



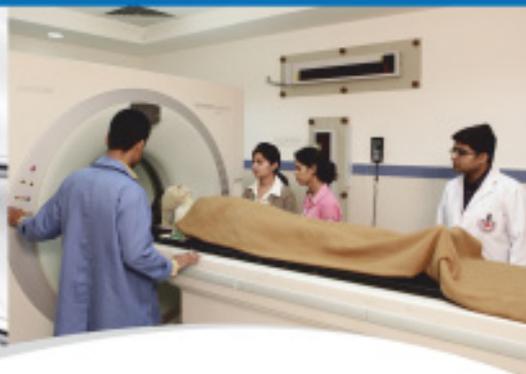
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Molecular Biology of Cancer



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From the Desk of Director Research

Cancer is viewed as a set of diseases that are driven by accumulation of genetic mutations which are considered the major cause of neoplasia. The process by which normal cells become progressively transformed to malignancy is now known to require the sequential acquisition of mutations which arise as a consequence of damage to the genome. This damage can be the result of endogenous processes such as errors in replication of DNA, the intrinsic chemical instability of certain DNA bases or from attack by free radicals generated during metabolism. DNA damage can also result from interactions with exogenous agents such as ionizing radiation, UV radiation and chemical carcinogens.

Consequent to Human Genome Sequencing and with the advent of Molecular Medicine and Bio-informatics tools, there has been greater understanding of cancer genetics and genomics which has led to development of several novel approaches to cancer diagnosis, prognosis and effective treatment and management of cancer. Recent introduction of next generation sequencing (NGS) technology have allowed better understanding of molecular genetic pathways and alterations in genes that are responsible for initiation, progression and metastasis of cancer.

Development of cancer detection biomarkers will be propelled by scientific discoveries and technological developments in how biomarkers are objectively measured (mutations, methylation, protein expression, molecular imaging). Moreover, advances in genomics, proteomics, molecular pathology and dissection of signaling pathways will generate many candidate biomarkers with potential clinical importance for their use in cancer staging, diagnosis, prognosis and development of personalized targeted therapy leading to improved patient care and survival.

This new discipline, by precisely identifying the molecular basis of the differences between normal and malignant cells, has created novel opportunities and provided the means to specifically target these modified genes. Successful use of these new therapies will rely upon a detailed knowledge of the genetic defects in individual tumors.

The present issue of the Cancer News spotlights the newer advances in the field of "Molecular Biology of Cancer" and features the regular articles, such as Special Feature, Guest Article, Perspective and In Focus. We are grateful to Prof. Bhudev C. Das, Chairman & Hargobind Khorana Chair Professor, Amity Institute of Molecular Medicine & Stem Cell Research, Amity University, Noida, for contributing the "Special Feature", and Dr. Deepa Philip, Specialist Registrar, Dept of Medical Oncology; Dr. Vikas Ostwal, Consultant Medical Oncologists, Dept of GI and Breast Medical Oncology, Tata Memorial Hospital, Mumbai for the "Guest Article" and Dr J. Sanil Manavalan, Assistant Professor of Medicine at Columbia University Medical Center for "Perspective".

Suggestions/ comments from the readers are welcome. Wishing our readers a Happy, Prosperous and Healthy New Year 2015!

Dr D C Doval

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SPECIAL FEATURE

WHAT'S NEW IN MOLECULAR GENETICS OF CANCER

Introduction

Cancer is the outcome of control proliferation of a cell initiated by alterations (genetic/epigenetic) in one or more genes, such as oncogenes and tumour suppressor genes that regulate cell proliferation, survival, and other homeostatic functions, leading to development of cancer. The alterations in genes may be induced by chemicals (e.g., from smoking or diet), radiation, and viruses or bacteria, and some individuals may inherit genetic mutations that predispose them to develop specific types of cancer. There is an increasing evidence that cancer is also driven by 'epigenetic changes' either by DNA methylation or by histone modifications, that leads to alterations in chromatin condensation thereby regulating expression of certain set of specific genes^{1,12}. We know now that there are about 17 signal transduction pathways and 2 stress response pathways which are conserved and are regulated by more than 20 protein factors or transcription factors mostly derived from host cell and bind to a gene's enhancer or promoter with the main aim of up or down regulating gene expression leading to development of cancer. Therefore, discovery of genetic and molecular biomarkers have been proved to be invaluable tools for early detection, reliable diagnosis, and effective treatment of cancer. Diagnostic and prognostic biomarkers are quantifiable traits that help clinical oncologists in identifying who is at risk, diagnose at an early stage, select the best treatment strategy, and monitor response to treatment.

Following Human Genome Sequencing and with the advent of Molecular Medicine and Bio-informatics tools, there have been greater understanding of cancer genetics and genomics that have led to development of several novel approaches to cancer diagnosis, prognosis and effective treatment and management of cancer. Recent introduction of next generation sequencing (NGS) technology have allowed better understanding of molecular genetic pathways and alterations in genes that are responsible for initiation, progression and metastasis of cancer.

Discovery of microRNAs and cancer stem cells along with the development of epigenomics, nanomedicine and cancer vaccines have provided the researchers and clinicians a new way to predict the risk of metastases,

systemic treatment resistance, and disease relapse in patients with cancer including novel approaches for prevention and targeted therapy.

In this article, we describe here some new frontiers in cancer genomics that have identified biomarkers which could be exploited for diagnosis, prognosis and treatment outcome and management of various cancers.

miRNA in Cancer

MicroRNAs (miRNAs or miRs) are a family of small non-coding RNA species (19-22 bases) that have been implicated in the control of many fundamental cellular and physiological processes such as cellular differentiation, proliferation, apoptosis and stem cell maintenance. miRNAs regulate gene expression by the sequence-specific targeting of mRNAs, leading to translational repression or mRNA degradation. Some microRNAs have been categorized as "oncomiRs" as opposed to "tumor suppressor miRs" Modulating the miRNA functions may provide excellent approaches for cancer therapy. Since a single miRNAs can bind to 100 different target transcripts, it has been estimated that miRNAs may be able to regulate up to 30% of the protein-coding genes in the human genome¹⁷. MicroRNA expression profiling also miRNA profiling were shown to be associated with tumour development, progression and response to therapy, suggesting their possible use as diagnostic, prognostic and predictive biomarkers.

miRNA as diagnostic biomarkers: Recognition of miRNAs that are differentially expressed between tumor tissues and normal tissues may help to identify those miRNAs that are involved in human cancers and further establish the possible pathogenic role of miRNAs in cancers¹¹. It is well known that miRNAs can be up-regulated or down-regulated in various human cancers. miR-21, miR-155, miR-10b, miR-29b-2 are up-regulated while miR-143, miR-145, miR-200 are down-regulated in breast cancer. miR-200a,b,c, miR-141 are up-regulated and miR-199a, miR-140, miR-145, miR-125b are down-regulated in ovarian cancer. Most aggressive oral tongue cancer shows exclusive over expression of miR-184 while oral squamous cell carcinoma shows miR-155 over expression. Over-expressed miRNAs may function as oncogenes by downregulating tumor-suppressor genes and/or genes that control cell differentiation or apoptosis, whereas the down-regulated miRNAs act as tumor-suppressor genes by negatively regulating oncogenes and/or genes that control cell differentiation or apoptosis⁵. Detection of miRNAs in saliva and other body fluids can also be used

as noninvasive and rapid diagnostic tool for the detection of cancer. miRNA biomarkers have revealed as a great potential in early diagnosis of cancer.

miRNA as prognostic biomarkers: miRNAs can also be utilized as prognostic markers to predict treatment/disease outcome. In lung cancer, miR-155 overexpression and let-7a down regulation were able to predict poor disease outcome. In gastric cancer a robust 7-miRNA signature can predict overall survival and relapse-free survival. Similarly, the low levels of miR-191 and high level of miR-193a were associated with a significantly shorter survival time as measured by Kaplan–Meier curves in melanomas³. Several miRNAs such as prognostic biomarkers are being discovered of variety of cancer.

miRNA profiling: miRNA profiling instead of gene profiling could be more reliably used for tumor classification, diagnosis and prognosis. Different platforms to assess the global expression of miRNA genes in normal and diseased tissues were developed. An extensive use of custom-made and then commercial miRNA microarrays, bead-based flow cytometric miRNA analysis methods and next generation of large-scale profiling method are represented by the high-throughput deep sequencing⁷. Genome-wide profiling showed that miRNA expression signatures (miRNome) allowed high accuracy information in different types of cancer.

miRNA in therapeutics: Experimental evidence demonstrates that the modulation of specific miRNA alterations in cancer cells using miRNA replacement or anti-miRNA technologies can restore miRNA activities and repair the gene regulatory network and signaling pathways, and in turn, reverse the cancer phenotype.

miRNA as regulator of signaling pathways: Aberrant expression of the epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor 2 (HER2) are features of many human tumors and are associated with disease progression, treatment resistance and poor prognosis. miRNA-7 and miRNA-331-3p reduce the Akt activity and thus directly regulate expression of EGFR and HER2, respectively in glioblastoma and prostate cancer.

miRNA reprogramming of cancer stem cells: Reprogramming the differentiated somatic cells with cocktail transcription factors (Oct4-Sox2-c-Myc-klf-4 or Oc4-Sox2-Nanog-Lin28) is the breakthrough in the stem cell biology. The miRNAs (miR-291-3p, miR-294, and miR-295) increase the efficiency of reprogramming by three transcription factors (Oct4, Sox2 and klf4), without

adding c-Myc. Certain miRNAs can also dramatically influence the fate of cancer stem cell thus allowing to overcome chemoresistance and relapse.

Hypermethylation of miRNA: The genetic alterations and failure of post-translational regulation might cause the dysregulation of subsets of miRNAs, but epigenetics alterations also appear to play an important role. In classical tumor suppressor genes, promoter CpG island hypermethylation occurs in genes involved in cell adherence, invasion, and angiogenesis, such as cadherins, tissue inhibitors of metalloproteins and thrombospondins. The epigenetic silencing of miRNAs (miR-127 and miR-124a) with tumor suppressor features by CpG island hypermethylation is also emerging as a common hallmark of human tumors.

Circulating miRNAs: new ace of intercellular communication: While majority of miRNAs are found intracellularly, a significant number of miRNAs have been observed outside of cells, including various body fluids. These type of miRNAs are known as circulating miRNAs which function as ‘extracellular communication RNAs’ that play central role in regulation of gene expression and the implication of miRNA-specific aberrant expression in the pathogenesis of cancer, including cardiac, metabolic, neurologic, immune-related diseases and many others. These miRNAs are stable and show distinct expression profiles in different fluid types. Recent studies have identified miRNAs in tumor tissues, plasma, saliva and urine. Some of the key molecular properties of these species include high stability in circulation and the ability to survive unfavorable physiological conditions. To-date, more than several tens of cancer have been investigated in which expression profiling of circulating miRNAs has revealed both diagnostic and prognostic utility for this class of biomarkers¹⁶. Circulating miRNAs have also been implicated in regulation of stem cells as well as cancer stem cells. Overall, circulating miRNAs have immense potential for refinement of the current processes for diagnosis, staging and prognostic prediction, and they may also serve as potential future therapeutic targets in the management of cancer.

