

World Journal of *Translational Medicine*

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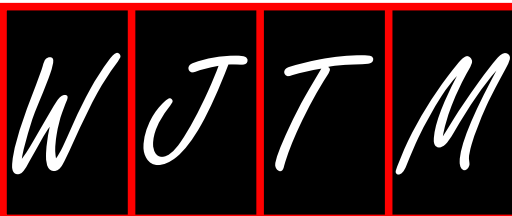
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Beyond the bedside: A review of translational medicine in global health

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syndrome, and non-communicable diseases. Laboratory research has excelled in many of these areas and is struggling in a few. Where successful therapies have been discovered there are often problems with appropriate use or dissemination to groups in need. Also, many diseases would be better prevented from a population health approach. This review highlights successes and struggles in the arena of global health, from smallpox eradication to the impending epidemic of cardiovascular disease, in an attempt to illustrate of the various phases of translational research.

Key words: Global health; Human immunodeficiency virus; Translational research; Vaccines; Cancer; Non-communicable diseases

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Core tip: This review summarizes efforts in translational research as applied to the major global health issues of vaccines, human immunodeficiency virus and acquired immunodeficiency syndrome and non-communicable diseases. Historical perspective as well as current efforts are presented in an effort to describe the success and challenges that are concurrent with translational medicine on the international stage.

Abstract

Translational research is a broad field of medicine with several key phases moving from scientific discovery to bench research and the hospital bedside, followed by evidence-based practice and population-level policy and programming. Understanding these phases is crucial when it comes to preventing and treating illness, especially in global health. Communities around the world struggle with a variety of health problems that are at some times similar and at others quite different. Three major world health issues help to outline the phases of translational research: vaccines, human immunodeficiency virus and acquired immunodeficiency

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INTRODUCTION

To address the breadth of translational research, the United States National Institutes of Health has recently endorsed a 5-phase model that describes the process

of moving from scientific discoveries to population health (Figure 1)^[1]. The process starts with scientific discovery of a problem or pathology, termed T0. From there, T1 and T2 encompass the classic “bench to bedside” process of finding a candidate treatment, test, or clinical intervention (T1) and then comparing safety and efficacy of the candidate against a placebo or existing therapy in randomized trials or other study designs (T2). Lastly, T3 research focuses on implementation and dissemination of evidence-based interventions and T4 examines population-level health impact and cost-effectiveness^[2].

The purpose of this review is to describe the impact of the phases of translational research on global health. Communities around the world suffer from health issues that are at times very different but can also be quite similar. Some therapies are readily available in resource-rich countries but scarce in less affluent countries. Other therapies simply do not work in certain parts of the world due to disease specificity or cultural issues. Though there are many blights, three major issues affecting the health of the global population are vaccine development, the human immunodeficiency virus and acquired immunodeficiency syndrome (HIV/AIDS), and non-communicable diseases (NCDs). Here we will review the history and current status of research in these areas, highlighting the role of various phases of translational research with respect to their effects on global health.

VACCINES

Early successes

The history of vaccine development and distribution exemplifies translational research. Edward Jenner discovered the smallpox vaccine in the 1790s by treating patients with “matter” from the sores of cowpox^[3]. This technique of sharing the live cowpox virus between patients lasted through the 19th century until the development of a live attenuated vaccine in the early 20th century^[4]. In the coming decades the World Health Organization (WHO) would sponsor a smallpox vaccination program, and eradication of the virus was formally declared in 1980 (the only other disease to be declared eradicated is Rinderpest, an RNA virus that affected cattle and water buffalo, mostly in Africa; vaccines for this virus were developed in the early 20th century and two major attempts at mass vaccination led to eradication in 2011).

A second vaccination “victory” resulted from the work of one of the fathers of bacteriology, Robert Koch, who demonstrated that the bacterium *Bacillus anthracis* was the cause of “wool-sorters’ disease” in 1876^[5]. Anthrax had plagued livestock for millennia, and humans involved in wool and hide processing were at risk of infection. Following this discovery, Louis Pasteur described a randomized controlled trial in which he treated livestock with an attenuated anthrax vaccine prior to inoculating them with a virulent strain

of the bacteria^[6]. The results were dramatic; 48 h after inoculation, all vaccinated sheep survived and all un-vaccinated sheep were dead. Virtual eradication was made possible by livestock quarantining and vaccination, but recent terror attacks using the anthrax spore have generated interest in newer vaccines^[7-9]. While the vaccine is only available for at-risk patients (veterinarians, researchers, certain military personnel, etc.) due to difficulties with production and storage, research is underway to develop a stable, needleless vaccine for widespread use^[10].

Works in progress

Worldwide, diarrheal illness is the second leading cause of death in children under the age of five years (760000 deaths each year)^[11]. There are many causes of diarrhea and a significant portion of the disease burden can be prevented through public health efforts to create safe drinking water and adequate sanitation. Infectious causes are well described, and three major sources have been the focus of vaccination efforts in recent decades.

Rotavirus is a leading cause of child mortality worldwide, especially in low-income regions; in children under the age of five in 2008, 5% of all mortality and over one-third of diarrhea-related mortality were attributable to rotavirus infection. Early experiments led to development of monovalent live-oral vaccines with variable success^[12]. In 1998 the rhesus rotavirus tetravalent vaccine (RRV-TV) was licensed for administration in children after successful trials. However, after several cases of bowel obstruction and intussusception following vaccine administration, the Centers for Disease Control and Prevention advised against using the vaccine and in 1999 the manufacturer withdrew the vaccine from market^[13]. Since that time, three live-attenuated oral vaccines have been approved for use. The monovalent (RV1) and pentavalent (RV5) rotavirus vaccines have been evaluated in several large trials and subsequently approved for use in most countries including the United States and the European Union^[14]. A third, the Lanzhou lamb rotavirus vaccine, has been approved for use in China only^[15].

Another major cause of diarrheal illness is typhoid fever, caused by *Salmonella enterica typhi* (*S. typhi*). Two vaccines, injectable (Vi PS) and oral (Ty21a), have shown efficacy and safety in clinical trials and field settings in Chile, Indonesia, and India, but are not ready for widespread immunization protocols^[16]. The Vi PS vaccine is non-immunogenic in children under 2 years, and many *S. typhi* strains are negative for the Vi polysaccharide. The Ty21a vaccine is not recommended for children under 5 years, and its acid-labile nature creates challenges with oral administration. Several newer typhoid vaccines are currently in phase 1-3 trials worldwide, but not licensed for use^[17].

Cholera, caused by *Vibrio cholerae*, is another area of focus for vaccine manufacturers. Dukoral®, an oral killed whole-cell vaccine, was licensed after

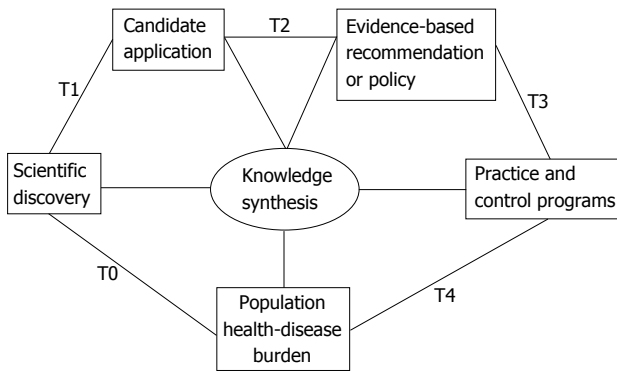


Figure 1 Epidemiology and the phases of translational research. T0: Scientific discovery research; T1: Translational research from discovery to candidate application; T2: Translational research from candidate application to evidence-based recommendation or policy; T3: Translational research from recommendation to practice and control programs; T4: Translational research from practice to population health impact. Source: Khoury *et al.*^[2]

a large randomized controlled trial in Bangladesh in 1990^[18]. The per-dose cost of United States \$5.25 was felt to be prohibitive for use in low-income regions, and subsequent development of Shanchol® at United States \$1.85 per dose was deemed fiscally feasible; Shanchol® use was then adopted after randomized controlled trials in Vietnam and India confirmed safety and immunogenicity^[19,20]. Four single dose, live attenuated oral cholera vaccines are in active clinical programs with hopes to improve efficacy, hasten onset and increase duration of protection^[21].

Ongoing challenges with "the big three"

Despite some vaccination success as a result of collaborative translational research implementation, malaria, tuberculosis (TB), and HIV/AIDS are three of the top ten causes of death worldwide^[22]. As such, much attention and funding has been directed towards finding vaccines for these diseases. Progress has been made, but there are still significant challenges.

There are approximately 250 million reported cases of malaria every year, including almost one million deaths in Sub-Saharan Africa, mostly in children^[23]. Many different vaccines are currently in various trials and they all face a similar challenge; *Plasmodium falciparum*, the causative agent of malaria, has a complex life cycle, with polymorphic antigens expressed in separate phases of the cycle^[24]. The best current vaccine candidate, RTS,S/AS01, is a combination of a portion of the circumsporozoite protein that helps the parasite invade human liver cells and the hepatitis B surface antigen, as well as the liposomal formulation adjuvant AS01. A phase II b trial of the RTS,S malaria vaccine showed safety and efficacy at 20 mo^[25] and phase III trials have demonstrated 31%-56% efficacy for one year, with protection from clinical malaria for at least 3.5 years^[26,27]. However, efficacy of the vaccine wanes with time and also varies based on the age of the vaccinated child^[28]. Despite mediocre results,

RTS,S will likely be the first malaria vaccine to receive regulatory approval^[29].

The bacille Calmette-Guérin (BCG) vaccine for *Mycobacterium TB* is one of the earliest developed vaccines and has been given to over four billion people to date^[30,31]. Despite this fact, TB kills 1.4 million people annually and drug-resistant TB is becoming a major problem^[32]. BCG protects infants from tuberculous meningitis and miliary TB, but is less effective against pulmonary TB in adolescents and adults. There are currently almost twenty candidate vaccines in various phases of clinical trials, all designed to prevent active TB disease^[32,33]. Some of these are live recombinant vaccines that have been genetically engineered for enhanced efficacy and/or safety, meant to replace BCG. Others are proteins or viral vector expressing antigens that are meant to serve as an immune booster following initial treatment with the BCG vaccine^[24]. A common challenge among trials evaluating TB vaccines is that the disease has a long latent period; thus, trialing preventative vaccines is slow and expensive^[31].

