cancer for which routine testing is performed are tumors that overexpress the HER2 growth factor receptor, as such tumors can be targeted with traztuzamab. In addition to these three well-recognized subgroups, results from more comprehensive expression profiling analysis has also revealed the existence of further subgroups. For example, it is now clear that ER+ tumors sometimes called ‘luminal’ tumors, reflecting the fact that they are likely to arise from luminal cells in the mammary epithelium) can be further subdivided into two more subtypes - Luminal A and Luminal B, that show very distinct patterns of patient prognosis. Genomic signatures have also been identified that can predict the mutational status of important cancer genes such as p53 in breast cancer, and evidence is further emerging that these breast cancer subtypes could also exhibit distinct responses to various forms of conventional cytotoxic therapy as well as targeted therapies such as MEK inhibitors. Besides subtypes based solely on gene expression patterns, recent work has also demonstrated that combining gene expression-based subtypes with DNA copy number information can further subdivide patients into even more homogenous populations. Such “integrative genomic” analyses will likely be required to address the rampant genomic complexity of solid tumors.

In contrast to breast cancer, consensus regarding specific molecular subtypes in many other solid tumors has yet to gain general acceptance. In our own specific research area of gastric cancer, several distinct molecular subtypes of gastric cancer have been reported, and correlative analysis of these subtypes to clinical information has identified expression signatures associated with differing histological subtypes and in some cases prognosis. However, few of these genomic predictors have been independently validated in reasonably large independent patient cohorts, and attempts at validation are confounded by differences in the patient populations from different countries and centres, including differences in treatment protocol. Furthermore, with the increasing number of genomic biomarkers linked to cell lineage, drug sensitivity and pathway activation status reported in the literature, there is also the oft-cited concern that different studies ostensibly targeting the same biological phenotype (e.g. prognosis in early stage breast cancer) tend to identify non-overlapping signatures. Such concerns, however, should be evaluated in the light of recent studies showing that apparently ‘distinct’ signatures tend to nevertheless target the subpopulations of tumors, and that consensus can be ultimately established through the rigorous cross-comparison of different data sets, as has recently been demonstrated for breast cancer and NSCLC. Ultimately, the acid test for the clinical robustness of such stratifications will have to occur in the form of well-designed randomized prospective control trails designed to robustly test the actual clinical utility of such proposed schema. In fact, there are already several trials that are already underway or being proposed. For example, the MINDACT trial involves the recruitment of 6,000 women across multiple centers with early stage breast cancer whose treatment will depend upon their expression profiles of the 70-gene prognostic classifier described above. Another exciting series of trials involves the use of genomic signatures to guide chemotherapy treatment in early-stage lung cancer. This is another clinically challenging disease where the standards of chemotherapy have not been established, and thus treatment of NSCLC...
patients is largely due to physician preference. Results from these trials will definitely establish if genomic technologies are at the stage where they are sufficiently robust and reproducible for the information to be interpreted in such a fashion that they actually guide clinical treatment.

FUTURE CHALLENGES AND OPPORTUNITIES

We close this review with a brief survey of some anticipated future challenges facing clinicians and researchers interested in the molecular stratification of cancer. With the increasing ability to characterize tumors at multiple levels including mutations, expression signatures, copy number aberrations, and epigenetic profiles, it will be critical to develop robust analytical systems whereby such multi-dimensional data can be integrated, even possibly with host genotype information, to define robust biological subtypes of cancer. Analysis of the independence and relationship between different genetic aberrations, such as the mutual exclusivity of EGFR and k-ras mutations in lung cancer, could also prove very useful in delineating major oncogenic signaling pathways and their sub-components in primary tumors. Another challenge arising from the notion that most, if not all, cancers comprise multiple subtypes is how to transform currently existing drug discovery programs, that traditionally have focused on identifying single drugs with efficacy in the majority of cancers, to identifying many drugs targeted for distinct cancer subtypes, in a rapid and cost-efficient manner. A third challenge arises when one considers that many of these studies have relied upon the molecular analysis of tumors, which can only be obtained through invasive means such as surgery or biopsies. It would be exciting to assess if similar patterns of cancer stratification could be inferred using profiles derived from more easily obtainable body fluids from cancer patients such as blood or stool. Finally, one also needs to grapple with recent findings suggesting that there exists in tumors a rare stem cell-like population that can prove highly drug resistant. Techniques to detect and classify such cancer stem cells will represent a key challenge in cancer classification, and success in doing so may represent the pivotal shift in our ability to treat cancer in a way that tangibly affects overall cancer outcomes.

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