Long Non-coding RNAs: A New Player in Cancer Research

Non-coding RNAs (ncRNAs) have distinct biological functions from that of small non-coding miRNAs and operate through defined mechanisms. ncRNAs, particularly long ncRNAs (lncRNAs), have essential

roles in tumorigenesis, and that lncRNA-mediated biology occupies a central place in cancer progression. With the number of well-characterized cancer-associated lncRNAs growing, the study of lncRNAs in cancer is now generating new hypotheses about the biology of cancer cells. This typically results in transcriptional repression, and many lncRNAs were first characterized by their repressive functions, including ANRIL, HOTAIR, H19, KCNQ1OT1, and XIST⁸. For clinical medicine, lncRNAs offer several possible benefits. lncRNAs, such as PCAT-1, commonly demonstrate restricted tissue-specific and cancer-specific expression patterns¹⁰. This tissue-specific expression distinguishes lncRNAs from miRNAs and protein-coding mRNAs, which are frequently expressed from multiple tissue types. ncRNAs may be superior biomarkers than many current protein-coding biomarkers, both for tissue-of-origin tests as well as cancer diagnostics.

Cancer Stem Cells (CSCs)

A subpopulation of cancer cells exists within the tumors that have the capacity to self-renew and to generate the more differentiated progeny which makes up the bulk of a tumor and have been termed as cancer stem cells, or tumor-initiating cells. The existence of CSCs has profound implications for cancer biology and therapy because eradication of CSCs is critical for achieving effective treatment and cure of cancer.

Cancer treatment by stem cell transplantation: A stem cell transplant replaces defective or damaged cells in patients whose normal blood cells have been crowded out by cancerous cells. Transplants can also be used to treat

hereditary disorders, such as sickle cell anemia, or to help patients recover from or better tolerate cancer treatment. Stem cells for transplant can be taken from the patient's own bone marrow before chemotherapy and then replaced after cancer treatment. This is a vital and often life saving treatment because chemotherapy destroys the bone marrow alongside cancer cells and the blood cells must be replenished for the patient's treatment to be successful. It is hoped that the molecular basis for this treatment can lead to similar treatments for other forms of cancer, allowing cancerous tissues in areas, such as the brain, to receive stem cells that replenish those that are damaged through radiation. Stem cells can also be obtained from a donor whose tissue most closely matches the patient or can be extracted from the placenta of newborn infants after birth and saved in special cord blood banks for future use.

CSC targeted treatment preventing relapse of cancer: Cancer stem cells resist chemotherapeutic drugs and can renew the various types of cells in the tumor thereby relapsing the disease. The drugs that can selectively target cancer stem cells offer great promise for cancer treatment, mainly in combination with chemotherapy (see Fig 1). It has been shown that metformin, a standard drug for diabetes inhibits the cellular transformation and selectively kills cancer stem cells in four genetically different types of breast cancer. The combination of metformin and well-defined chemotherapeutic agent doxorubicin, kills both cancer stem cells and non-stem cancer cells in culture. Furthermore, this combinatorial therapy reduces tumor mass and

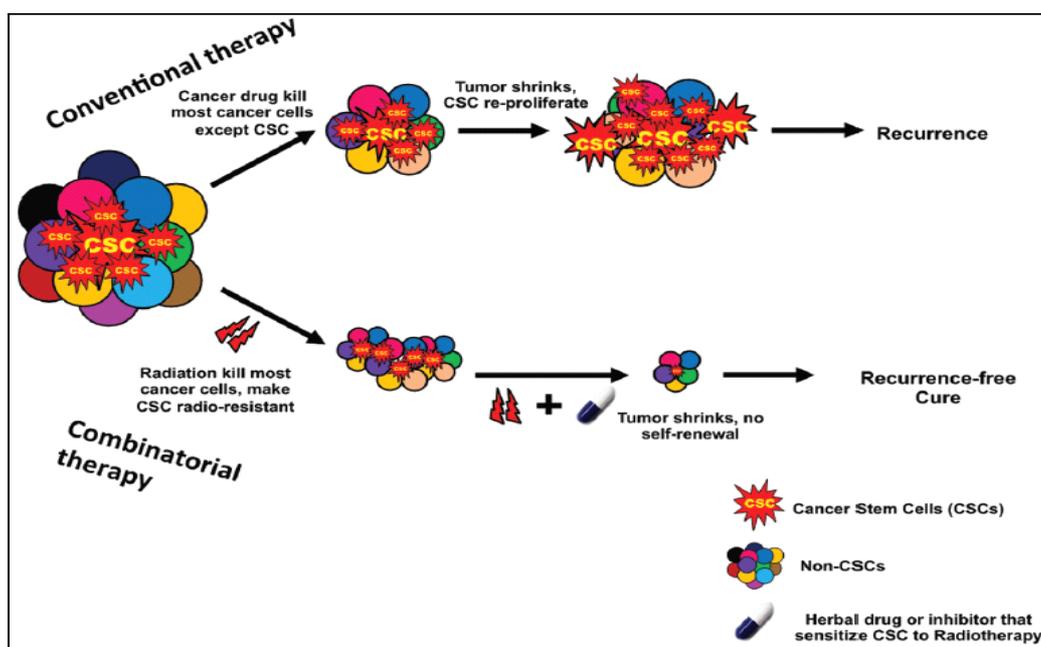


Fig1: Diagram demonstrating how to target cancer stem cells

prevents relapse much more effectively than either drug alone in a xenograft mouse model. Mice seem to remain tumor-free for at least two months after combinatorial therapy with metformin and doxorubicin is ended. Therefore it has been suggested that the combination of metformin or any CSC sensitizing agent (eg curcumin) and chemotherapeutic drugs can be used to improve treatment of patients with breast or other cancers⁹. There are some agents that specifically reduce CSCs, such as salinomycin, which reduces CSCs by >100-fold relative to paclitaxel, a commonly used breast cancer drug. Global gene expression analyses show that salinomycin treatment results in the loss of expression of breast CSC genes previously identified in breast cancer tissues.

Understanding cancer stem cell biology and signaling can encourage the development of drugs and cancer specific treatments. Identification and characterization of CSCs for every possible tumor are of paramount importance for development of new therapeutic avenues.

Human iPSCs: potential clinical applications in cancer: The induced pluripotent stem cell (iPSC) research has significantly changed our perspectives on regenerative medicine and cancer research by providing a unique tool to derive disease-specific stem cells for study. The human induced pluripotent stem cells (hiPSCs) can be derived from direct reprogramming of human somatic cells to a pluripotent stage through ectopic expression of specific transcription factors. These cells have two important properties, self-renewal capacity and ability to differentiate into any cell type of the human body. The generation of hiPSCs has increased their potential use as novel candidates for disease modeling, drug screening, regenerative medicine and cell based therapy. It opens new opportunities for understanding the mechanisms of disease in the production of new disease models, in drug development/drug toxicity tests, gene therapies, and cell replacement therapies. Stem cell therapies using patient-specific iPSCs would be free from immune rejection and ethical issues¹⁹. Patient specific iPSCs need to be derived from diseased tissue portions (i.e. hepatocyte within liver cancer) and not the tissues which do not carry any pathogenetic events. It is hoped that the generation of safe and effective iPSCs for use in cell therapy as well as in disease modeling and drug screening will be achieved in the near future for their clinical application.

Circulating Tumor DNA

Dying tumor cells release small pieces of DNA into blood stream called cell-free circulating tumor DNA (ctDNA) which can be used for cancer, diagnosis,

progression and monitoring prognosis. The levels of ctDNA can be detected using personalized profiling by various methods, including next generations sequencing. The levels of ctDNA increases in blood as the cancer stage progresses from Stage I to IV⁴. In a study of 206 colorectal cancer patients, patients with lower blood level of ctDNA survival was longer than those with higher levels of ctDNA. However, in some patients' with cancer ctDNA had first responded to a specific gene targeted therapy, but progressed while still being treated. It happened because of new somatic mutations occurring which inhibit the drug action on cancer cells. In such cases, the screening for mutations in ctDNA, both before and after therapy can help in finding new mutations and provide valuable information to clinicians when tumors are no longer responsive and a different treatment strategy is necessary. Thus, ctDNA appears to be an extremely effective and advantageous biomarker as it is found in the blood, provides a semi-invasive, less risky and alternative method to repeated tumor biopsies to monitor tumor progression. This suggests that by simply measuring the level of ctDNA in a patient's blood could serve as a way to determine the stage and treatment required for that tumor.

Oncogene Expression Profiling

Oncogene profiling is the measurement of the expression of thousands of genes at once, to create a global picture of cellular function. These profiles can distinguish between cancer and normal cells and show how the cells react to a particular treatment. Four technologies are considered in this evaluation: two are based on gene expression profiling and two on immunohistochemistry. The two gene expression profiling are MammaPrint (Agendia) and Oncotype DX (Genomic Health). Three are based on immunohistochemistry (also referred to as protein expression profiling in the diagnostics assessment report): IHC4 (academic sponsor) and Mammostrat (Clariant). HERmark is also very useful HER2 status of breast cancer patients.

The MammaPrint assay by Agendia analyzes expression of 70 genes from an early-stage breast cancer tissue sample to correctly stratify the patients into low risk or high risk of cancer recurrence within 10 years after diagnosis. The low risk can patients safely avoid chemotherapy and have excellent clinical outcomes while high risk patients should receive chemotherapy. Thus, it will help clinician to make a more informed decision about whether to utilize chemotherapy or other treatments to reduce the risk of recurrence.

Another assay Oncotype DX analyzes profile of 21 genes and calculates a recurrence score number between 0 and 100; the higher the score, the greater the risk of recurrence. This test is used to estimate a woman’s risk of recurrence of early-stage, hormone-receptor-positive breast cancer, as well as how likely she is to benefit from chemotherapy after breast cancer surgery. The Oncotype DX test is also used to estimate a woman’s recurrence risk of DCIS (ductal carcinoma in situ) and/or the risk of a new invasive cancer developing in the same breast, as well as how likely she is to benefit from radiation therapy after DCIS surgery.

The IHC4 test assesses levels of four key proteins in a breast cancer sample, ie ER, PgR, HER2 and Ki-67. The IHC4 score is calculated from the percentage of cells positive for Ki67 and PgR (0–100%), the Histoscore (a measure of the percentage of cells positive multiplied by intensity, range 0–300) for ER status, and the tumor HER2 status (expressed as positive or negative).

The Mammostrat test uses five immunohistochemical markers (SLC7A5, HTF9C, P53, NDRG1, and CEACAM5) to stratify patients into risk groups. These markers are independent of one another and do not directly reflect either proliferation or hormone receptor status. The current version of the test provides categorical classification of breast cancer sub-type, and quantitative values for ESR1/ER, PR/PgR, ERBB2/HER2, proliferation, and Luminal score (ER-pathway). The test uses formalin-fixed paraffin-embedded tissue sections.