HIV kills two million people annually and infects approximately 7000 people per day, making it one of top causes of death worldwide^[34]. Naturally, a significant portion of the world's research dollars are directed toward treating and preventing this disease. Challenges facing researchers looking for a vaccine against HIV include: global variability of HIV, lack of a validated animal model with appropriate immune response, large variety of infected cells that develop as a result of HIV genome integration into the host's DNA, and destruction of immune cells by HIV^[24]. Phase III trials of most vaccines have failed to show efficacy^[35,36] or reduce viral loads^[37,38], and some have actually shown increased HIV infection among vaccine recipients^[39]. The most exciting results are from a randomized controlled trial comparing placebo to a recombinant canarypox vector vaccine (ALVAC-HIV) and two boosters of a recombinant glycoprotein 120 subunit vaccine (AIDSVAX B/E). In a population of greater than 16000 healthy Thai volunteers, this AIDSVAX B/E showed 31% vaccine efficacy vs placebo by reducing the cumulative probability of infection, but did not reduce viral loads^[40]. Nevertheless, finding a safe and effective vaccine against HIV is proving to be one of the most daunting tasks in research today^[41].

Targeted efforts in resource-poor environments

Even when early-phase translational research has found a safe and effective vaccine for a given disease there are still challenges to widespread availability, and the major barrier to vaccine development for low-income regions is cost^[42]. Patients in poor countries cannot afford new, expensive vaccines and pharmaceutical companies are not incentivized to invest capital in developing treatments that will be too expensive for the prospective customers to afford^[43]. Not only that, but the opportunity costs of delaying more profitable projects

are not appealing to industry. To solve this problem, major institutions in global health came together in 1999 to create the Global Alliance for Vaccines and Immunization (GAVI)^[44]. One aim of the alliance is to support new vaccine research, as outlined by their approach to meningitis.

The North African “meningitis belt” is an area that includes countries from Senegal to Ethiopia with a population of around 350 million people^[45]. Annual outbreaks in these countries claim hundreds to thousands of lives and are caused mostly by *Neisseria meningitidis* (*N. meningitidis*) serogroup A^[46]. Controlling these outbreaks requires identifying the culprit strain of *N. meningitidis* and producing a specific polysaccharide vaccine to treat the population at risk. These polysaccharide vaccines are poorly immunogenic in young children, do not prime immunologic memory, and do not lead to “herd immunity”^[47]. To combat this problem, in 2001 the Bill and Melinda Gates Foundation gave United States \$70 million to the WHO to establish the Meningitis Vaccine Project^[48]. The goal was to eliminate epidemic meningitis in Africa through the development of a serogroup A meningococcal conjugate vaccine that would cost less than United States \$0.50 per dose. Vaccine development began in 2003, clinical phase 1-3 trials were completed, and in 2010 MenAfriVac™ was licensed for use in populations aged between one and 29 years; large-scale immunization campaigns began immediately. In 2011, no case of meningococcal A disease occurred in a vaccine recipient in Burkina Faso and the percentage of meningococcal infections in Niger due to serogroup A dropped from 98.6% to less than 2%^[49].

The GAVI alliance has also addressed hepatitis B virus (HBV) in China. HBV is a significant problem in low-income countries, and China accounts for up to half of the HBV-related deaths worldwide^[50]. Over 260000 people die annually in China from HBV-related liver cancer and cirrhosis. A full 60% have a history of infection, and around 10% are chronic carriers^[51]. However, because of high costs, vaccination rates were substantially higher in major cities and wealthy provinces in Eastern China. In 2002, China added HBV vaccination to its National Immunization Programme, and at the same time the China Ministry of Health teamed with GAVI to start the China-GAVI project with a goal of providing free HBV vaccination to people in the poor and western provinces of China. From 1997-2003, overall vaccination coverage increased from 70.7% to 89.8% and timely coverage increased from 29.1% to 75.8%. In the 22 provinces targeted by the China-GAVI Project, timely coverage increased from 64% in 2004 to 81% in 2006, and complete coverage increased from 52% in 2001 to 92% in 2006^[52]. National HBV vaccination programs have had similar effects in other countries^[53] and have been shown to greatly reduce the incidence of hepatocellular carcinoma in these populations^[54].

These examples of meningitis and HBV illustrate

the benefit of targeted funding towards developing specific therapies or distributing existing therapies to a population in need. Fifty years after the Sabin and Salk vaccines were developed, polio has been eradicated in much of the world and vaccine campaigns are now addressing the few remaining countries with recently documented cases^[55,56]. The Bill and Melinda Gates Foundation has given United States \$1.5 billion to the Children's Vaccine Program for research initiatives in malaria, TB, diarrheal diseases, measles, hookworm, and meningitis^[56]. GAVI is targeting the 74 poorest countries in the world with a three-fold approach: improving vaccination infrastructure, purchasing necessary vaccines, and supporting research and development^[56].

For many diseases, notably HIV, TB, and malaria, challenges in vaccine development still need to be overcome. For many others, effective vaccines exist and simply need to be distributed effectively. Through this combination of research and distribution it is possible to use discoveries in the lab to prevent and even eliminate the burden caused by these historically tragic diseases.

HIV/AIDS

In 1981 scientists discovered HIV as the causative agent of AIDS, typified by uncommon opportunistic infections in otherwise healthy young men^[57]. Since then, highly active antiretroviral therapy (HAART) has been proven to significantly reduce morbidity and mortality by suppressing HIV replication and improving CD4⁺ T cell counts. Population studies in developed as well as developing countries have shown a significant effect of HAART treatment on reductions in both viral load as well as HIV transmission and new diagnoses^[58-63]. The WHO has made evidence-based recommendations for HIV treatment and prevention^[64] and the international community has contributed substantially through organizations such as Global Fund to Fight AIDS, TB and Malaria and PEPFAR, the President's Emergency Plan for AIDS Relief^[65]. Despite this progress, over two million people per year become newly infected with HIV worldwide^[66].

A major barrier to defeating HIV is the highly mutagenic and drug-resistant nature of the virus^[64]. The availability of fixed-dose combination pills and simplified treatment schedules can decrease resistance development by increasing adherence to HAART regimens, but resistance is still developing and can be difficult to monitor^[67]. Genotypic testing and viral load monitoring are often not available in resource-limited settings due to cost-constraints and lack of adequate technology. As such, in resource-limited settings the WHO recommends monitoring early warning signs associated with developing drug-resistance: adherence to first-line regimens, changing regimens, inconsistent filling of prescriptions, and missing appointments^[67]. Diagnosis of drug resistance in resource-limited settings is a clinical observation and research is now investigating empiric second- and third-line HAART

options for patients with suspected resistance^[64].

Ultimately, prevention will be the only way to definitively eradicate HIV. Efforts in vaccine development were discussed previously, but another strategy being investigated is prompt treatment of exposed or at-risk individuals with HAART to prevent viral transmission^[66]. Treatment of mothers and children in the peri-natal period has been demonstrated to safely and effectively reduce HIV transmission at birth and during breastfeeding^[68-70]. HIV post-exposure prophylaxis (PEP) taken within 72 h of an occupational exposure has been shown to prevent transmission in the great majority of cases^[71]. Animal trials and observational studies in humans also demonstrate a benefit for non-occupational exposures such as sexual encounters and intravenous drug use^[72]. There is promise of using antiretroviral therapy as pre-exposure prophylaxis for certain high-risk groups, but phase I - III trials have shown variable protection from HIV transmission, likely due to poor drug adherence^[73]. Potential problems with widespread availability of HIV PEP include increased drug resistance, risky behavior, and decreased cost-effectiveness^[66]. To address these issues, dozens of trials in a variety of countries are either planned or ongoing^[74].

NCD EPIDEMIC

In the year 1900, the three leading causes of death in the United States were pneumonia, TB, and diarrhea/enteritis. These diseases caused one third of all deaths, of which 40% were among children under five years of age. Over the next century scientists would discover microorganisms and their role in infectious disease as well as determine ways to treat them. Subsequently the burden of disease has shifted; pneumonia, influenza, and HIV were responsible for 4.5% of deaths in the United States in 1997. Conversely, 54.7% of deaths in that year were a result of heart disease and cancer^[75].

This shift in disease burden is not unique to the United States or even wealthy, industrialized countries. The incidence of many NCDs such as cardiovascular disease (CVD), cancer, and diabetes is growing so fast in developing countries that many have called it an epidemic^[76,77]. From 1909 to 1999, global mortality caused by cancer and CVD increased from 15% to 53%^[78]. In China, for example, the percentage of mortality attributable to CVD tripled from 1957-1990^[79]. The causes are many and include a worldwide surge in life expectancy, lifestyle changes, urbanization, altered diets, increased tobacco use, poor fetal and childhood nutrition, and diminished physical activity^[77].

Epidemiological studies in developing countries have highlighted the substantial presence of risk factors for CVD, many of which are modifiable^[80-83]. These risk factors are less prevalent in developing countries than in developed nations and the incidence of NCDs is lower as well. Nonetheless, incidence is increasing and developing nations are also at risk of a NCD epidemic^[84]. This provides a unique opportunity to halt disease

progression in these regions. Research from developed countries highlights the benefits of preventative medicine in population-based interventions^[85], and national public health programs have successfully improved population health in developed as well as developing countries by disseminating information regarding risk factors^[86,87]. Social education is especially necessary to confront cultural misconceptions in areas where health professionals are distrusted and obesity is seen as a sign of affluence^[88].

When prevention fails, management of affected or high-risk individuals will always be necessary. While there are many known treatments for diabetes, hypertension, hypercholesterolemia, and other chronic diseases, the incidence of these diseases continues to increase both in the United States and worldwide^[89-91].

Another challenge to curbing this epidemic is delivery of appropriate therapy. For example, the results of the β -Blocker Heart Attack Trial were published in the United States in 1981, and 15 years later only 62.5% of patients who had had a myocardial infarction were appropriately being prescribed beta-blockers^[92]. Despite wide availability of an inexpensive, safe, efficacious intervention, less than two-thirds of patients receive appropriate treatment.

Studies in United States have shown health benefits using one-on-one lifestyle teaching^[93] and even text and email reminders^[94] to encourage patients to exercise, modify diet, and take medications as instructed. However, these tactics may not apply to resource-limited countries with insufficient supplies of doctors and medicines^[95]. As is the case with vaccines, governments in these countries can improve health by investing in cost-effective initiatives to develop and provide medications for their citizens at an affordable price^[96-98]. Developing regions have had success improving the management of NCDs by focusing on primary care systems improvements and non-physician-led community initiatives^[95,99-102].

Prevention and management of NCDs is a complicated problem, and the challenges faced by developing and developed countries are both similar and different. There is a unique opportunity in the developing world to prevent an epidemic that is currently evolving^[84]. Risk factors are increasing, but the prevalence of NCDs in developing countries is still quite low, and research has shown that prevention, risk factor modification, and policy change can prevent the NCD epidemic from equaling others the world is currently battling.

Ethical issues

The considerations regarding ethical translational research in global health are diverse. Clearly, the exploitation of economically disadvantaged individuals is egregious, but there are many nuances to consider. When a resource-rich country funds research in a resource-poor country, how do you define the standard of care? Is it wrong to inject live malaria parasites into HIV-positive patients to study the effect CD4⁺ T cell counts, even in an area where malaria is endemic^[103]?