HERmark is a proprietary diagnostic test that accurately quantifies HER2 total protein levels and HER2:HER2 homodimerization in patients with breast cancer. HERmark is highly sensitive and can detect HER2 at levels from 2,500 to over 1 million receptors

per cell – 7 to 10 times more sensitive than IHC. Several such predictive biomarkers have also been developed (see Table 1) which have led to paradigm shift toward personalized cancer treatment.

Epigenomics: A New Frontier in Cancer Research & Therapy

During the past decade, more focus has been given to the role of molecules that affect chromatin dynamics, i.e, global DNA methylation and post-translational modifications of histones proteins in cancer cells. It has given a new discipline of “cancer epigenome,” showing heritable abnormalities that occur in the absence of DNA sequence alterations in the genome². Presently, there are four FDA-approved drugs with an “epigenetic mode” of action being used in clinics: (1) DNA methyltransferase (DNMT) inhibitors 5-azacytidine (Vidaza); (2) decitabine (20-deoxy-5-azacytidine, dacogen); (3) histone deacetylase (HDAC) inhibitors suberoylanilide hydroxamic acid (SAHA, Zolinza), and (4) romidepsin (Istodax). Numerous other DNMT and HDAC inhibitors are also being developed and evaluated in preclinical studies, and clinical trials. 5-Azacytidine and decitabine have been successful in treating myelodysplastic syndrome and myeloid leukemias¹⁵, whereas, SAHA and romidepsin are currently being used for the treatment of cutaneous T-cell lymphoma. FDA-approved cancer therapy drugs that primarily target DNA methylation and global histone modifications, are being increasingly used in clinical practices, and additional leads are being found and evaluated. Genomic and epigenomic profiling and epigenetic biomarkers are being exploited maximally due to the advent of next generation sequencing technologies and bioinformatics tools. So, successful cancer treatment would require both genomic and epigenomic information of tumor.

Table: Predictive biomarkers for drug response in different human cancers

Biomarker	Cancer type	Drug therapy	Drug target
HER2 (gene amplification)	Breast	Trastuzumab	HER2
Estrogen receptor (protein expression)	Breast	Tamoxifen	Estrogen receptor
BCR-ABL (gene translocation)	CML	Imatinib, dasatinib, nilotinib	BCR-ABL
EGFR + KRAS (KRAS mutation)	CRC	Cetuximab, panitumumab	EGFR
EGFR (kinase domain mutation)	NSCLC	Erlotinib, gefitinib	EGFR
PML-RAR (gene translocation)	APL	All trans retinoic acid	PML-RAR
BRCA1/2 (mutation)	Breast	Olaparib, veliparib	PARP
BRAF V600E (mutation)	Melanoma	Vemurafenib	BRAF
ALK (rearrangements)	NSCLC	Crizotinib	ALK

Abbreviations: APL, acute promyelocytic leukemia; CML, chronic myeloid leukemia; CRC, colorectal cancer; NSCLC, non-small-cell lung cancer

Source: Nat. Rev. Clin. Oncol. Doi:10.1038/nrclinonc.2011.121

Mitochondrial DNA Mutations in Cancer: Causality or Association?

Although genetic and epigenetic changes are most important, mitochondrial (mt) alterations are also emerging as new molecular markers. Analysis of frequent mtDNA mutations in different stages of cancer progression, strongly suggests its functional relation with tumorigenesis. Mitochondria are a power house of cell that generates ATP through oxidative phosphorylation. Human mtDNA is a 16.6 kb circular double-stranded DNA molecule, which is present at a high copy number per cell and encodes 13 polypeptides involved in respiration and oxidative phosphorylation, 2 rRNAs and a set of 22 tRNAs which are essential for protein synthesis. Increased mtDNA mutations are reported in various types of cancers. With next-generation sequencing or Mitochip¹⁸ combined with rapid high-throughput methods help in early detection of mtDNA mutations for targeted prevention, or therapy or early detection of cancer.

Cancer Exosomes: ‘Micro Factories’ that Aid in Tumor Growth

The 2013 Nobel Prize in Physiology or Medicine was jointly awarded to three scientists for their discovery of vesicle transport in cells, and its role in physiology, diagnostics and therapy. Exosomes are now believed to be present in all body fluids, and represent a new way of cell signaling. Exosomes are small secreted vesicles that can transfer their content to recipient cells. Increasing evidence suggests that tumor-derived exosomes can confer either antitumorigenic or protumorigenic effects. All exosomes contain a cellular stew of smaller components including proteins, messenger RNA (mRNA) and miRNAs. Recent studies have shown that exosomes may be used to encapsulate and protect exogenous oligonucleotides for delivery to target cells as they are natural cell-derived nanocarriers, are immunologically inert and possess an intrinsic ability to cross biological barriers. However, the crucial components of exosomes and their role in carcinogenesis are still largely unknown.

Cancer Vaccines

Vaccines are designed to boost the body’s natural ability to protect itself through the immune system from dangers posed by damaged or abnormal cells, such as infected cells or cancer cells. FDA has approved two vaccines, Gardasil and Cervarix, that protect against infection by the two types of high risk HPV 16 and 18 that cause approximately 70 percent of all cases of cervical cancer worldwide⁶. In addition, Gardasil protects against infection by two additional HPV types, 6 and 11,

which are responsible for about 90 percent of all cases of genital warts in males and females. The FDA has also approved a cancer preventive vaccine that protects against HBV infection. Chronic HBV infection can lead to liver cancer. The original HBV vaccine was approved in 1981, making it the first cancer preventive vaccine to be successfully developed and marketed. Today, most children in the United States are vaccinated against HBV shortly after birth (US Centers for Disease Control and Prevention, 2005).

Sipuleucel-T (Provenge®) is the vaccine used to treat advanced prostate cancer in which hormone therapy is not effective. For this vaccine, immune system cells are removed from the patient’s blood and are exposed to chemicals that turn them into special immune cells called dendritic cells. They are also exposed to a protein called prostatic acid phosphatase (PAP), which should produce an immune response against prostate cancer. The dendritic cells are then given back to the patient by infusion into a vein (IV). This process is repeated twice more, 2 weeks apart, so that the patient gets 3 doses of cells. Back in the body, the dendritic cells help other immune system cells attack the prostate cancer. Dendritic cell vaccines are also being employed for personalized treatment of cancer.

PET- CT (Positron Emission Tomography-Computerized Tomography)

The availability of accurately aligned, whole-body anatomical (CT) and functional (PET) images could have a significant impact on diagnosing, staging the malignant disease, identifying localizing metastases, response to treatment, and looking for follow-up recurrence. It can help clinicians to decide on the most appropriate cancer treatment, and also provide an indication on the effectiveness of ongoing chemotherapy. PET is a medical imaging technique in which a small amount of a radioactive tracer (positron-emitting radionuclide) is given to the patient, normally by injecting it into a vein. Different types of tracers have been developed for imaging with PET, but ¹⁸F-2-fluoro-2-deoxy-d-glucose (FDG), an analog of glucose is currently the only agent approved by the FDA. The use of FDG to image glucose metabolic rate takes advantage of the observation, that malignant cells have higher rates of aerobic glycolysis than normal tissues and thus utilizes more glucose to meet its energy needs. CT uses X-rays to produce images of the body. Other radiotracers like choline labeled with (18)F or (11)C, (11)C-acetate, and (18)F-fluoride have also demonstrated promising results. A recent study has shown that PET scans have great potential in predicting

the prognosis for patients such as inoperable lung cancer. PET scans add a new dimension to a physician's ability to determine patients who need additional cancer therapies for better management of cancer¹³. ¹⁸F-FDG PET-CT imaging is an efficient technique to detect breast cancer recurrence.

Next Generation Sequencing (NGS) & Pathway Discovery for Personalized Targeted Cancer Therapy

NGS technology, a simplification method of Human Genome Sequencing, is quickly replacing other technologies as the gold-standard in cancer diagnostics. NGS method compares the genetic makeup of cancerous cells to that of normal cells in order to determine which genes are altered in cancerous cells and that may affect disease outcome. NGS provides significantly higher sensitivity than traditional techniques, and permits the discovery of rare somatic mutations that occur in cancer cells at much lower frequencies, and many of which can be identified as important cancer drivers.

Patients have benefited enormously from targeted cancer therapy and avoided the harmful side effects of other cancer therapies. But a large number of patients still suffers from a certain cancer type, since they don't have good response to targeted cancer therapy. In order to cure the patient, it is important to change the therapeutic strategy from targeted therapy to personalized therapeutics. Blood is very suitable to serve as the source of personalized therapeutics because it is easy and noninvasive to obtain from individuals. Circulating tumor cells (CTCs) from cancer patients, have been used as the biomarker for diagnosis and prognosis¹⁴.

Recently, scientists have developed high-throughput, gene-by-gene oncogenic activity assay, which rapidly identifies the final mutations within a given patient tumor. It identifies tumor-specific driver mutations, either by next generation sequence or by functional assay that detects dysregulated activation of signaling pathways. This allow to identify functionally impactful driver mutations, and quantify the effect of the microenvironment on the pathways and then to examine the impact of a candidate drug on a specific tumor. Assigning functional significance to specific driver mutations is critical for tailoring successful treatments with the trial of possible drugs. NGS provides correlation information between DNA mutations and available drugs. Drugs can be incubated in the live cells and monitors the effects of the different drugs on the activation of signaling pathways. This is to help physicians to tailor treatment to their patients by developing personalized medicine. It helps in identifying and prioritizing new drug targets with minimum effort.

Nanotechnology in Cancer Treatment

The use of nanotechnology in cancer treatment offers some exciting possibilities, including the possibility of destroying tumors with minimal damage to healthy tissue and organs. This approach helps in detection and elimination of cancer cells before they form tumors, enhance drug localization, increase drug efficacy, and potentially decrease chances of multidrug resistance. The use of nanoparticles for drug delivery, tumor therapy, and tumor follow-up using different imaging modalities. For example, tumor-killing agent called tumor necrosis factor alpha (TNF) is attached to a gold nanoparticle along with Thiol-derivatized polyethylene glycol (PEG-THIOL), which hides the TNF bearing nanoparticle from the immune system. This allows the nanoparticle to flow through the blood stream without being attacked. Nanoparticles can be modified in numerous ways for diagnosis and treatment of cancer.

Currently used NPs in cancer therapeutics include dendrimers, liposomes, lipid NPs (LNPs), polymeric NPs (PNPs), micelles, protein NPs, ceramic NPs, viral NPs, metallic NPs, and carbon nanotube (CNTs) that have shown encouraging results in cancer therapy. Despite extensive research on NP systems for cancer therapeutics, there are only a few nanoparticulate pharmaceutical drug delivery systems (NDDSs) approved by the US FDA. The NDDSs that have been approved include liposomal doxorubicin (Myocet; Elan Pharmaceuticals, Cedar Knolls, NJ), PEGylated liposomal doxorubicin (Doxil; Ortho Biotech, and Caelyx; Schering-Plough), PEGylated liposomal daunorubicin (DaunoXome; Diatos), and the recently approved albumin-bound paclitaxel-loaded NPs (Abraxane; Abraxis Bioscience). One of the major challenges in cancer treatment, such as multidrug-resistance (MDR), can also be overcome by these nano formulation drugs. Though the emerging field of nanomedicines has shown great promise, all newly developed nanoparticles, whether used as carriers for drugs, therapeutic agents, or imaging agents, will need to be thoroughly characterized physiochemically, pharmacologically, and immunologically before they can be approved for use in humans. The nanoparticle size, uniformity, and consistency between batches also need to be tightly regulated. Nanotechnology provides hope in developing new ways for cancer detection, diagnosis, and therapy that could be tailor made for each individual's tumor molecular profile, and qualifies for personalized therapies.

Future Perspective

Development of cancer detection biomarkers will be propelled by scientific discoveries and technological

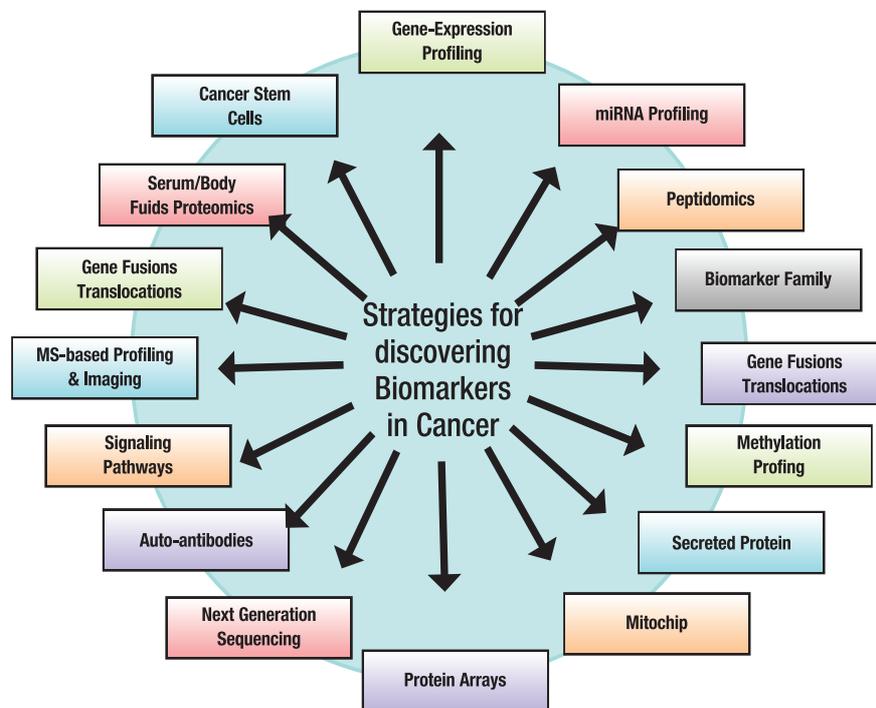


Fig 2. Emerging technologies for biomarker discovery in cancer

developments in how biomarkers are objectively measured (mutations, methylation, protein expression, molecular imaging). Moreover, advances in genomics, proteomics, molecular pathology and dissection of signaling pathways will generate many candidate biomarkers with potential clinical importance for their use in cancer staging, diagnosis, prognosis (see Fig 2) and development of personalized targeted therapy leading to improved patient care and survival. Nevertheless, we need to travel still a long way to achieve complete cure for cancer.

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GUEST ARTICLE

PHARMACOGENETICS IN CANCER THERAPEUTICS: FROM DNA TO DRUGS- AN ELUSIVE DREAM OR IMMINENT REALITY

Sir. William Osler, the father of modern medicine has rightly said, “If it were not for the great variability among individuals, medicine might well be a science, not an art.” We live in an era of Personalised or Precision Medicine now. We have shifted from the paradigm of “One Size Fits All” to the paradigm of “Personalised Medicine” where we aim at offering the right medicine to the right person at the right dosage at the right time. pharmacogenetics forms the backbone of Personalised/ precision medicine. Currently, the USFDA has included pharmacogenetic information update in the package insert of over 30 anticancer agents. In this review we will attempt to give an overview on the role of pharmacogenetics in cancer therapeutics with the aim of improving efficacy with reduced toxicity.

Pharmacogenetics/Pharmacogenomics?

Pharmacogenomics: It is the science that allows us to predict a response to drugs based on an individual’s genetic makeup. [Felix Frueh].

- How genes affect.. the way our body processes drugs (pharmacokinetics)...the interaction of drugs with receptors (pharmacodynamics)...the treatment efficacy and adverse side effects.

Pharmacogenetics: A subset of ‘pharmacogenomics’. The role of genetics in drug responses [Vogel. 1959].

Why is it Important in Cancer Therapeutics?

1. Medicine is more personal in oncology than in any other branch of medicine. Our primary aim being [Primum Non Nocere, do no harm], it becomes very important to balance the risk/toxicity with benefits of our treatment.
2. With advances in molecular oncology, we now know that every cancer is not the same. Each individual harbours not just a cancer but different cancers. There is so much of tumor heterogeneity. “It is more important to know what sort of person has a disease than to know what sort of disease a person has” (Hippocrates). Unique to oncology is the fact that two related but different genomic systems (tumor and germline genomes) need to be studied to improve

treatment efficacy and reduce toxicity.

3. Chemotherapeutic agents are drugs with very narrow therapeutic index. They can be very dangerous if not used prudently with pharmacogenetic makeup in consideration. Hence it is important and necessary to pre-emptively predict untoward side effects.
4. Chemotherapeutic drugs are quite expensive both to take and to make. So the use of ineffective drugs will be a waste of resources.
5. It helps to determine appropriate dosing for an individual, balance the toxicity and benefit, and explain variable treatment responses and to choose novel drug treatments.

Where to do Pharmacogenetic Studies?

PGx information of 24 biomarkers are available in the drug labels for 30 FDA-approved anticancer agents. These biomarkers include gene variants, functional metabolizing enzyme deficiencies, expression changes, chromosomal abnormalities and many others. Based on the level of scientific evidence support, these markers have been presented differently in different sections of the FDA-approved drug labels. The level of FDA recommendation comprise: ‘mandatory’ – if the biomarker appears in ‘boxed warning’ or ‘contraindications’; ‘recommended’ – if the biomarker appears in ‘indications and usages’ or is clearly stipulated; or ‘proposed’ – if the biomarker is mentioned in another section of the package insert, such as ‘warning and precautions’ and ‘clinical pharmacology’. This classification method is not an official FDA definition. Therefore, all PGx markers included in the drug package inserts are of value. For those markers indicated as ‘mandatory’ or ‘recommended’, clinical action should

Table: Anticancer Drugs Approved by FDA with Labelling Regarding Pharmacogenomic Biomarkers

BIOMARKERS WITH PHARMACOKINETIC EFFECT	
TPMT	6MP, 6TG
UGT1A1	IRINOTECAN, NILOTINIB
BIOMARKERS WITH PHARMACODYNAMIC EFFECT	
EGFR	CETUXIMAB, ERLOTINIB, GEFITINIB, PANITUMUMAB
KRAS	CETUXIMAB, PANITUMUMAB
ABL	IMATINIB, NILOTINIB, DASITINIB
C-KIT	IMATINIB
HER-2	TRANSTUZUMAB, LAPATINIB
ER	TAMOXIFEN

be considered. When incorporating these PGx markers into guiding cancer chemotherapy, improved efficacy or reduced toxicity have been observed.

How to do Pharmacogenetic Studies?

Pharmacogenetic discovery research: Two approaches are commonly used in PGx discovery research: the candidate gene approach and genome-wide studies.

- The candidate gene approach focuses on one or a few genes involved in drug metabolism, transport or targeting pathways. This method has been developed based upon advances in pharmacology occurring since the 1950s. Classical examples include Irinotecan and *UGT1A1* polymorphisms, Tamoxifen and *CYP2D6* polymorphisms, EGFR tyrosine kinase inhibitors and *EGFR* polymorphism.
- Genome-wide association studies include all genes and noncoding sequences of the human genome, assuming that all genetic materials have equal chances to affect drug responses. It examines common genetic variations for a role in drug response by genotyping large sets of genetic variations across genome. It is either “discovery-based” vs “hypothesis-based”. It relates genetic variations to clinical outcome and identifies associations in genes not previously suspected. Pathway based studies examine biologically plausible associations between certain individual polymorphisms and clinical outcomes. It usually combines multiple related genetic variants to reveal otherwise undetectable effects of individual variants on clinical outcome.

Discovery for optimization of anticancer therapy: It identifies novel drug targets or pathways related to the drug or disease. It in turn helps to give us to have a more comprehensive understanding of the mechanism of the drug. For example, the almost miraculous discovery of Imatinib which changed the therapeutic landscape of CML wouldn't have been possible without pharmacogenetic studies on mutations and drug targets.

When to do Pharmacogenetic Studies?

1. Classical examples of chemotherapeutic agents:

6-mercaptopurine & TPMT: 6-mercaptopurine is an anticancer antimetabolite that is used to treat leukemia and lymphoma. The clearance of 6-mercaptopurine is subject to the function of TPMT. Functional deficiency in TPMT would increase the level of 6-mercaptopurine *in*

vivo and causes serious myelosuppression. Due to the high rate of polymorphisms in its coding sequence, activity of TPMT varies greatly in a population. So far, more than 20 genetic variants in TPMT have been identified. Among them are rs1800462 (G>C), rs1142345 (A>G) and rs1800460 (G>A), which are three variations that were found to reduce TPMT enzyme activity, which led to relatively high levels of 6-mercaptopurine and severe toxicity in the human body. Therefore, the FDA has recommended genotyping of TPMT SNPs prior to the usage of 6-mercaptopurine. If any of the three SNP sites carry the variant allele that leads to TPMT deficiency, substantial dose reduction of 6-mercaptopurine should be considered. Even though the FDA has not given the details of dose reduction, another source suggested 10% of original dose for homozygous TPMT deficient patients and 50% of that for heterozygous patients.

2. Capecitabine & DPD: Capecitabine is a prodrug of 5-FU that has been prescribed for the treatment of metastatic breast and colon cancers. *In vivo*, capecitabine is activated through a series of catalytic enzymatic activities to form 5-FU. DPD is one of the enzymes that control the rate-limiting step in 5-FU inactivation in the liver. Early PGx research with candidate gene approaches suggested the association of 5-FU treatment outcomes and the germline variations in *DPD*, with reduced DPD activity corresponding to longer 5-FU half-life and increased risk of toxicity. To-date, more than 30 SNPs and insertions/deletions have been found in/near the *DPD* gene. To date, only three variants have been consistently reported to be significantly associated with grade ≥ 3 5-FU toxicities studies. It was also reported that approximately 50–60% of patients carrying these three genetic variants in *DPD* developed severe 5-FU toxicity.