Is it fair to randomly assign some malnourished men to receive vitamin-fortified bread and others standard bread when they normally would not have the fortified option anyway^[104]? When conducting HIV vaccine trials in high-risk populations, is it necessary to provide condoms or safe-sex counseling^[105]? Is it ethical to test a new therapy against subjects who go untreated because they cannot afford medicine which is standard of care^[106]? Early-phase translational research has the potential to harm subjects, and that risk increases when crossing international boundaries^[107].

Another ethical conundrum is the notion of disproportionate profiting from discoveries made in resource-poor countries host^[107]. Not only does it seem wrong to expose patients to a potentially life-altering treatment they could never afford, but such discoveries can further exacerbate international disparities in health, as well as create inequalities in care within the host country^[107]. However, international research partnerships can also improve the quality of care in host nations. For over 30 years Cornell has collaborated with the Haitian Ministry of Health on research that started with AIDS and TB but has since expanded to include maternal-child health, family planning, cancer prevention and treatment, immunization, and education. In that time they have successfully reduced rates of HIV and other sexually transmitted infections, increased the number of patients on HAART, and trained thousands of medical personnel, all the while enjoying uninterrupted NIH support since 1983 and generated more than 150 peer-reviewed publications^[108]. By funding symbiotic partnerships it is possible not only to generate research data but also to improve population health and reduce the "implementation gap" that plagues global health^[109].

CONCLUSION

The history and current struggles of research in vaccine development, HIV, and NCDs emphasize the importance of the five phases of translational research. Many vaccines have been successfully discovered and their effectiveness proven, some are works in progress, and all must have the potential to be efficiently delivered to populations in need. Treatment for HIV has evolved rapidly and become increasingly effective, but HIV is far from eradicated. Therapies for NCDs are well studied in the developed world, but there is much work to be done in prevention and population health in both developing and developed countries. Moving from clinical observation to bench research to bedside intervention is a hallmark of academic medicine in resource-rich countries. However, stopping at the bedside will not improve population health. Region-specific epidemiologic research can highlight needs, opportunities, and challenges that vary due to economic and cultural differences between communities, and goal-directed funding and research can find solutions to these problems. Research in developing countries can inform policy in developed countries, and vice-versa. As

the field of global health grows, research and resources will be shared more efficiently and the successes of smallpox and polio will be translated to HIV and cardiovascular disease.

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Epigenetics and DNA methylation in cancer

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Abstract

Epigenetic is the study of those alterations regulating gene expression without altering DNA sequence and inherited by transmission through cell division. Mutational and epimutational events that alterate cellular growth and division are combined in carcinogenesis. Advances in genome and epigenome-wide analysis identify DNA hypomethylation, hypermethylation of tumor suppressor genes, aberrant histone modifications and/or specific miRNA expression profiles to contribute to tumor initiation and progression. The major challenge for cancer researchers is to enlighten the complex relationship between the epigenetic and genetic machinery in order to

optimize combined therapies, reducing chemoresistance and minimizing adverse effects in cancer patients. In this review we will cover many distinct aspects of epigenetic phenomenon. Firstly, we will globally explain the most common epigenetic events and their effects on gene expression regulation. Secondly, we will review the evidence of the correlation between epigenetics and cancer progression, focusing in particular on the effect of aberrant hypo- and hyper-methylation. We will also consider the main methods currently used for methylation analysis, covering both locus-specific technologies and genome-wide analysis. Finally, we will discuss the introduction of novel epigenetic drugs in combination with conventional treatments in order to develop more effective cancer therapies. Such information could help in understanding the important role of epigenetics in cancer.

Key words: Epigenetics; DNA methylation; Cancer; Regulation of transcription; Prognostic markers

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Core tip: Carcinogenesis occurs through a combination of mutational and epimutational alterations involving key pathways in cellular growth and division. Tumour cells exhibit two main differences from normal cells in DNA methylation: a global reduction in DNA methylation and the hypermethylation of specific sequences, mainly CpG islands, that cause the transcriptional silencing of tumour suppressor genes, thus directly driving the carcinogenic. In this review, we'll focus on our current understanding of this process, aiming to discuss how the analysis of cancer methylomes and the re-expression of epigenetically silenced genes have potential uses in developing more effective cancer therapies.

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INTRODUCTION

In the early 1940s the word “epigenetics” was introduced in the biological vocabulary to describe those phenomena that traditional genetics could not completely explain. Conrad Waddington (1905-1975) defined epigenetics as “the branch of biology which studies the causal interactions between genes and their products which bring the phenotype into being”. Today the most common definition for “epigenetics” is “the study of changes in gene function that are mitotically and/or meiotically heritable and that do not entail a change in DNA sequence”^[1].

The epigenetic field covers chromatin-based events that regulate DNA-templated processes. Specific chromatin-modifying enzymes highly regulated modifications to both DNA and to histones, proteins involved in DNA packaging into structural units defined nucleosomes^[2]. Table 1 describes the most well-known DNA and histone modifications and their correlated functions.

Epigenetic modifications play critical role in regulating DNA transcription, repair and duplication. Genomic alterations leading to deregulated expression patterns in chromatin regulators could be responsible for cancer induction and progression^[2].

Earliest studies on gene expression and DNA methylation indicated the possible link of epigenetics to cancer, as detailed in the history of cancer epigenetics by Feinberg *et al.*^[3] and confirmed by recent results from the International Cancer Genome Consortium (ICGC). The analysis of genomes of various cancers allowed us to identify recurrent somatic mutations causing a loss or gain of function in tumour suppressor genes and in oncogenes, respectively. The so called “driver” mutations often present at a high prevalence, are recurrently found in various tumours^[1]. Recent studies identified many of these mutations also in numerous epigenetic regulators. Feinberg hypothesised that epigenetic changes may induce genetic alterations causing cancer initiation and/or progression.

Differently from somatic mutations, epigenetic changes occur without changing DNA sequence; they include chromatin structure variations, due to methylation or histone variants, nucleosome remodelling and non-coding regulatory RNAs changes (Figure 1).

It is now well demonstrated that epigenetic events are heritable changes in gene structures aimed to perpetuate altered activity states^[4]. These alterations in the state of chromosomal regions are called epimutations and could play a significant role in carcinogenesis as they have been commonly found in epigenetic regulators.

The first epigenetic mark studied in correlation with cancer was aberrant DNA methylation causing deregulation in normal gene expression^[5]. DNA methy-

lation is a covalent chemical change that causes the addition of a methyl (CH₃) group at the 5' carbon position of a cytosine ring. The presence of methyl groups determines the turning off of gene transcription and thus the silencing of these genes. The methylation pattern is inherited by the daughter cells during mitosis, allowing maintenance of gene transcription regulation after replication and generating a stable gene silencing mechanism.

A family of DNA methyltransferases (DNMTs) regulate DNA methylation in the CpG dinucleotide by catalyzing the addition of CH₃ groups from S-adenosyl-L-methionine to the 5' position of cytosine. Methyl-binding domain (MBD) proteins (MeCP2, MBD1, MBD2, and MBD4) are able to bind to methylated CpGs, causing transcriptional silencing^[1]. Aberrant methylation frequently occurs in cancer and the most common types of alteration are hypo-, hypermethylation and loss of imprinting (Table 2).

In somatic cells DNA methylation mainly occurs at cytosines usually concentrated in islands (CGIs) which frequently correspond to the promoters of tumour suppressor genes (TSGs) (Figure 2A), which are unmethylated in normal cells.

In cancer cells methylation levels are frequently reduced in specific repetitive elements or in target chromosomal regions (Figure 2B). *LINE-1* elements hypomethylation has been described in colorectal, urothelial and hepatocellular cancers, disrupting normal patterns of gene expression. Moreover, *Alu* elements are hypomethylated with *LINE-1* elements in prostate adenocarcinomas, pancreatic endocrine tumors, and carcinoid tumors^[6]. Hypomethylation of these elements is strongly linked to tumorigenesis through insertional mutagenesis, genomic rearrangements, deletions or inversions causing genomic instability and gene activation^[7]. Although hypomethylation has been clearly correlated to cancer development, the chemical process resulting in the removal of methyl groups (demethylation) and its role in gene regulation are still unclear. The family of enzymes Ten-eleven translocation [TET (TET1, TET2 and TET3)] has been identified to be active in initiating demethylation. They are 2-oxoglutarate-/Fe(II)-dependent oxygenases that convert the 5-methylcytosine (5mC) into 5-hydroxymethylcytosine with mechanisms still not well described^[8].

DNA hypermethylation is the most well studied abnormality of DNA methylation. This method of gene inactivation is the most common mode used by cancer cells to silence TSGs, thus affecting DNA repair, apoptosis, angiogenesis, cell cycle regulation, and capability of invasion. TSGs that are cancer-specifically silenced by CpG island hypermethylation of their promoters are, for example, retinoblastoma, *CDKN2A* (*p16*), *hMLH1*, and *VHL* genes^[6]. However, hypermethylation could also hit DNA repair genes and transcription factors indirectly affecting downstream targets, thus leading to genetic errors and tumori-

Table 1 Most common chromatin modifications with their reader motifs and function

Chromatin modification	Nomenclature	Chromatin-reader motif	Attributed function
DNA modification			
5-methylcytosine	5mC	MBD domain	Transcription
5-formylcytosine	5fC	Unknown	Unknown
5-hydroxymethylcytosine	5hmC	Unknown	Transcription
5-carboxylcytosine	5caC	Unknown	Unknown
Histone modification			
Acetylation	K-ac	Bromodomain Tandem PHD fingers	Transcription, repair, replication, and condensation
Methylation (lysine)	K-me1, K-me2, K-me3	Chromodomain, tudor domain, MBT domain, PWWP domain, PHD fingers	Transcription and repair
Methylation (arginine)	R-me, R-me2s, R-me2a	Tudor domain	Transcription
Phosphorylation (serine and threonine)	S-ph, T-ph	14-3-3, BRCT	Transcription, repair and condensation
Phosphorylation (tyrosine)	Y-ph	SH2	Transcription and repair
Ubiquitylation	K-ub	UIM, IUIM	Transcription and repair
Sumoylation	K-su	SIM	Transcription and repair
ADP ribosylation	E-ar	Macro domain, PBZ domain	Transcription and repair
Deimination	R→Cit	Unknown	Transcription and decondensation
Propoline isomerisation	P-cis↔P-trans	Unknown	Transcription
Crotonylation	K-cr	Unknown	Transcription
Propionylation	K-pr	Unknown	Unknown
Butyrylation	K-bu	Unknown	Unknown
Formylation	K-fo	Unknown	Unknown
Hydroxylation	Y-oh	Unknown	Unknown
O-GlcNAcylation (serine and threonine)	S-GlcNAc; T-GlcNAc	Unknown	Transcription

Adapted by Dawson *et al*^[2], 2012. 5mC: 5-methylcytosine; 5hmC: 5-hydroxymethylcytosine; 5caC: 5 carboxylcytosine; 5fC: 5 formylcytosine; me1: Monomethylation; me2: Dimethylation; me3: Trimethylation; me2s: Symmetrical dimethylation; me2a: Asymmetrical dimethylation; Cit: Citrulline; MBD: Methyl-CpG-binding domain; PHD: Plant homeodomain; MBT: Malignant brain tumor domain; PWWP: Proline-tryptophan-tryptophan-proline domain; BRCT: BRCA1 C terminus domain; UIM: Ubiquitin interaction motif; IUIM: Inverted ubiquitin interaction motif; SIM: Sumo interaction motif; PBZ: Poly ADP-ribose binding zinc finger; SH2: Src Homology 2.