Cetuximab and panitumumab are two anti EGFR monoclonal antibodies that were designed to inhibit the growth and survival of tumor cells with overexpressed EGFR in colon and head and neck cancers. However, these drugs were found to be inefficient in some patients, even though they did have the mutated EGFR. Later on, several research teams reported the association between the resistance of cetuximab/panitumumab and KRAS mutations. Not surprisingly, if KRAS is actively mutated, the inactivation of EGFR by cetuximab or panitumumab will have no beneficial effect in curing KRAS-induced cancers. A

Pharmacogenetic test on the KRAS gene has been recommended by the FDA before prescribing cetuximab and panitumumab in the treatment of colon, lung, and head and neck cancers. According to the drug label, only patients with EGFR-expressing colon cancer and KRAS mutant negative (wild-type) are to be treated with these drugs.

4. Crizotinib & EML4-ALK: Crizotinib is an ALK inhibitor approved to treat non-small-cell lung cancer (NSCLC). The fusion EML4-ALK gene is found in 3–5% of NSCLC patients. This EML4-ALK fusion is a constitutively activated kinase and leads to carcinogenesis. The drug resistance to crizotinib was soon observed in certain patients. Later PGx research found the drug resistance is linked to several tumor-specific genetic mutations in ALK. Currently, the FDA has issued ALK positive as detected by an FDA-approved test as the indicator for prescribing this drug to treat patients with locally advanced or metastatic NSCLC. However, the FDA has not yet recommended PGx testing of these drug-resistant variants.

5. Irinotecan and UGT1A1 polymorphism: It is a topoisomerase-1 inhibitor which is a prodrug in itself. It requires activation to its active form SN-38. Hepatic UGT1A1 caused glucuronidation and inactivation of the active drug which is then excreted in bile and urine. Variant alleles like UGT1A1*28 leads to significant increased amounts of active drug leading to life threatening toxicities like diarrhea and leucopenia.

6. Platinum agents: Polymorphisms in excision repair enzymes like ERCC-1 and glutathione dependent enzymes like glutathione S transferases are important in predicting response with Cisplatin.

7. Tamoxifen and CYP2D6 polymorphisms: Tamoxifen is converted to varied metabolites mainly endoxifen and 4OH tamoxifen by CYP isoforms. There have been many studies correlating this pharmacogenetic association with tamoxifen resistance and efficacy.

8. Polymorphisms in drug transporters: MDR1 (P-glycoprotein, ABCB1) P-glycoprotein (PGP), encoded by the MDR1 gene (ABCB1), is the best-characterized ATP-binding cassette (ABC) transporter. PGP is involved in the transport of many chemotherapeutic agents like Adriamycin, Paclitaxel. This membrane efflux transporter is also found in normal tissues, such as the hepatocytes, kidney, small intestine, colon, adrenal glands, and capillary endothelium of the brain and testes. Multiple MDR1 polymorphisms have been described to occur in various allelic combinations.

9. Thymidylate synthase and 5-FU: One of the primary mechanisms of action of 5-FU is the inhibition of thymidylate synthase (TS) by FdUMP. Clinical resistance to these TS-targeted agents has been linked to over expression of TS in tumor. In rectal cancer patients there a very significant correlation has been reported between TSER genotypes and tumor downstaging after pre-operative chemoradiation. TSER genotyping may be useful in selecting patients who are likely to respond to treatment with 5-FU or its analogues.

Future perspective: Pharmacogenetics is a promising field in Personalised/precision medicine. It has the potential to reduce the life-threatening toxicity and improve therapeutic efficacy prior to administration of chemotherapy. Though promising there are many challenges and unanswered questions before bringing it to the front stage of standard clinical practice.

1. Environmental factors and concomitant medications may act as potential confounding factors affecting pharmacokinetics and pharmacodynamics making the situation more complex.
2. The concept of tumour heterogeneity makes Pharmacogenetic studies more complex. It would be judicious to study both the primary and metastatic disease.
3. Practical genotyping needs to be developed. It has to be a test that is economical, cost effective, easily available to clinicians and straightforward to interpret the results. A combination of multiple biomarkers and tests in a common test platform would be more economic and feasible.
4. More comprehensive platforms including genetic and epigenetic platforms like Mrna, miRNA, DNA methylation and histone acetylation may be the norm of the future.
5. Mitochondrial genome has also attracted attention and importance due to its pivotal role in metabolism, cell differentiation and cell signaling.

Conclusion

With the deciphering of the human genome, science can proudly say that now we know how God wrote the Book of Life. But we humans have to be humble to say that we still don't know how to read that Book. We need to go from the Bench to the Bedside and then back to the Bench again.

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PERSPECTIVE

EPIGENETICS IN CANCER

Introduction

Cancer has traditionally been viewed as a set of diseases that are driven by the accumulation of genetic mutations that have been considered the major causes of neoplasia (1). However, we now know that the disruptions of epigenetic regulatory mechanisms are also common in cancer (2,3). The role of genetic mechanisms in the pathogenesis of cancer is relatively straightforward: mutation of tumor suppressors and/or oncogenes causes either loss or gain of function and abnormal expression. The role of epigenetic mechanisms is a bit more complex and is determined by the chromatin structure including DNA methylation, histone variants and modifications, nucleosome remodeling as well as small non-coding regulatory RNAs (4). We now know that the genetic and epigenetic mechanisms are not separate events in cancer but they intertwine and take advantage of each other during tumorigenesis. Alterations in epigenetic mechanisms can lead to genetic mutations, and genetic mutations in epigenetic regulators lead to an altered epigenome (5).

Epigenetics and Tumorigenesis

Epigenetics is the study of stable and heritable changes in gene expression that are not caused by changes in the DNA sequence (6). The term “epigenetics”, coined by Conrad Waddington, was originally used to describe heritable changes in a cellular phenotype that were independent of alterations in the DNA sequence. Currently, it is most commonly used to describe chromatin-based events that regulate DNA-templated processes (7).

Epigenetic inheritance is important in many physiological processes, including differentiation, silencing of chromosomal domains such as the X chromosome of female mammals (Xi), stem cell plasticity, aging and genomic imprinting. Genomic imprinting is a “parent-of-origin” specific allele silencing or relative silencing of one parental allele compared with the other parental allele. This process is maintained by differentially methylated regions within or near imprinted genes. Epigenetic abnormalities also provide information about many pathophysiological conditions, including tumorigenesis (8).

Tumorigenesis is regarded as the process whereby cells undergo a change involving uncontrolled proliferation, a loss of checkpoint control tolerating the accumulation of chromosomal aberrations and genomic aneuploidies, and mis-regulated differentiation. It is commonly thought to be triggered by at least one genetic lesion, such as a point mutation, a deletion or a translocation, disrupting either oncogenes or tumor suppressor genes (9). In cancer cells, oncogenes are activated through dominant mutations or overexpression

of a gene, while tumor suppressor genes become silenced. Accumulation of aberrant epigenetic changes, such as DNA methylation, histone modifications and chromatin remodeling, is also associated with oncogenesis. Thus, neoplastic transformation is a complex multistep process that involves the random activation of oncogenes and/or silencing of tumor suppressor genes, through genetic or epigenetic events, and is referred as the “Knudson two-hit” theory (1).

Chromatin provides the scaffold for the packaging of our entire genome and is a macromolecular complex of DNA and histone proteins. The basic functional unit of chromatin is the nucleosome. It contains 147 base pairs of DNA, which is wrapped around a histone octamer, with two each of histones H2A, H2B, H3, and H4. In general and simple terms, chromatin can be subdivided into two major regions: (A) heterochromatin, which is highly condensed, late to replicate, and primarily contains inactive genes; and (B) euchromatin, which is relatively open and contains most of the active genes. Studies have demonstrated that all the components involved in the coordinated regulation of the nucleosome are subject to covalent modification, which fundamentally alters the organization and function of these basic structures of chromatin (10).

Modifications to DNA and histones are dynamically laid down and removed by chromatin-modifying enzymes in a highly regulated manner. There are now at least four different DNA modifications (2, 11) and 16 classes of histone modifications (12,13). These modifications can alter chromatin structure by altering noncovalent interactions within and between nucleosomes. They also serve as docking sites for specialized proteins with unique domains that specifically recognize these modifications. These chromatin readers recruit additional chromatin modifiers and remodeling enzymes, which serve as the effectors of the modification.

The information conveyed by epigenetic modifications plays a critical role in the regulation of all DNA-based processes, such as transcription, DNA repair, and replication. Consequently, abnormal expression patterns or genomic alterations in chromatin regulators can have profound results and can lead to the induction and maintenance of various cancers

Epigenetic Pathways Connected to Cancer

DNA Methylation: Many human genes contain CpG rich regions (CpG islands) at their transcription start sites and are normally unmethylated. Methylation of cytosine of a CpG dinucleotide by DNA methyl transferase (DNMT) enzyme results in repression of gene expression (14). Aberrant methylation of tumor suppressor genes is one of the earliest events in the initiation of tumorigenesis.

The methylation of the 5-carbon on cytosine residues (5mC) in CpG dinucleotides was the first described covalent modification of DNA and is perhaps the most extensively characterized modification of

chromatin. DNA methylation is primarily noted within centromeres, telomeres, inactive X-chromosomes, and repeat sequences (2,15). Although global hypomethylation is commonly observed in malignant cells, the best-characterized epigenetic alterations in cancer are the methylation changes that occur within CpG islands. CpG islands occupy approximately 60% of human gene promoters, most of which are constitutively expressed genes and are defined as a 1000-kb stretch of DNA with GC content greater than 50%. CpG island methylation plays an important role in transcriptional regulation, and it is commonly altered during malignant transformation (2, 15).

Three active DNMTs have been identified in higher eukaryotes. DNMT1 is a maintenance methyltransferase that recognizes hemimethylated DNA generated during DNA replication and then methylates newly synthesized CpG dinucleotides, whose partners on the parental strand are already methylated (16). Conversely, DNMT3a and DNMT3b, although also capable of methylating hemimethylated DNA, function primarily as de novo methyltransferases to establish DNA methylation during embryogenesis (17). DNA-hemimethylation is when only one of two (complementary) strands is methylated. A hemimethylated site is a single CpG that is methylated on one strand, but not on the other. DNA methylation can inhibit gene expression directly, by inhibiting the binding of specific transcription factors, and indirectly, by recruiting methyl-CpG-binding domain (MBD) proteins. These include MBD1, MBD2, MBD3, and MeCP2. These in turn function to recruit histone-modifying enzymes to coordinate the chromatin-templated processes (18).

Although mutations in DNA methyltransferases and MBD proteins have long been known to contribute to developmental abnormalities (15), it is only recently, based on sequencing of cancer genomes, we have become aware of somatic mutations of these key genes in human malignancies. Examples include: the presence of recurrent mutations in DNMT3a in up to 25% of patients with acute myeloid leukemia (AML). DNMT3a, is also mutated in, myeloproliferative diseases (MPD) and myelodysplastic syndromes (MDS). In addition to its catalytic activity, DNMT3a has a chromatin-reader motif, the PWWP (proline-tryptophan-tryptophan-proline) domain, which may aid in localizing this enzyme to chromatin. Somatic mutations in cancer may also affect this domain (19). Importantly, these mutations are invariably heterozygous and are predicted to disrupt the catalytic activity of the enzyme. Moreover, their presence appears to impact prognosis (20).

In cancers, hypomethylation is often associated with oncogenes. c-Myc, a transcription factor that acts as an oncogene, is one of the widely reported hypomethylated genes in cancers. Hypomethylation at specific promoters can activate the aberrant expression of oncogenes and induces loss of imprinting (LOI). The most common LOI

event due to hypomethylation is insulin-like growth factor 2 (IGF2), which has been reported in a wide range of tumor types, including breast, liver, lung and colon cancer (21, 22). S100P in pancreatic cancer, SNCG in breast and ovarian cancers and melanoma-associated gene (MAGE) and dipeptidyl peptidase 6 (DPP6) in melanomas are well-studied examples of hypomethylated genes in cancer (12, 23).

In addition, the transcriptional inactivation caused by promoter hypermethylation affects genes involved in the main cellular pathways: DNA repair [hMLH1 (mismatch repair gene 1), MGMT (O6-methylguanine-DNA methyltransferase), WRN (Werner syndrome, RecQ helicase like), BRCA1 (breast cancer 1)], cell cycle control (p16 INK4a, p15 INK4b, RB), Ras signaling [RASSF1A [Ras association (RalGDS/AF-6) domain family member 1], NORE1A], apoptosis [TMS1 (target of methylation-induced silencing 1), DAPK1 (death-associated protein kinase), WIF-1, SFRP1], metastasis [cadherin 1 (CDH1), CDH13, PCDH10], detoxification [GSTP1 (glutathione S-transferase pi 1)], hormone response (ESR1, ESR2), vitamin response [RARβ2 (retinoic acid receptor β2), CRBP1] and p53 network [p14 ARF, p73 (also known as TP73), HIC-1] among others. This provides tumor cells with a growth advantage and increases their genetic instability and aggressiveness (24, 25).

Histone Modification

Histone modifications influence chromatin structure which plays an important role in gene regulation and carcinogenesis (26). Chromatin consists of DNA, histones, and non-histone proteins condensed into nucleoprotein complexes and functions as the physiological template of all eukaryotic genetic information. Histones are small basic proteins containing a globular domain and a flexible charged NH₂ terminus known as the histone tail, which protrudes from the nucleosome. Regulation of gene expression occurs through posttranslational modifications of the histone tails provided by covalent modifications, including acetylation, methylation, phosphorylation, ubiquitination, sumoylation, proline isomerization, and ADP-ribosylation (12, 27 & 28). Posttranslational modifications to histone tails govern the structural status of chromatin and the resulting transcriptional status of genes within a particular locus. These modifications are reversible and are controlled by a group of enzymes, including histone acetyltransferases (HATs) and deacetylases (HDACs), methyltransferases (HMTs) and demethylases (HDMs), kinases, phosphatases, ubiquitin ligases and deubiquitinases, SUMO ligases and proteases which add and remove such modifications (12, 28). Euchromatin is characterized by high levels of acetylation and trimethylated H3K4, H3K36 and H3K79. On the other hand, heterochromatin is characterized by low levels of acetylation and high levels of H3K9, H3K27 and H4K20 methylation (29-31). Studies have shown that histone

modification levels are predictive for gene expression. Actively transcribed genes are characterized by high levels of H3K4me3, H3K27ac, H2BK5-azacytidine (H2BK5ac) and H4K20me1 in the promoter and H3K79me1 and H4K20me1 along the gene body (31). Genome-wide studies have revealed that various combinations of histone modifications in a specific genomic region can lead to a more 'open' or 'closed' chromatin structure resulting in the activation or repression of gene expression (25, 29).

Histone modification patterns are also altered in human tumors. Recent studies have shown that histone modification levels are predictive for gene expression. Actively transcribed genes are characterized by high levels of H3K4me3, H3K27ac, H2BK5ac and H4K20me1 in the promoter and H3K79me1 and H4K20me1 along the gene body (31). Loss of acetylation is mediated by HDACs that have been found to be over-expressed or mutated in different tumor types. Aberrant expression of both HMTs and HDMs is observed in various cancer types (32). For example inactivating mutations in the histone methyltransferase SETD2 and in the histone demethylase UTX and JARID1C are found in renal carcinomas (32, 33). H3 acetylation and H3K9 dimethylation can discriminate between cancerous and nonmalignant prostate tissue and H3K4 trimethylation can predict occurrence of prostate-specific antigen serum level elevation after prostatectomy for cancer (34). EZH2 (enhancer of zeste homolog 2) expression is an independent prognostic marker that is correlated with the aggressiveness of prostate, breast and endometrial cancers (35).

Non-Coding RNAs

MicroRNAs (miRNAs) are small non-coding RNAs of ~22 nucleotides and are involved in post translational gene silencing by controlling mRNA translation into proteins (36). miRNAs induce heritable changes in gene expression without altering DNA sequence and thus contribute to the epigenetic landscape. In addition, miRNAs can both regulate and be regulated by other epigenetic mechanisms (34). Cancer development and miRNA profiles are now being used to classify human cancers (34, 36, 37). Approximately, 1000 miRNA genes have been computationally predicted in the human genome with each miRNA targeting multiple protein-coding transcripts. It has been predicted that miRNAs regulate the translation rate of more than 60% of protein-coding genes (38), and participate in the regulation of cellular processes. Like mRNAs, miRNAs are mainly transcribed by RNA polymerase-II although miRNA synthesis is known to occur by RNA polymerase-III in those miRNAs that reside near tRNA, Alu and mammalian-wide interspersed sequences (34). The first association between altered expression of miRNA and cancer development was described in chronic lymphocytic leukemia with chromosome 13q14 deletion. This deletion deregulates miRNA-15 and miRNA-16 (39). Most of the targets of these two miRNAs are

involved in cell growth and cell cycle. The let-7 is one of the most widely studied miRNA families in cancer. Alterations of let-7 function have been described in several human cancer types, including carcinomas of the head and neck region, lung, colon, rectum and ovary. It acts mainly as a tumor-suppressor miRNA (34). miRNA-145 is a well-known tumor-suppressor miRNA down regulated in many human cancers owing to aberrant DNA methylation of its promoter and/or p53 mutations (40). This miRNA is a pluripotency repressor which regulates silencing of OCT, SOX2 and KLF4 in human embryonic stem cells; these genes are required for cell self-renewal and pluripotency maintenance (41). Interestingly, it is becoming apparent that the expression of epigenetic regulatory enzymes, such as DNMT, HATs, and HMTs, can be controlled by miRNAs (42). In particular, the miRNA-29 family can directly regulate the expression of DNMTs such that downregulation of this family of miRNAs in small-cell lung cancer results in increased expression of DNMT3a and 3b causing a global genomic hypermethylation and specific methylation-induced silencing of tumor-suppressor genes, such as FHIT and WWOX (43, 44).

Despite the promise of epigenetic therapies, in most cases available therapies lack specificity. Epigenetic drugs targeting DNA methylation or DNMTs show considerable cytotoxicity because these drugs cause global demethylation by passive demethylation or trapping of DNMTs. Such unintended consequences have limited the use of these drugs for prolonged periods of time. Because HDACs and HATs are part of macromolecular protein complexes, targeting them can also lead to unintended consequences. The lack of specificity is in keeping with the fact that epigenetic modifications are not stand alone processes, with synergistic interactions between and within marks increasing complexity of regulatory control. The inhibitors designed to target HMTs and HDMs mainly target cofactors and/or cofactor binding sites, leading to a considerable degree of non-specificity in light of the vast array of enzymes using the same co-factors to catalyze multiple processes. Thus, a more comprehensive structural analysis is needed to identify unique domain(s) and residues critical to the catalytic mechanisms used by these enzymes. Increased understanding of the histone code and the macromolecular protein complexes involved in epigenetic regulation will help refine the targeting of epigenetic therapies. This will involve the design of so called smart drugs that will target only specific epigenetic modifications, either alone or in combination with other marks. Thus, unless our understanding of the epigenome continues to improve and the key regulators of epigenetic control are identified, therapies targeting epigenetic modifications within the human genome will remain of limited value and restricted for widespread dissemination in the clinical setting.

Conclusion

Epigenetics is a rapidly expanding field, and the study of epigenetic regulation in cancer is emerging. Disruption

of the epigenome is a fundamental mechanism in cancer, and several epigenetic drugs have been proven to prolong survival and to be less toxic than conventional chemotherapy. The epigenetic modification patterns associated with the development and progression of cancer are potentially clinically useful. Despite significant advances, challenges remain, including a lack of predictive markers, unclear mechanisms of response and resistance, and rare responses in solid tumors. The development of DNA methylation markers may prove useful for early cancer detection, establishing a diagnosis of cancer, or predicting the prognosis in cancer cases. Recent advances in epigenomic approaches allow mapping of the methylation/acetylation state and miRNA levels in the genome with high accuracy, which may help in the identification of biomarkers for various diseases. Understanding the molecular events that initiate and maintain epigenetic gene silencing could lead to the development of clinical strategies for the prevention and therapy of cancer.

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IN FOCUS

CIRCULATING TUMOR CELLS: A MULTIPURPOSE TOOL FOR BETTER CANCER MANAGEMENT

Circulating tumor cells (CTC) are the cells shed from the primary tumor which gain access to circulation and are prone to seeding the hospitable sites to generate metastasis. Their presence portends poor prognosis, signifying metastasis or potential for metastasis. At least in breast, colon and prostate these cells gain access to circulation even before presentation. No wonder, these cancers are considered systemic diseases from beginning. Besides being part of the tumor once, these carry the genetic signatures of the primary tumor albeit with the diversity created by clonal evolution. It is also possible that only the worse and the most mobile clones circulate which otherwise may get obscured in the enormous population of lesser villains at the primary site. Recognizing these may mean learning the worst capabilities of the foe. Irrespective, these cells once enumerated and harvested supply us with precious information on several counts as listed below:

- a.) Prognosis
- b.) Prediction
- c.) As liquid biopsy to test for molecular biomarkers and detect acquired mutations following targeted therapy.
- d.) To monitor response to therapy (intermediate end points of response to therapy) and intermediate end point in pharmacodynamics and drug discovery.

e.) As companion to imaging in determining need and benefit of surgery.