Table 2 Abnormal DNA methylation patterns in cancer cells and related consequences

DNA hypomethylation	Consequence
Global hypomethylation	Reactivation of endoparasitic and repetitive genomic sequences Chromosomal and genomic instability
Hypomethylation of gene bodies	Activation of incorrect sites of transcription initiation
Loss of promoter methylation	Activation of metastasis and tumour promoting genes
DNA hypermethylation	Consequence
Promoter CpG island (CpGI) methylation	Tumour-suppressor gene silencing Inhibition of transcription factors suppressors
Loss of imprinting	Abnormal transcriptional inactivation Deregulation of imprinted genes

Adapted by Cock-Rada *et al*^[8], 2013.

genesis^[7].

A less studied epigenetic event is the loss of parental allele specific monoallelic expression of genes, the so-called loss of imprinting (LOI); this may be caused by hypomethylation of one of the two parental alleles (Figure 3). Insulin-like growth factor 2 LOI has been associated with an increased risk of colorectal cancer^[5,9] and other neoplasias. Data demonstrated that LOI can also cause tumour suppressor gene silencing; for example, *ARHI*, a candidate breast tumour gene, shows aberrant allele-specific silencing. Moreover, *LIT1*, an untranslated RNA, undergoes LOI in about half of patients with Beckwith-Wiedemann syndrome, determining downregulation of *CDKN1C* (which encodes KIP2, also known as p57)^[3]. Table

3 shows some of the most well known genes epigenetically regulated in cancer.

ABERRANT CGI HYPERMETHYLATION IN CANCER AND INACTIVATION OF TSGS

The fact that aberrant hypermethylation in cancer causes TSG silencing is supported by three important evidences: (1) hypermethylation has been observed alongside inherited germ line mutations and could be the specific "hit" that completely disables TSG activity (*i.e.*, *CDNK2A-p16/ARF*); (2) in sporadic cancers the tissue specificity of TSG hypermethylation causes predisposition in the specific tissues as the

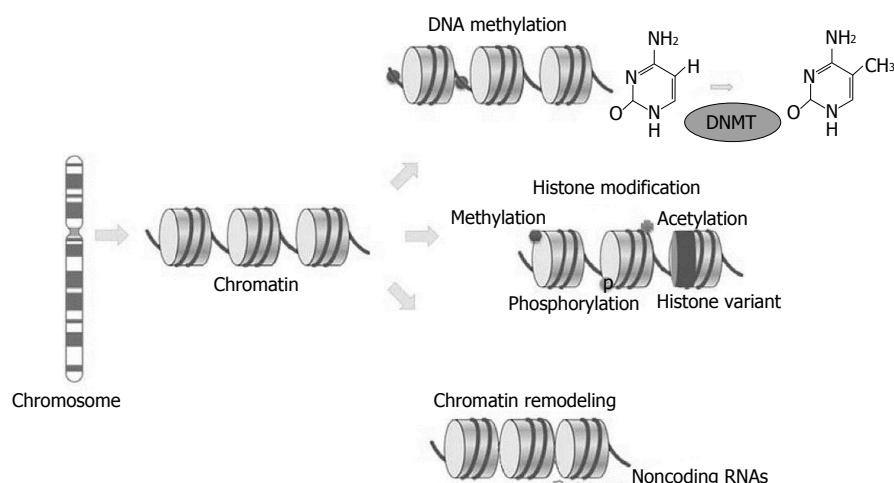


Figure 1 Epigenetic mechanisms. Variations in chromatin structure without DNA sequence modifications by (1) DNA methylation; (2) histone modifications methylation, phosphorylation and acetylation; (3) histone variant composition (dark); and (4) chromatin remodeling (sparse or dense nucleosome occupancy), and noncoding RNAs. DNMT: DNA methyltransferases. Adapted by Choi *et al*^[1], 2013.

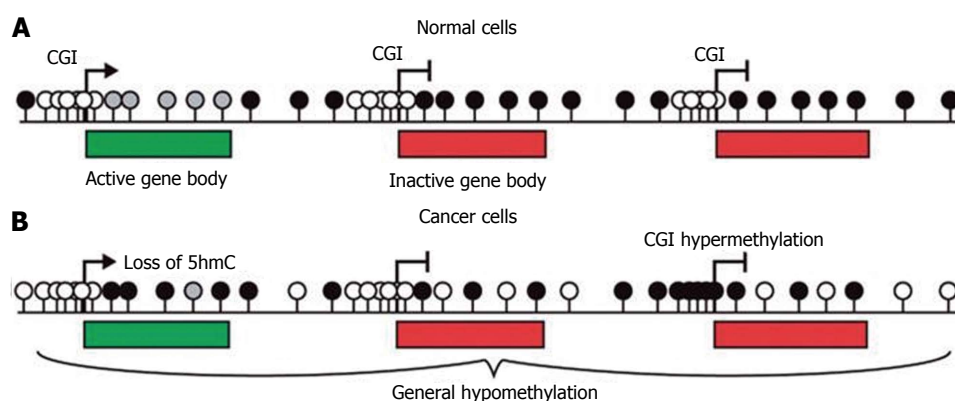


Figure 2 The methylation scenario of normal and cancer cells. A: In the mammalian genome the amount of CpGs is low and most these sites are methylated (black lollipops). CGIs are usually located in gene promoters and are generally unmethylated (white lollipops), irrespective of gene expression status. The bodies of active genes are enriched in hydroxymethylated CpGs (grey lollipops); B: In cancer cells both DNA methylation and hydroxymethylation are reduced even if some CpG island have been found to be aberrantly hypermethylated. Adapted by Sproul *et al*^[4], 2013. 5hmC: 5 hydroxymethylcytosine.

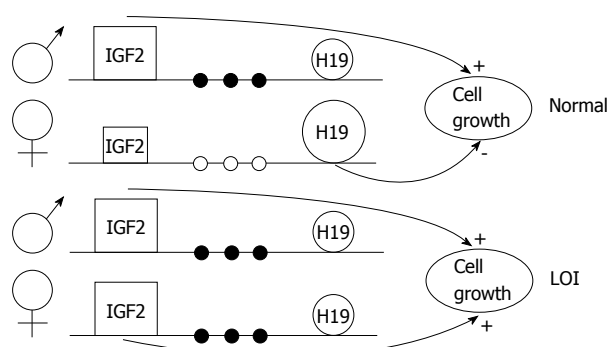


Figure 3 Model of loss of imprinting of insuline-like growth factor 2, H19 and methylation of the H19 promoter in Wilms' tumor. In normal cells, the paternal IGF2 and maternal H19 genes are expressed (shown large). Several sites upstream of H19 are methylated on the paternal allele (filled circles) and unmethylated on the maternal allele (open circles). In tumors with LOI, the maternal chromosome reverses to a paternal epigenotype, with a paternal pattern of methylation of the H19 promoter, IGF2 turned on, and H19 turned off, causing increased cell growth. LOI of H19 on the maternal chromosome, when it occurs, could occur independently or could be influenced by events in the paternal chromosome. Adapted by Steenman *et al*^[9], 1994. LOI: Loss of imprinting; IGF2: Insuline-like growth factor 2.

inherited mutations in these same genes. For example, *MLH1* mutations and hypermethylation predispose to colorectal cancer, the latter been limited to colorectal tumoural tissues. Similarly, *BRCA1* mutations predispose to breast and ovarian tumours and hypermethylation is limited to those tissues; and (3) the strongest evidence that aberrant DNA hypermethylation contributes to silencing of TSG in cancer is that demethylation of promoters is able to reactivate those genes. Many studies demonstrate the ability of 5-aza-2'-deoxycytidine to cause DNMT1 degradation and methyltransferase maintenance, leading to reactivation of hypermethylated gene promoters^[4].

The mechanism(s) responsible for aberrant promoter hypermethylation in cancer are still unclear. However, genomewide analyses of normal and tumour cells demonstrate two principal processes: active mechanisms targeting specific factors to CGIs, or passive ones deriving from a loss of protection against *de novo* methylation.

Over-expression or increased activity of DNMTs

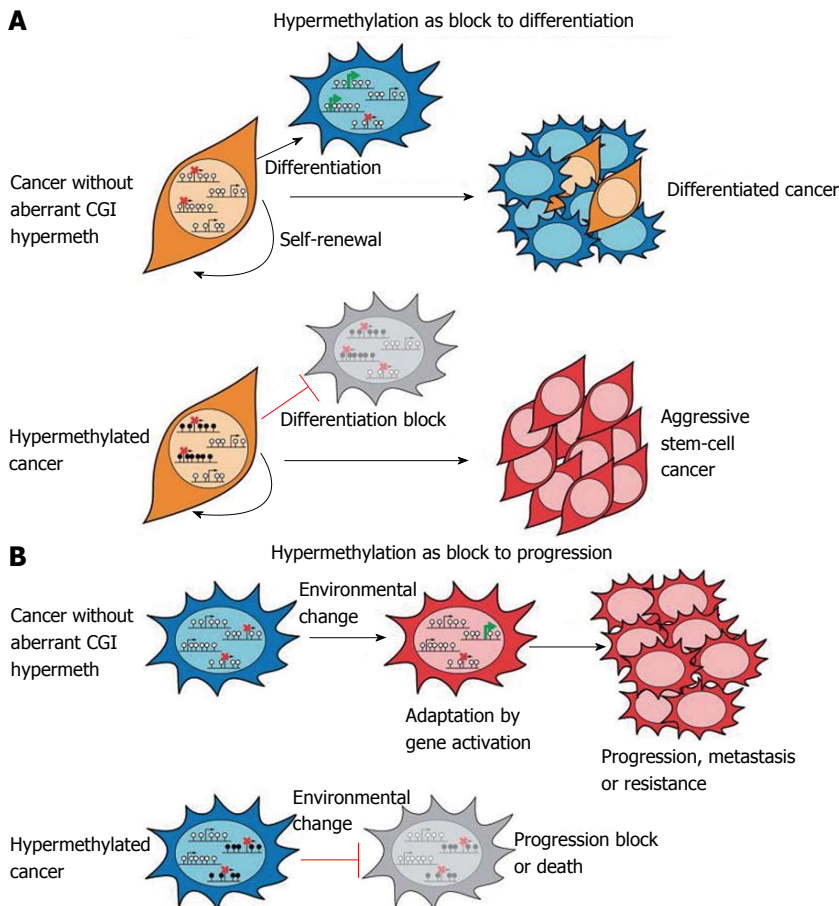


Figure 4 Hypermethylation consequences in cancer. A: Key genes required for normal cellular differentiation become hypermethylated in cancer, resulting in a block to their activation and to normal differentiation processes. Thus cancer cells express a more aggressive, stem-cell like phenotype; B: Hypermethylation of repressed CpG island promoters might prevent the activation of genes facilitating survival in changing conditions such during metastatisation. Thus, widespread hypermethylation might restrict the potential for epigenetic adaptation and result in block to progression. Adapted by Sproul *et al*^[4], 2013.

might cause aberrant CGI hypermethylation. Many studies initially reported DNMTs to be increased, but more recently it has been attributed to cell cycle regulation or to an increased number of cycling cells.