Before embarking upon the discussion on role of CTC, one may be interested to know the methods to assay these cells. These cells, like proverbial “needle in the haystack” are too few to be counted by routine methods of blood cell enumeration. Rough estimates suggest that one tumor cell reaches circulation for every $10^5 \times 10^7$ tumor cells and this one too is diluted amongst billions of haemic cells providing a final concentration of 1/million to 100million cells in bloodstream. That is the problem. However, ingenious use of several attributes of these cells and sound principle of physical sciences have allowed many platforms to enrich these cells as a first step. Some use immunoaffinity using magnetic beads usually coated with epithelial cell antibody(s) like EPCAM, MUC1 or by placing columns coated with the similar antibodies and creating turbulence to enhance contact time for antigen-antibody binding to occur and improve cell capture. Others rely on size and deformability using microfluidics and else using density based centrifugation methods for enrichment. Next step of enumeration uses flowcytometry, molecular methodology or electrochemical properties for counting. The basic methodology of CTC enumeration is depicted in Fig 1.

Several methods (Table 1) have been launched and used in research settings. However, lack of analytic validity leads to variable counts which preclude comparison across platforms and has been a source of consternation. It is also yet undetermined as to how the CTC count or the harvested cells themselves can be

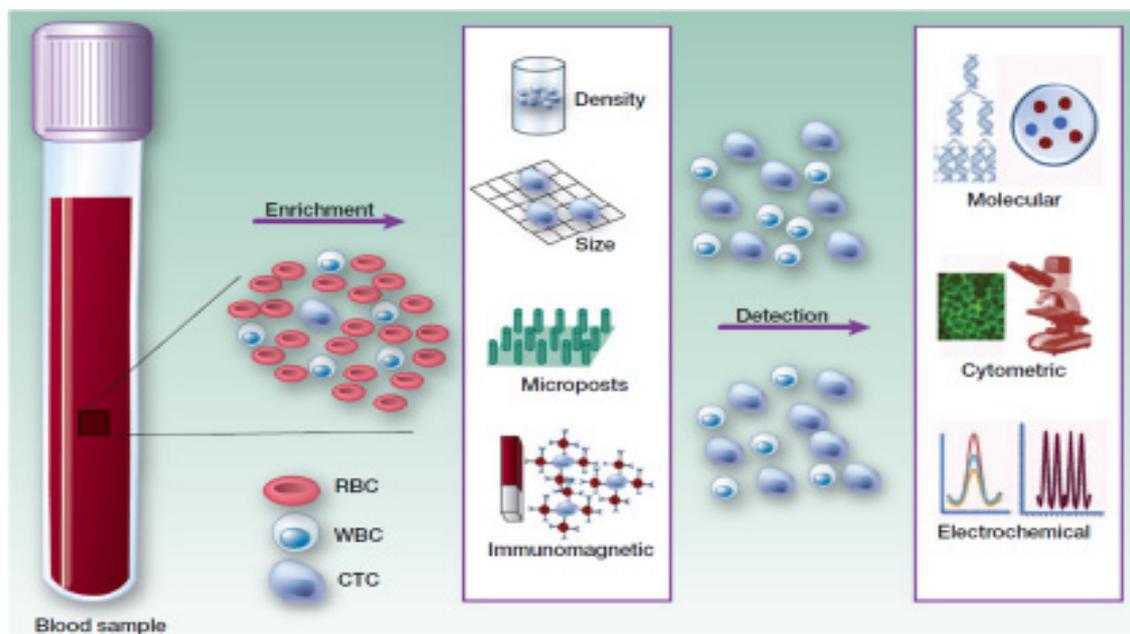


Fig 1: Principle of CTC counting depicts stages of enrichment and enumeration

Table 1

Platform	Vendor/ Developer	Methodology
Immunoaffinity Assay: Positive Selection using Antibody(s) against Epithelial Cell Antigen		
Cell Search	Veridex	Epcam-coated beads based positive selection using magnetic beads followed by staining and image analysis. Clinically validated in metastatic breast, colorectal & prostate cancer. Only FDA approved platform.
Adna Test	Adnagen	Immunomagnetic bead enrichment (EPCAM, MUC-1, mesothelin) followed by nested PCR
Anti-EPCAM, Anti-CK Antibody	Glenn Deng, Stanford University	CTC enrichment assay using the combination of anti-CK and anti-EPCAM antibodies
Cell Collector	Gilupi	Functionalized structured medical wire coated with anti-epcam antibodies placed directly into the blood stream of a patient via an indwelling catheter. Stays in the arm vein for 30 minutes and thus enables the capture of CTSCs in vivo.
Biofluidica ctc	Biofluidica	EPCAM coated chip to capture EPCAM expressing cells followed by elution & electrical counting
Epispot	Laboratoire de virologie	Initial depletion of CD45 followed by EPCAM expressed selection
Microfluidic Devices		
Oncocoe	Biocept	Biotin-tagged antibodies that bind selectively to CTCs
Clearcell	Clearbridge	Label-free technology that uses lateral traps to capture tumor cells based on size and deformability
Herringbone- chip	Daniel Haber and Mehmet Toner	Microvortices are used to significantly increase the number of interactions between target CTCs and the antibody-coated chip surface
De novo Sciences Jetta 400	Wayne Klohs Sunitha Nagrath Gil Omenn David Parkinson Ken Pienta	CTC isolation is achieved by flowing a sample over a proprietary designed set of 56,320 microfluidic capture chambers. The Systems will then characterize the cells for downstream analysis.
Size based devices		
Screencell	Screencell	Microporous membrane filter allows size selective isolation of CTCs
Cellsieve	Creatv microtech	Lithographically fabricated filters with precision pore dimensions
Size and Deformability		
Parsortix	Angle	Uses size and deformability using a wier-type step filter
Density		
Oncoquick	Greiner bio one	Porous barrier density gradient centrifugation technology
Immunomagnetic and physical properties		
Magsweeper	Stanford University	Immunomagnetic enrichment of target cells. Individual extraction of isolated cells based on their physical characteristics

utilized for clinical decision making and what shall be the impact of such decision(s) on the outcome of therapy. A lot needs to be learnt before CTCs become an immaculate example of translational medicine. All said, CTCs hold enormous promise as multifunctional biomarkers in cancer management and in drug development by becoming intermediate endpoint of response determination.

Role of Circulating Tumor Cells in Prognosis

A major issue in cancer management is establishing an accurate prognosis, ie, anticipating course and outcome of the disease. This is the question that not only dogs the physician but also the patient. Currently, the ability to provide an accurate prognosis depends largely on the TNM stage supported in several organs by additional morphological features like angiolymphatic emboli, depth of invasion, pattern of invasion at leading edge, perineural invasion and margins of resections. The list keeps expanding, underlining the fact that perfect formula for prognostication has not been achieved.

Can “Circulating Tumor Cells” (CTCs) provide an improved prognostic exactness? A German group studied 35 women with “early breast cancer” & enumerated their CTCs prior to any treatment, of whom 17 tested positive for CTC and 18 tested negative. Followup data showed that the group that tested negative for CTC had a median overall survival of 125 months. In contrast, the group with 5 or more CTC/7.5 ml of blood had a median overall survival of only 61 months.

In one more study reported in *Lancet Oncology* which included 302 chemo-naïve patients with stage I to III operable breast cancer undergoing surgery for their primary tumours between February 2005 and December 2010 at MD Anderson Cancer Center, Houston showed that “Detection of one or more circulating tumor cells at start of therapy predicted both decreased progression-free survival (log-rank $P = .005$; HR = 4.62, 95% CI = 1.79–11.9) and overall survival (log-rank $P = .01$; HR = 4.04, 95% CI = 1.28–12.8)”. At 2 years, progression-free survival was 87% among the 73 patients with one or more circulating tumour cells versus 99% in the 229 patients with none. Progression-free survival was 79% among the 29 patients with two or more CTCs and 69% among the 16 patients with three or more CTCs. Overall survival was 99% in patients with no CTCs, 94% in patients with one or more CTCs, 89% in patients with two or more, and 81% in patients with three or more.

This stark difference in OS in early breast cancer also extended to metastatic breast cancer as published by Cristofanilli et al. The study population of 177 received either chemotherapy or hormone therapy, either as first-line or a subsequent line of therapy. A cut off threshold of ≥ 5 CTC/7.5 ml was reported as binary notation capable of conferring prognostic significance. CTC positivity (≥ 5 CTC/7.5 ml) was independently associated with a poorer prognosis before initiation of the new line of therapy but also after 3 or 4 weeks of treatment. Combining these two sequential CTC counts, patients with a baseline CTC count ≥ 5 CTC/7.5 ml but < 5 CTC/7.5 ml on treatment had a much better prognosis than those with a persistent CTC count ≥ 5 CTC/7.5 ml. In contrast, patients who developed ≥ 5 CTC/7.5 ml after initiation of the new line of therapy converted to a poor prognosis. Subsequent analyses of this cohort suggested that the prognostic value of CTCs was independent of early assessment by conventional clinical and imaging criteria.

In a similar study at M. D. Anderson Cancer Center; CTCs were counted in 151 patients of metastatic breast cancer. These patients were also evaluated for other prognostic cancer markers like hormone receptor and HER2 status along with CA 27.29. Cases with 5 or more circulating tumor cells (CTCs) had a median overall survival of 13.5 months. The median overall survival for those with less than 5 CTCs was above 29 months. The research group rested their case stating that CTCs have superior and independent prognostic value.

Further, recent research indicates that CTC evaluation can be used to predict prognosis for men with prostate cancer. Researchers at Thomas Jefferson University compared the levels of CTC in 37 men with metastatic prostate cancer. Their findings were noteworthy. For men with 5 or more CTCs, the median overall survival was only 8.4 months while for men with less than 5 CTC the median overall survival was 48 months. Yet another study measured CTCs in 55 men with a rising PSA after surgery for prostate cancer. A rising PSA after surgery is strongly predictive of prostate cancer recurrence. Radiation therapy was administered to 15 patients. Of these prostate cancer patients, 60% who were CTC positive had progression of the disease during radiation therapy, while there were no disease progressions in the CTCs negative group. Additional studies have confirmed these results.

In another global meta-analysis of 12 articles containing survival outcomes and clinical characteristics and 15 articles containing only clinical characteristics of

lung carcinoma, demonstrated hazard ratio (HR) for OS predicted by pretreatment CTCs was 2.61 [1.82, 3.74], while the HR for PFS was 2.37 [1.41, 3.99]. The HR for OS predicted by post-treatment CTCs was 4.19 [2.92, 6.00], and HR for PFS was 4.97. Subgroup analyses were conducted according to histological classification and detection method. Odds ratio (OR) showed the appearance of pretreatment CTCs correlated with the lymph node status, distant metastasis and TNM staging while post-treatment CTCs correlated with TNM staging only. The authors concluded that detection of CTCs in the peripheral blood indicates a poor prognosis in patients with lung cancer.

Similar results have been replicated in colorectal and pancreatic cancer. In a study by Romiti A, published in *J Gastrointest Liver Dis*, a total of 75 colorectal cancer patients were enrolled, including 54 stages I-III and 21 stage IV patients. Overall, 21 (28%) patients had a positive CTC count at baseline, significantly associated with a worse prognosis as compared to a negative status (OS: 36.2 vs 61.6 months; $P = 0.002$). CTC count remained positive after chemotherapy in 22.4% of the patients and it was an independent prognostic factor of OS ($P = 0.03$; Hazard Ratio: 3.55; 95% CI: 1.1-11.5). The authors surmised that the presence of CTCs is associated with a reduced survival in colorectal cancer patients.