Moreover, methylome analyses demonstrate that hypermethylation is not random, but hit specific set of genes. The promoters of such genes seem to be relatively poor of retrotransposons (transposable elements that duplicate *via* RNA intermediates and are reverse-transcribed and inserted at new genomic locations) compared with hypermethylation-resistant promoters^[4].

EPIGENETIC PLASTICITY OF CANCER CELLS

Many results have demonstrated epigenetic plasticity of cancer cells, suggesting that hypermethylation could act as a block to differentiation and to progression. This derives from studies reporting gene expression profiles of aggressive tumours to be similar to those of embryonic stem (ES) cells. Thus, it has been proposed that in cancer the hypermethylation in ES polycomb repressive complexes targets might impact differentiation and maintain stem-cell-like state (Figure

4A). Moreover, hypermethylation could act in cancer progression (Figure 4B)^[4]. In fact, dissemination of tumour from the primary site requires the re-modelling of gene expression profiles. Moreover, drug resistance might result from secondary activating mutations and/or epigenetic alterations. Hypermethylation causing gene repression might provide a protection to these events and favour cancer progression^[4].

EPIGENETIC REGULATION OF EPITHELIAL-MESENCHYMAL TRANSITION

Epithelial-mesenchymal transition (EMT) and the otherway process, mesenchymal-epithelial transition, (MET) are important during cellular growth and in physiological tissue repair (wound healing) but they also play a crucial role in carcinogenesis. In normal tissues, many intercellular junctions (desmosomes, adherens, tight junctions) ensure tissue homeostasis and stability, linking epithelial cells together and to the extracellular matrix. In particular conditions, such as physiological circadian changes or tissue loss or damage, epithelial cells can acquire a mesenchymal phenotype, including

Table 3 Genes that are epigenetically regulated in cancer

Cancer-associated pathway	Gene
Cell cycle	Rb, p16 ^{INK4a} , p16 ^{INK4b} , 14-3-3, cyclin E, p14 ^{ARF}
Signal transduction	<i>ErbB2</i> , <i>RASSF1</i> , <i>LKB1/STK11</i> , <i>APC</i>
Apoptosis	<i>DAPK</i> gene, <i>Caspase-8</i> gene
DNA repair	<i>MGMT</i> , <i>MHL1</i> , <i>BRCA1</i> , <i>FNACF</i>
Carcinogen metabolism	<i>GSTP1</i> gene
Hormonal response	Oestrogen receptor gene, progesterone receptor gene, <i>RAR-β</i> gene
Senescence	<i>TERT</i> , <i>TERG</i>
Invasion/metastasis	<i>TIMP-3</i> gene, <i>E cadherin</i> gene, <i>VHL</i> gene
Transcription	Runx3, Twist, Er α , Er β , PR, RAR, vitamin D receptor
Drug responsiveness	Glutathione S-transferase, thymidylate synthase

Adapted by Choi *et al.*^[1], 2013.

an intermediate stem cell phenotype, such as in embryogenesis. Recently, many studies have aimed at understanding the role of EMT and MET in cancer progression and, in particular, in the initial processes of tissue invasion and extravasation. EMT, in fact, is minutely regulated by networks of activating/deactivating signalling pathways and also by epigenetic alterations (DNA methylation, histone modifications and by miRNAs). For this reason, anomalies in regulating those mechanisms might cause cancer initiation and progression, depending on the capability of cells to react to external and internal stimuli.

One of the main mechanisms used by epithelial tumor cells to convert into de-differentiated, mesenchymal cells is by silencing epithelial genes, such as E-cadherin, and losing cell-cell contacts. Loss of E-cadherin happens in early tumor progression, so that the EMT process is strictly related to metastatic invasion. The replacement of E-cadherin by N-cadherin (cadherin switching)^[10] depends on multiple cellular signaling mechanisms [Hedgehog, Wnt, Notch, transforming growth factor β , fibroblast growth factor (FGF), epidermal growth factor and platelet-derived growth factor]. Moreover, many epigenetic events are involved in the EMT program and are responsible of the silencing of specific epithelial markers, leading the epithelial cells to be aggressive and invasive (Figure 5)^[11].

DNA METHYLATION IN ANGIOGENESIS AND METASTASIS

During tumorigenesis, cells acquire metastatic potential following angiogenesis, induction of cell surface metalloproteases, decrease in the expression of cell-cell adhesion molecules, and increased expression of cell surface receptors that aid in motility. E-cadherin and α -4 integrins, two of the most common cell adhesion receptors, are silenced by methylation in several cancers^[12]. Similarly, intracellular basement membrane proteins (*i.e.*, NID1 and NID2) are also

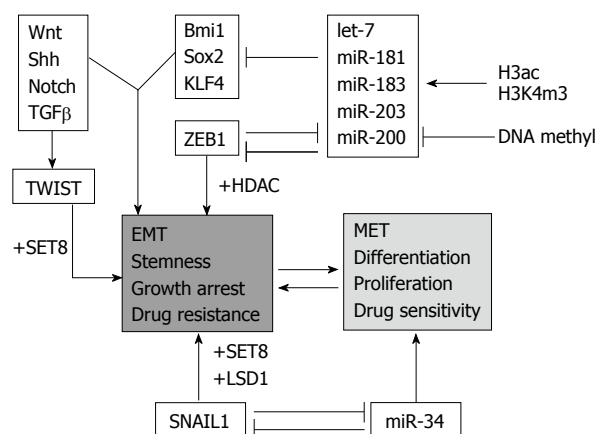


Figure 5 Molecular regulators regulating epithelial vs mesenchymal cell phenotypes and reverse process. In gray/italicised factors/processes involved in epigenetic control are highlighted. ac: Acetylation; EMT: Epithelial-mesenchymal transition; MET: Mesenchymal-epithelial transition; miR: miRNA; m3: Trimethylation; Shh: Sonic hedgehog; TGF β : Transforming growth factor beta; KLF4: Kruppel-like transcription factor4; HDAC: Histone deacetylase inhibitor; LSD1: Lysine-specific demethylase 1. Adapted by Kiesslich *et al.*^[11], 2013.

silenced by methylation in cancer. Therefore, it is evident that epigenetics could also play a critical role in the metastatic process^[13]. The phenomenon of metastasis is a complex process involving several distinct steps: tumor cells, supported by angiogenesis, infiltrate the basement membrane.

Aberrant methylation of metastasis initiation genes could be responsible of tumor invasiveness (for a detailed review, refer to Cock-Rada and Weitzman, 2013^[8]).

Several genes have been identified which regulate the metastatic process, can predict prognosis and metastasis and are used in daily clinical practice^[8]. These genes are usually involved in regulation of extracellular matrix (ECM) and angiogenesis, regulation of cell adhesion and invasion, and repressive and activating histone modifications.

In particular, in the beginning of tumour progression, cellular matrix metalloproteinases (MMPs) are able to degrade the ECM for angiogenesis. The loss of MMP regulation and release of angiogenic stimuli (FGF-2 and vascular endothelial growth factor) contribute to this process^[14]. Tissue inhibitor of metalloproteinase 2 (TIMP-2) is a MMP inhibitor suppressed in some solid and lymphoid tumours by CpGI hypermethylation^[15,16]. TIMP-3 was also found to be silenced by DNA methylation in gastric and oesophageal cancers and to correlate with poor survival^[17].

Cells migrate through the ECM, invade adjacent structures and traverse into lymphatic or blood vessels, so that are able to disseminate to distant sites, form micrometastases and eventually colonise the new organ with macrometastases (Figure 6).

EPIGENETICS AND RADIATION BIOLOGY

Exposure to ionizing radiation (IR) could cause alteration in gene expression, deregulation of cell

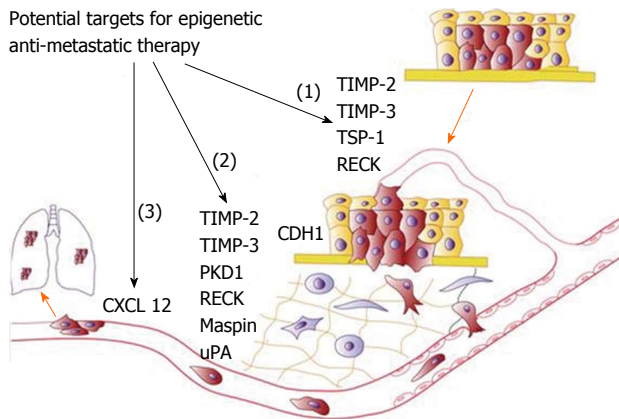


Figure 6 Schematic representation of progressive steps from initial tumour formation to establishment of metastasis include (1) tumour growth, angiogenesis and localised invasion; (2) intravasation and survival; and (3) extravasation and formation of distant tumours. For each step some genes promoting these processes and regulated by DNA methylation are indicated. Adapted by Cock-Rada *et al.*^[6], 2013. TIMP-2: Tissue inhibitor of metalloproteinase 2.

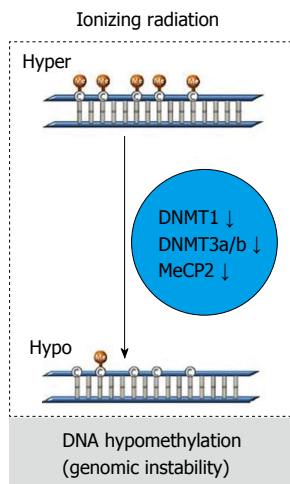


Figure 7 The figure represents the general change of global DNA methylation after radiation exposure in cancer cells. Radiation might induce a decrease in DNA methyltransferases, including DNA methyltransferase 1 (DNMT1), DNMT3a, DNMT3b, and methyl CpG binding protein 2 leading to global DNA hypomethylation and genomic instability. Adapted by Kim *et al.*^[6], 2013.

cycle, and apoptosis. Nonetheless, although several studies demonstrate that IR could also alter DNA methylation^[6], the epigenetic events following IR still need to be defined at the molecular level.

In vivo studies show that IR causes a dose-dependent and sex/tissue-specific global hypomethylation, together with a decrease in methyltransferases (DNMTs; DNMT1, DNMT3a, and DNMT3b) and methyl CpG-binding protein (MeCP2) level (Figure 7). Thus, radiation exposure could be strictly correlated to DNA hypomethylation patterns resulting in genomic instability^[6].

Interestingly, recent studies in colon cancer report a correlation between the DNA methyltransferase inhibitor 5-aza and radio-sensitivity^[18]. In breast cancer cells, fractionated IR caused DNA methylation alterations at specific loci (*TRAPP9*, *FOXC1*, and *LINE1*)^[19]. More

recently, it has been shown that radiosensitive and radioresistant cancer cells present differential DNA methylation alterations^[20]. Nevertheless, the epigenetic mechanisms at the basis of those alterations and site-specificity in DNA methylation, is still not clear and requires further studies, with the final aim of identifying useful methylation target for developing cancer targeting therapies^[6].

EPIGENETIC REGULATION OF miRNAs IN CANCER

Currently, many studies are focusing on the role of microRNAs (miRNAs) in cancer development and metastasis. Here we refer the reader to several excellent recent reviews^[21-25].

miRNAs are small, non-coding RNAs involved in post-transcriptional gene expression regulation through binding to complementary sequences in the 3'-untranslated region of messenger RNAs (mRNA). This interaction leads to mRNA cleavage or inhibited protein synthesis, thus reducing protein expression of the targeted gene. When affecting expression of oncogenes and tumor suppressor genes, common breakpoints and fragile sites (preferential sites of chromatid exchange, deletion, translocation, amplification, or integration of plasmid DNA and tumor-associated viruses), the up- or down-regulation of miRNAs could be critical for tumorigenesis and cancer progression. Indeed, a large set of aberrantly regulated miRNAs have been already identified in several tumor entities, although the biological mechanisms at the basis of miRNA regulation are still poorly studied^[21].

Recent studies demonstrated that many miRNAs could act as TSGs, but others are frequently over-expressed in human tumors possibly exerting a tumorigenic function. For example, miR-17-92 cluster shows an oncogene function, is transactivated by the *c-MYC* oncogene, and accelerates lymphomagenesis in murine models^[26]. Moreover, miR-155 has been shown to induce leukemia in transgenic murine models and plays a critical role in inflammation and immune response^[27]. miR-21 has been found in several tumor types as a regulator of important TSGs such as *PTEN1* and *PDCD4*^[25].

On the basis of their correlation with cancer, miRNAs are divided as: oncogenic, tumor-suppressive, and "context-dependent" miRNAs^[1]. In cancer cells, the loss of miRNA regulation could activate oncogenes or repress target tumor suppressor genes. Moreover, mutations could occur also in miRNA sequences, leading to lack of recognition of its binding target and thus to oncogene activation and/or tumor suppressor repression. miR-155, miR-21, and miR-17 to -92 are, for example, oncogenic miRNAs and their expression has been found to be amplified in several tumor types; furthermore, tumor-suppressive miRNAs (miR-146, -15 and -16) appear to be down-regulated in cancers.

miRNA mutations are also known to target epigenetically modifying enzymes, such as EZH2 and DNMT3. Alterations of miRNA expression, including miR-101 and miR-29, may cause extensive alterations in histone acetylation or DNA methylation of other miRNAs that target oncogenes and TSG. Since a correlation between miRNA expression and tumorigenesis has been demonstrated, miRNA might be useful therapeutics, replacing tumor-suppressive miRNA or targeting the oncogenic ones^[1].

The study of the influence of DNA methylation on miRNA transcription on a genome-wide level has been hampered by poor miRNA promoter annotation. Recently, large collaborations (ICGC and The Cancer Genome Atlas), have created extensive data sets of genetic, epigenetic, and transcriptome profiles of different tumor entities and cell lines. Furthermore, the Encyclopedia of DNA Elements (ENCODE) consortium profiled a variety of cell lines for 12 histone modifications and variants including H3K4me3 and acetylation of histone 3 at lysine 9 (H3K9ac) to disclose regulatory regions in the human genome^[28].

The resulting data allow us to extend the knowledge on tissue-specific and ubiquitous miRNA promoters. Analysis of 329 miRNA promoters revealed that 300 overlapped with or were close to a DNase I-hypersensitive site. All these analyses might permit us to estimate the number of tissue-specific miRNA promoters as suggested for miR-21^[21].

For example, miR-9-1 has been associated with a CpG island 200 bp upstream and has been found to be hypermethylated in breast cancer, melanoma, and head and neck cancer. Also, miR-200 family members have been found to be near to a CpG island^[29]. CpG island methylation correlated with down-regulated miRNA expression in breast and prostate cancer cell lines^[30]. Moreover, a correlation between loss of miRNA expression and acquisition of mesenchymal features have been observed in tumour progression^[21].

The ENCODE consortium is about to publish the genomewide DNA methylation data, completing analysis of epigenetic regulation of all gene classes including miRNAs in cell lines. In addition, cancer methylomes are analyzed and will be made publicly available by the ICGC which, for example, provided the methylomes of patients with chronic lymphatic leukemia. In conclusion, integrating data sets from different sources will enable scientists to estimate the global influence of DNA methylation on the regulation of miRNA and their aberrant behaviour in cancer^[21].

Furthermore, there has been demonstrated a possible role of miRNAs in IR-induced response *in vitro* and *in vivo*. Indeed, in murine models IR cause sex- and tissue-specific alterations in miRNA expression^[6].

miRNAs are likely epigenetically regulated but it is already well known that they can also affect expression of epigenetically regulated genes by targeting key enzymes responsible for epigenetic reactions. This group of miRNAs is called epi-miRNAs (Figure 8)^[25].

ANIMAL MODELS OF CARCINOGENESIS

Recently, many *in vivo* models of carcinogenesis have been developed in order to investigate epigenetic mechanisms and cancer progression. These models are usually derived from transgenic manipulation or toxicant exposure, inducing a tissue-specific cancer. As a consequence, these models could be useful in characterizing molecular pathways of carcinogenesis and elucidating the contribution of epigenetic and genetic alterations transforming carcinoma *in situ* to metastatic disease^[7].

Recently, these mouse models have been used to study the efficacy of epigenetic-modifying drugs (*i.e.*, 5-azacytidine, decitabine and zebularine), as well as to determine their toxicity, by treating xenograft mice and evaluating tumour size or metastasis formation. However, these results cannot be directly translated into the clinic, since because tumour biology and response to drugs in mice may be substantially different from patients^[8]. In oncology, the greatest challenge is the integration of human and animal results from translational research. This integration may shed light on how or when epigenetic dysregulation could occur in tumor and how environmental and dietary hits may influence the tumour phenotype. Additionally, these studies will provide information on susceptibility to therapy that target epigenetics (DNA demethylating agents, histone deacetylases inhibitors, or a other promising epigenetic therapies currently in trials). Thus, investigation of genetic and epigenetic profiles in cancer patients is a crucial step in the improvement of any personalized cancer therapy^[7].

EPIGENETICS AS A SOURCE OF BIOMARKERS

A biomarker is an indicator of normal biological processes, pathogenic processes, or pharmacologic response to therapeutic intervention. Biomarkers have many valuable applications in disease detection and monitoring, even if the validation and qualification of biomarkers for use with patients is time-consuming. Currently, many validated biomarkers should be used for personalized therapy. In cancer, several biomarkers have been used to reflect the extent of tumor growth and metastasis or as tools for screening and monitoring of disease. For example, our group identified *TFPI2* gene as a novel biomarker of metastatic melanoma, demonstrating that its methylation correlates with metastatic state of the disease. Moreover, we observed that circulating, methylated *TFPI2* DNA was undetectable in sera from healthy individuals but detectable in sera from patients with primary and metastatic melanomas. In particular, the presence of methylated *TFPI2* DNA in serum was strongly associated with metastatic disease, thus defining *TFPI2* a sensitive and specific biomarker of metastatic melanoma^[25].

New biomarkers, useful in clinical oncology and

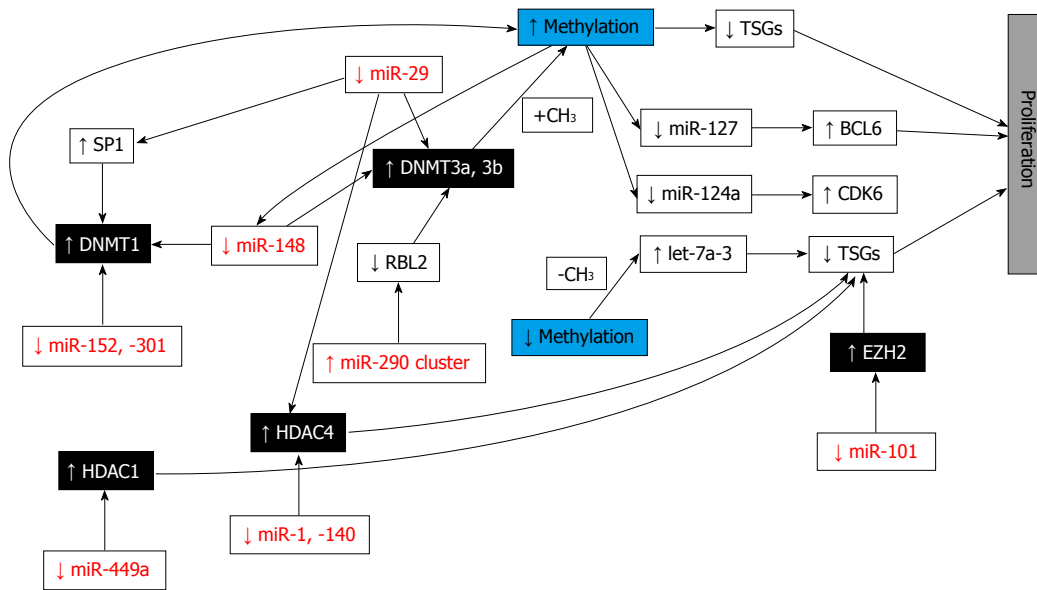


Figure 8 Epi-miRNA functions in cancer cells. Epi-miRNAs (in red) directly target epigenetic effectors (black boxes) and indirectly affect the expression of epigenetically regulated miRNAs and protein coding genes (white boxes), contributing to carcinogenesis. TSGs: Tumor suppressor genes; DNMT: DNA methyltransferase; HDAC: Histone deacetylase; EZH2: Enhancer of zeste homolog 2; BCL6: B-cell CLL/lymphoma 6; CDK6: Cyclin-dependent kinase 6; SP1: Sp1 transcription factor; RBL2: Retinoblastoma-like 2 (p130); CH₃: Methyl group. Adapted by Fabbri *et al.*^[23], 2013.

based on DNA methylation, are coming from epigenomic analyses. As a consequence of the large set of alterations in methylation discovered in different tumours, a myriad of DNA methylation-based biomarkers of several human neoplasia have been reported, principally involving hypermethylation of tumor suppressor CGIs^[5].