Role of CTCs has also been evaluated in pancreatic and neuroendocrine carcinoma with same prognostic implication albeit with different threshold cut-off.

The value of CTCs in forecasting prognosis appears to be on solid terra-firma. "As the number of cells increases, so does the risk". If you have more cells in circulation, the odds of developing distant metastases increase. That message appears loud. Yet, it must be admitted that no recommendation or guidelines have been crafted for evidence based usage of CTCs for prognostication.

Role in Prediction (Likely Response to Therapy)

The predictive role of CTCs has largely been studied in breast and especially in relation to HER2 expressing tumors. a) In a study conducted by Mario Giuliano at Fox Chase Hospital, Philadelphia of the 148 patients with HER2 non amplified disease who were treated with chemotherapy: 64 (43.2%) received combination chemotherapy, 45 (30.4%) received single-agent chemotherapy, and 39 (26.4%) were treated with chemotherapy plus bevacizumab. The treatments were

selected according to patient characteristics (such as age, co-morbidity) and the traditional predictive markers in use at the time of therapy administration. A hypothetical predictive value for CTCs, comparing different treatments combination, was compared with low (< 5) or high (≥ 5) baseline CTC counts. Combination chemotherapy was superior to single-agent chemotherapy, in terms of PFS, in both the CTC groups, although the benefit provided by combination regimens was primarily confined to patients with CTCs ≥ 5 . With respect to OS, combination chemotherapy was superior to monochemotherapy only in patients with CTCs ≥ 5 , but the heterogeneity between the two subgroups was not statistically significant. Furthermore, the association of chemotherapy with bevacizumab was superior to monochemotherapy, regarding PFS, but only in patients with a high baseline CTC count (HR = 0.88, 95% CI = 0.42 to 1.83 in patients with CTCs < 5 ; and HR = 0.28, 95% CI = 0.12 to 0.64, in those with CTCs ≥ 5 ; test for heterogeneity $P = 0.04$).

Alteration in Receptor Status in MBC

The estrogen receptor (ER), progesterone receptor (PgR) and HER2 receptor are the three biomarkers used in breast cancer management. ER, PgR and HER2 expression help make an informed decisions regarding therapy. ER and/or PgR positive disease earn Endocrine therapy while Trastuzumab and other anti HER2 drugs are used for tumors overexpressing HER2. Currently, treatment decisions at the time of MBC relapse are generally made based on the receptor status of the primary breast cancer. However, discordance in receptor status between primary tumor and disease recurrence has been observed in up to 10% of patients. It is either because of clonal evolution, a consequence of an unstable genome of the cancer cells or due to an already existent clone of HER2 positive cells overwhelmed at primary site by their rarity but observed in circulation because of its higher mobility and aggressive potentials. How shall those patients be treated who are negative for HER2 amplification at primary site but exhibit amplification in CTCs? As yet, there are minimal data addressing this issue. Meng et al, retrospective study on 24 patients with MBC and HER2" primary tumour, reports that four of nine patients with HER2+ CTCs at the time of metastatic disease received trastuzumab. Of these, one had rapid remission of symptoms and complete response on imaging, two patients had partial responses and one no response.

The question remains open and continues to beg the answer. DETECT III randomised phase III trial (NCT01619111) shall answer this question. This

multicentre study compares standard therapy with or without lapatinib in patients with MBC after HER2⁺ primary tumor but with HER2⁺ CTCs. Should the DETECT III trial demonstrate an advantage for the addition of anti-HER2 therapy in the setting of aberrant HER2 gain, treatment for patients might also potentially expand to include other anti-HER2 agents.

In a study, presented at 2012 ASCO Annual Meeting, Dr Lucci’s team reported finding HER2-positive cells in the blood of patients with HER2-negative disease. The team reports that “It is actually a parallel study, where we took the blood sample and we looked at the markers on that blood sample to see if the HER2 status of the circulating tumor cells is the same or different from the primary tumor. What we found was that in a significant number of patients, you can find changes in the circulating cells—either HER2 amplification or HER2 loss—that are different from the primary tumor.” This suggests that either: (1) We have to look at the primary tumours more carefully to ascertain if they are really HER2-negative or -positive; or (2) Some patients may have a change in the HER2 status of certain cells released into the circulation. We don’t currently know exactly why these HER2-positive cells are found in circulation, but the images are quite clear, and thus it opens up a whole new area for research. That is something that could affect treatment in the very near future, and we are continuing that study currently.”

At present one can, therefore conclude that predictive significance of CTCs is not well defined and there is no hard evidence to recommend search for predictive biomarkers in CTCs. But let us accept ‘it is just the beginning’ and more shall follow with concrete recommendations.

CTC as Liquid Biopsy

Imaging allows obtaining a biopsy from most metastatic sites sans a few and is relatively cheap and standard of care but, fraught with serious morbidity occasionally and a rare fatality. In light of this, analysis of biomarkers on CTC is an attractive option and has been alluded to as liquid biopsy (Table 2). Take the example of non small cell lung carcinoma on targeted TKI for EGFR that develops acquired resistance. In such a situation, obtaining a biopsy from site of progression will be highly resented by the patient. He may agree readily if same can be checked out on CTCs. Serial assessment of biomarker status therefore can only be realistically obtained from a less invasive procedure like harvested CTCs. The barriers to use of liquid biopsy however are many like cost, availability, validated platforms and clinical validity and utility of test outcomes. But, needless to say that concept is appealing.

Monitoring Response to Therapy

Monitoring response to therapy in breast cancer at diagnosis, after one cycle of chemotherapy and at the end of one year has been used to prognosticate. But, can the CTC count after one cycle of chemotherapy be used to monitor response and make decision on effectiveness of therapy and bring in early change if ineffective? SWOG S0500 is a phase III trial that studied treatment decision made based on blood levels of tumor cells in women with metastatic breast cancer receiving chemotherapy. The primary objectives of the trial being: i) whether women with metastatic breast cancer and elevated circulating tumour cells (CTCs) (≥ 5 per 7.5 mL of whole blood) after 3 weeks of firstline chemotherapy derive increased overall survival from changing to an

Table 2: Merits and Demerits of Liquid Biopsy versus Conventional Biopsy

Tissue biopsy	Liquid biopsy
Invasive, infrequent morbidity & rare fatality	Minimally invasive
Monitoring treatment response/disease course with multiple biopsies impractical. Not patient centric	Easy. Patient friendly. Not likely to be resented by patient
Good material. Can be used for several analyses	Low yield will mean assessment of a few or single analyte.
No specialized analytical equipment required	Specialized analytical equipment required
Can be performed at the vast majority of treatment centers	Can only be performed in certain laboratories equipped for CTC analysis
All evaluations for biomarkers have well established clinical utility	Clinical utility of biomarker testing on CTCs not as yet established

alternative chemotherapy regimen at the next course rather than waiting for clinical evidence of progressive disease before changing to an alternative chemotherapy regimen; ii) whether these patients derive increased progression-free survival (PFS) from changing to an alternative chemotherapy regimen at the next course rather than waiting for clinical evidence of progressive disease before changing to an alternative chemotherapy regimen; iii) confirm previous findings that patients with < 5 CTCs per 7.5 ml of whole blood on initial screening have longer median OS and PFS than patients with < 5 CTCs per 7.5 ml of whole blood; iv) determine the prognostic value of sequentially collected CTC values in these patients; v) Compare toxicity between patients with and without elevated CTCs after 3 weeks of first-line chemotherapy and between the two randomized treatment arms. This study confirms the prognostic significance of CTCs in patients with MBC receiving first-line chemotherapy. For patients with persistently increased CTCs after 21 days of first-line chemotherapy, early switching to an alternate cytotoxic therapy was not effective in prolonging OS. However, the survival was superior in those who had lower count at 21 days following first cycle of chemotherapy.

This important study therefore confirms the prognostic significance of the CTCs but fails to confirm the value of switching regime following persistence of raised counts post first cycle of chemotherapy.

It can therefore be concluded that while CTCs at 21 days following first cycle of chemotherapy are of prognostic significance and have the potential for intermediate endpoint in pharmacodynamics the count is of no value currently in making therapy altering decision.

As Companion to Imaging in Determining Need and Benefit of Surgery

Investigators have shown that CTCs evaluation may be more accurate than imaging used to evaluate the effectiveness of treatment in metastatic breast cancer. In a pioneering work performed in 2006, metastatic breast cancer patients had imaging tests done before and 10 weeks after they began therapy. The results of the imaging tests were reviewed by two independent radiologists. CTCs were measured 4 weeks after the start of therapy. The results were amazing. The group that responded to treatment based on imaging tests but had 5 or more CTCs suffered a poorer outcome than the cohort with CTCs counts below 5 but less definite response on imaging. These findings suggest that the levels of CTCs were far more important at predicting

survival compared to the actual visual changes noted on imaging tests. Additionally, there was a 15% disagreement in the interpretation of the imaging tests between the two radiologists, compared to less than 1% variation in the results of CTC testing. The precision of CTCs enumeration coupled with superior response predictor demonstrates the potential of CTCs *vis a vis* radiologic studies, and seems to be a more robust predictor of survival than is radiographic response.

Conclusion

CTCs enumeration at several timelines is a valuable prognostic marker. The value however, as predictive marker has yet not solidified. The increasing use of precision molecules in treatment of cancer and acquired resistance thereof may propel the use of liquid biopsy to seek secondary mutations. Whether the liquid biopsy can reliably and accurately mirror the changes in the tumor sites is a question that needs to be answered. However, this seems to be an important potential use. With thousands of targeted molecule in development an early intermediate endpoint will be handy in speedy launch of newer drugs. This proposition seems plausible and can provide giant leap in growth of targeted therapy.

The methods of enumeration and harvesting CTCs are many but lack analytical validity which in any case is by comparison to cell search system (the only FDA approved system) which itself has received criticism for relying on EPCAM based positive selection the expression of which may actually be suboptimal during epithelial-mesenchymal transformation. A new system 'Denovo Jetta-400,' is pending approval by FDA which has the potential to separate CTCs on the basis of physical attributes with identification and biomarker evaluation on the harvested material. Such new systems which permit flexibility in assessment of CTCs may hold the future. Aptamers binding DNA/RNA of interest may also offer improvement over selection by capturing cellular antigens which may express variably during epithelial mesenchymal transformation.

There are several challenges to making CTCs as multifunctional cancer biomarker but such challenges also provide opportunity for innovative and ingenious discoveries. The limit of science is decided by the ability of the man's mind to think and once the mind is seeded by a new thought, the answers will emerge. History bears testimony to this fact.

(Dr Anurag Mehta, Director Laboratory Services, Dept of Pathology)

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