DNA methylation is an epigenetic event that usually occurs in specific genes or in viral genome regions that are quite promising as independent diagnostic and prognostic markers. Several of these markers could be in common with two or more cancers, while others appear to be tumor-specific, providing an opportunity to determine the origin of metastases of uncertain origin. Moreover, information derived by new biomarkers could help in distinguishing similarities or differences between diseases. However, there is a growing need for evaluation and selection of the most appropriate biomarker sets, standardisation of the methods for assessment of each type of alteration, and clinical validation^[31]. This could hamper and delay implementation of useful epigenetic biomarkers.

We suggest that the readers refer to a detailed review concerning the discovery and validation of clinically relevant DNA methylation biomarkers in cervix and prostate cancers^[32].

VALIDATED METHODS FOR METHYLATION ANALYSIS AND CLINICAL SIGNIFICANCE

One of the open questions in the epigenetic field is which method of analysis of DNA methylation should be the standard in order to show evidence of clinical utility.

Healthy cells show a specific DNA methylation

pattern; however, alterations in this pattern, such as hypomethylation or hypermethylation, can lead to diseases, including cancer. Methylation status is currently used to classify and characterize cancers and could be of clinical significance at three levels: detection, prognosis, and prediction of treatment responses. In recent years, different methods have been developed to identify aberrant methylation signatures and may be used to identify specific biomarkers useful for tumor subtypes classification. All these technologies have been commonly classified as: (1) global approaches for detection of gross DNA methylation; (2) locus-specific methods for analysis of specific methylated CpG regions; and (3) genome-wide approaches developed to identify methylation hot-spots in the whole genome sequence (Figure 9)^[33].

Two of the most used analyses in DNA methylation are methylation-specific polymerase chain reaction (PCR) (MSP) and bisulphite sequencing PCR. These methods needed an initial bisulphite reaction converting unmethylated cytosines to "uracil" bases read as thymidines (T) after amplification by PCR. This allows to not modify methylated cytosines ("C") in 5mCpG dinucleotides that remain "C". Thus, a hypothetical bisulphite-converted sequence of 5'-AATCmCGTACTmCGCCTG-3' would be read as 5'-AATTCGTATTCGTTTG-3', where the Ts *in italics* derive from unmethylated Cs, whereas methylated CpG remains CpG (here underlined). After bisulphite transformation, DNA could be analysed to specifically distinguish between methylated and unmethylated cytosines.

In MSP, two distinct set of primers containing at least two CpG dinucleotides within the primer sequences are used: U primers detect unmethylated CpGs while M primers detect methylated CpGs. In a

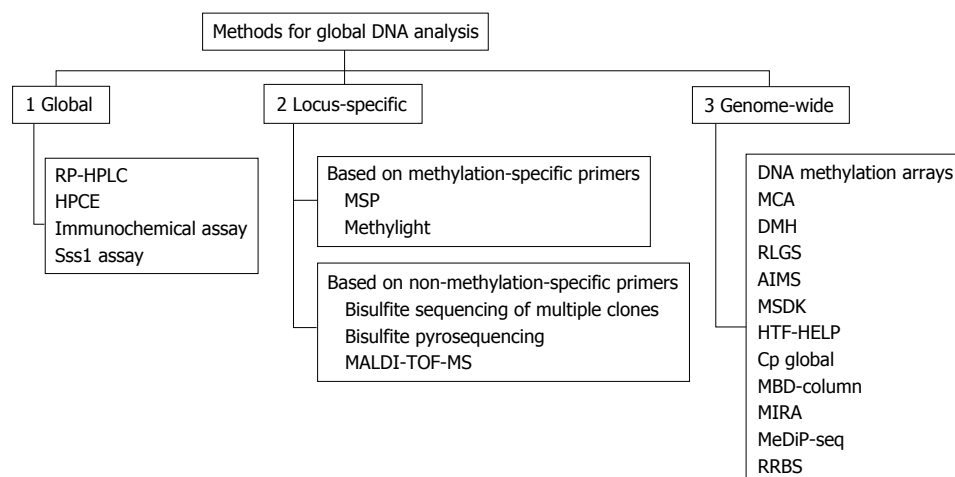


Figure 9 Methods for genomic DNA methylation analysis, classified as global, locus-specific and genome-wide. In the case of locus-specific approaches, techniques were divided depending on the use of methylation-specific primers or not. RP-HPLC: Reverse-Phase high-performance liquid chromatography; HPCE: High performance capillary electrophoresis; Sss1 assay: Methyl group acceptance assay; MSP: Methylation-specific polymerase chain reaction (PCR); MALDI-TOF-MS: Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; MCA: Methylation CpG island amplification; DMH: Differential methylation hybridization; RLGS: Restriction-landmark genomic scanning; AIMS: Amplification of inter-methylated sites; MSDK: Methylation-specific digital karyotyping; HTF-HELP: HpaII tiny fragment enrichment by ligation-mediated PCR; MBD-column: Methylated DNA binding column; MIRA: Methylated CpG island recovery assay; MeDiP-seq: Methyl-DNA immunoprecipitation and sequencing; RRBS: Reduced Representation Bisulfite Sequencing. Adapted by Toraño *et al.*^[33], 2011.

methylated sample, only M primers produce a PCR band but not U primers; vice versa in samples not methylated. Requiring a simple PCR machine, MSP is adequate in the analysis of large numbers of clinical samples and has been successfully used in tumour methylation studies. The main limit of MSP is that the result obtained is purely qualitative. For quantification of methylation levels, bisulphite sequencing PCR has been developed. In this method, after PCR, amplified DNA need to be appropriately cloned into a vector and then 5-10 clones independently sequenced in order to read all CpG sites included in the amplified sequence, giving a global representation of the cellular methylation status^[34].

More recently, many methods focusing on specific single-CpG have been developed, such as combined bisulfite restriction analysis (COBRA)^[35], MethyLight^[36], and bisulfite pyrosequencing^[37]. In COBRA bisulfite conversion and PCR amplification are maintained; then, PCR product are digested by restriction enzyme in a methylation-dependent manner. Digestion proceeds in the recognition sequence only if the CpG site is protected from bisulfite conversion by methylation. Thus, the presence of restriction products indicates methylation in the PCR amplicon. MethyLight is a bisulfite-dependent, fluorescence-based, quantitative real-time PCR method for DNA methylation. This technique includes specific priming combined with methylation-specific fluorescent probing, allowing one to sensitively detect very low frequencies of hyper-methylated alleles.

Another method based on chemical modification of genomic DNA with sodium bisulfite is pyrosequencing. This technique allows quantification of methylation at individual CpG dinucleotides into an amplified DNA fragment. Different from MSP, primers for amplification are designed from regions which contain no CpG

dinucleotides and differences between methylated and unmethylated sequences are seen only after pyrosequencing. The main advantage of this method is that it allows high-resolution analysis of methylation and detection of small changes in methylation at each CpG, and also in samples containing large amounts of normal DNA.

All the above-mentioned methods are sensitive, specific, and relatively inexpensive, but none allows one to analyse the whole genome, which includes about 28 million CpGs. For a global analysis, recent microarray-based methods have been designed, including direct hybridization^[38], methylated DNA immunoprecipitation (MeDIP)^[39] and HELP assay (HpaII tiny fragment enrichment by ligation-mediated PCR)^[40]. Direct hybridization to CpG island arrays is able to detect DNA methylation in several CpG sites. It is based on the use of methylation-specific oligonucleotides arrayed on glass slides, detecting all possible methylation in target genes. MeDIP is a genome-wide method based on an antibody that recognises 5-methylcytosine in methylated DNA sequences. This technique is used for either array-based hybridization (MeDIP-chip) or high-throughput sequencing (MeDIP-seq). However MeDIP presents a significant limitation: restricted resolution typical of array-based technology. The HELP assay is comparative isoschizomer profiling of DNA methylation. DNA is digested by HpaII in parallel with MspI (resistant to DNA methylation), and then the HpaII and MspI products are either amplified by ligation-mediated PCR and hybridized using separate fluorochromes to a customized array, or directly sequenced^[41].

All these methods based on next generation sequencing technology which produces a huge amount of information on methylomes. Generally, genome-wide technologies are very useful for genome-wide DNA methylation analysis but they are relatively expensive

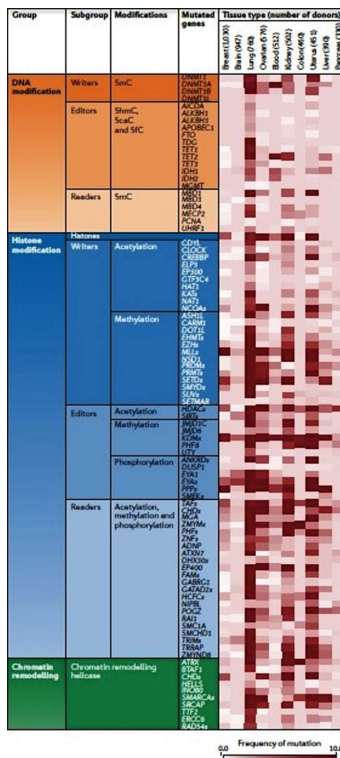


Figure 10 Some of the most known mutated genes classified in groups based on DNA modification, histone modification and chromatin remodelling enzymes. The number of analysed tumour tissues is given. Several epigenetic enzymes present high frequencies of mutations in distinct tumor types. These data are not adjusted for chromosomal instability or mutator phenotypes, hence the frequencies reflect a combination of probable driver mutations in epigenetic regulators and the background mutation rate for the tumour type. 5caC: 5 carboxylcytosine; 5fC: 5 formylcytosine; 5hmC: 5 hydroxymethylcytosine; 5mC: 5 methylcytosine. Adapted by Plass *et al*^[42], 2012.

and cannot be currently introduced in routinely clinical studies (Table 4). However, methylation profiling could be a useful tool to better understand the biological mechanism at the basis of tumorigenesis and provide insight into prevention strategies to reduce the burden of cancer.

MUTATIONS IN REGULATORS OF THE EPIGENOME

Thanks to advances in sequencing technologies thousands of cancer genomes and methylomes have been re-sequenced and new coding-gene mutations, genetic rearrangements, DNA copy-number alterations and alterations in either regulatory sequences or epigenetic patterns have been discovered. In addition, abnormalities in epigenetic enzymes and pathways, including DNA methylation or demethylation, histone modification, and chromatin remodelling processes, have been highlighted (Figure 10). For example, novel gene mutations have been uncovered in different tumours (*IDH1* or *DNMT3A* in acute myeloid leukemia, mitochondrial succinate dehydrogenase in paragangliomas or gastrointestinal stromal tumours, AT-rich interactive domain 1A-ARID1A in NSCLC; CREB-binding protein-CREBBP, E1A-binding protein p300

Table 4 Comparison of methylation arrays *vs* ultra-deep sequencing for DNA methylation analysis

	Methylation arrays	Ultra-deep sequencing
CpG coverage	+	+++
Sensitivity	+++	++/+ (antibody-based)
Time consuming	++	++
Data analysis	+++	+
High-throughput	+++	+
Price	++	+ / ++ (price decreasing)

Adapted by Toraño *et al*^[33], 2011.

and MLL in small-cell lung cancer; H3F3A in paediatric glioblastoma; and MLL2 and SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin (SMARCA4) in medulloblastoma^[42].

A major challenge for researchers will be to investigate the role of these mutations in tumorigenesis.

ROLE OF SNPS ON EPIGENETIC REGULATION AND CANCER

Beside the discovery of new mutations, genome-wide association studies have identified various single nucleotide polymorphisms (SNPs) correlated with increased risk of cancer. Interestingly, those SNPs are preferentially located in functional enhancers in ES cells and might confer cancer susceptibility by altering the cellular chromatin setting. In fact, a correlation between genetic variations and in gene expression changes have been demonstrated to involve chromatin accessibility of transcription factor (TF) binding sites, such as SNPs (CpG SNPs) that create or delete CpGs and influence the binding of specific TFs. Further and deeper studies on this association could reveal the functional link among epigenetic, genetic variation and phenotype^[43].

METHYLTRANSFERASE INHIBITORS IN CANCER THERAPY

The major clinical impact of the rising knowledge of epigenetic mechanisms is the possibility of defining epigenetic cancer therapy which inhibit methylation events in order to increase therapeutic efficacy. Recently, many epigenetic-modifying drugs have been introduced in combination with standard chemotherapy treatments in cancer patients. Nonetheless, those drugs may lack specificity, since they modulate global expression more than being gene-specific. However, different from other drugs, they could be able to restore TSG expression or loss-of-function phenotypes; thus, combined therapy could be a good therapeutic strategy.

Unfortunately, it is evident that epigenetic biology is complex; indeed, there are quite a number of scientific and pragmatic challenges, many of which are summarized in Table 5^[44].

Currently, the most common epigenetic drugs are the DNMT inhibitors azacytidine and decitabine,

Table 5 Epigenetic drug discovery challenges

Category	Issues
Target selection	Few activating mutations, translocations or syntethic lethal relationships known limited high-quality antibodies to epigenetic proteins and histone marks (<i>e.g.</i> , confirm target expression linkage of target to mark) Biology driving cancer phenotype unknown or poorly understood Post-translation modification of histone <i>vs</i> non-histone substrates by "epigenetic" targets unclear
Chemistry	Existing chemical librairies may not have adequate diversity to provide goog strating points Few crystal structures solved; are structrues relevant if not reflecting complete complex?
Assay development	Few reference compunds to establish assy signal window, sensitivity, reproducibility Are binding or enzyme configured to properly reflect physiological context? Production of active enzymes is difficult, may require multimeric complex and specific sunstrate (nucleosome, histone, non-histone)
<i>In vivo</i> biology	Limited high-quality antibodies to epigenetic proteins and histone marks (quantify mark or target gene product) Histone marks and target genes slow to change, require longer-duration studies to assess engagement (PD biomarker) May necessitate higer compund requirement to conduct studies, earlier optimatation of PK properties than traditional paradigm May require novel models for tumors with mutation or traslocations
Toxicology	Acute and/or chronic liabilities of specific isoform targeted epigenetic therapies currently unknown Knockout animal data limited; inducible knockouts, dominant negatives preferred but more scarce and technically challenging
Clinical	Identify and implement appropriate patient selection markers, more challenging if not activating mutation (overexpression, gene profile?) Identify and implement suitable PD marker (posttranslational modification or mark, target gene, surrogate tissue or tumor?) Epigenetic changes at metastatic sites can differ from primary tumor, which should be targeted clinically?

Adapted by Campbell *et al*^[44], 2014.

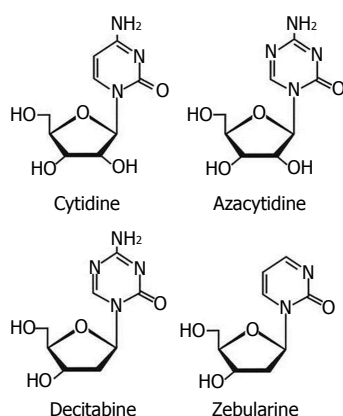
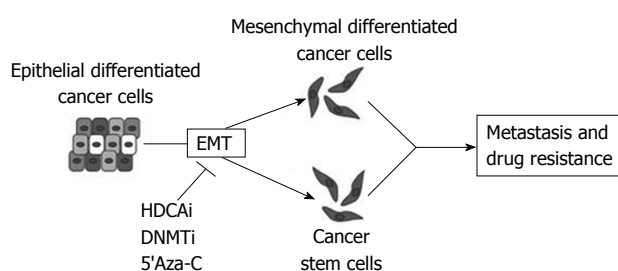
**Figure 11** DNA methyltransferase inhibitors, analog to nucleoside.

Figure 12 Different classes of epigenetic drugs could inhibit epithelial-mesenchymal transition which plays a crucial role in tumor progression generating both mesenchymal differentiated and stem cancer cells. HDCAi: Histone deacetylase inhibitor; DNMTi: DNA methyltransferase inhibitor; 5'Aza-C: 5-azacytidine are given as examples of demethylating agent. Adapted by Kiesslich *et al*^[41], 2013.

although their clinical efficacy is limited by toxicity and chemical instability. Zebularine [1-(β -D-ribofuranosyl)-1,2-dihydropyrimidin-2-one] is a DNMT inhibitor characterized by more stability and less toxicity with a

inhibitory effect on cytidine deaminase (Figure 11).

All these drugs are based on the rationale that, unlike genetic mutations, epigenetic alterations is potentially reversible, thus being an attractive target for cancer therapy.

Since hypermethylation of tumor suppressor genes and overexpression of DNMTs are crucial events for tumor progression, the possibility of de-methylating DNA sequences seems a good strategy for cancer therapy. DNMT inhibitors, in fact, can allow re-expression of aberrantly silenced genes and restore their normal function. Azacytidine (Vidaza; Celgene) and decitabine (5 aza 2' deoxycytidine) (Dacogen; SuperGen) have been approved by the Food and Drug Administration (FDA) for current management of acute myeloid leukemia and myelodysplastic syndrome. Azacytidine has also been approved by the FDA and the European Medicines Agency for use against chronic myelomonocytic leukemia. More recently these two drugs have also been introduced in clinical trials in patients with solid tumors.

Zebularine is a novel member of the nucleoside DNMT inhibitor family, not yet used routinely in clinical practice. Although much *in vitro* data show good results, especially in terms of less toxicity compared to azacytidine or decitabine, zebularine use for future clinical trials is needed^[45].

Moreover, recent studies have also demonstrated a possible use of epigenetic-modifying drugs in targeting invasion, metastasis and drug resistance, all involving EMT (Figure 12)^[11].

CONCLUSION

It is already well known that cancer is a heterogeneous disease and an integrated genome, epigenome, and

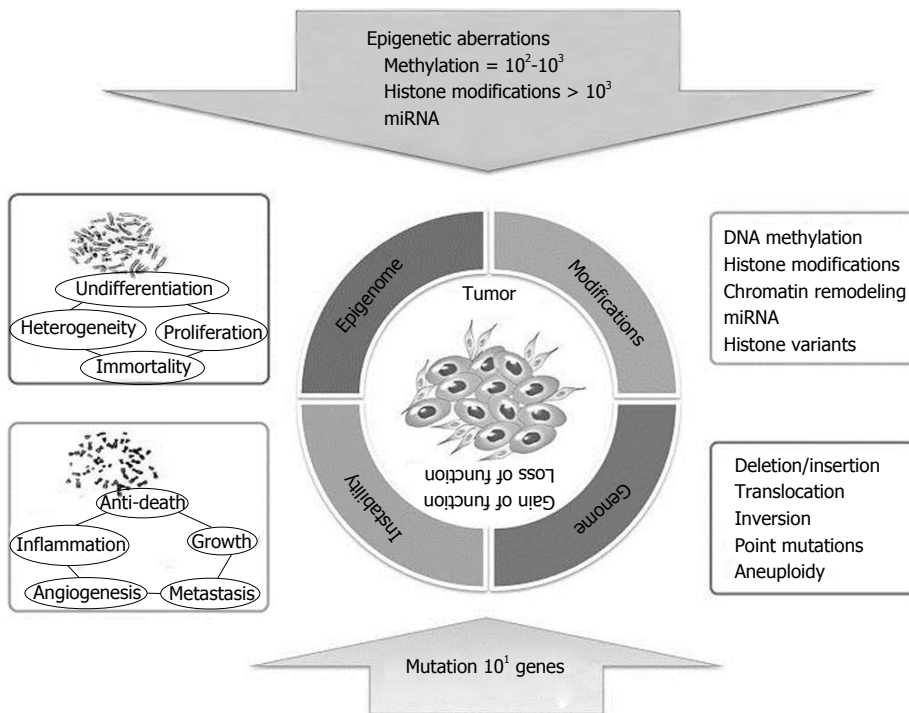


Figure 13 Epigenetics and genetics cooperates in cancerogenesis. Adapted by Choi *et al*^[1], 2013.

transcriptome analysis seems necessary to help clinicians to find good therapeutic strategies to treat this complex disease.

Only in recent years have researchers begun to integrate data deriving from both genetic and epigenetic alteration analyses, including mutations, CNVs, structural changes, epigenetic profiles, and expression changes in both coding and non-coding RNAs. Thanks to these studies, it is now well accepted that epigenetic abnormalities can play a crucial role in tumor initiation and development. In addition, it is now recognized that tumor cells present epigenetic silencing at higher frequency than mutations. Indeed, in tumour cells, hyper- or hypomethylation, histone modifications, and miRNA expression dysregulation are present in thousands of genes, while mutations affect only tens of genes, although all of them determine gene inactivation (Figure 13).

Epigenetic events can be useful biomarkers for detecting disease and predicting therapeutic efficacy. The epigenome undergoes all the above described during tumor initiation driving tumor cell heterogeneity, and consequently progression. Notably, those patterns are stable but reversible depending on the cellular environment, while mutations remain irreversibly locked into the cancer genome. For this reason, epigenetic events could be “drugable” targets for reversing epimutational effects and associated phenotypes.

Basing on the hypothesis that epigenetic agents may enhance sensitivity to conventional drugs (e.g., platinum or taxane chemotherapy), many efforts have been made for using epigenetic agents to re-sensitize tumours recurrent or refractory to first-line treatment.

Advances in genome-wide methylation analyses

and the combination of new epigenetic drugs with conventional therapies could offer new hope for cancer patients, providing in the near future more effective patient-tailored treatments.

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Therapeutic targets in gastrointestinal stromal tumors

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Abstract

Gastrointestinal stromal tumors (GISTs) are the most common type of mesenchymal tumor of the gastrointestinal tract. The tumorigenesis of GISTs is driven by gain-of-function mutations in *KIT* or platelet-derived growth factor receptor α (*PDGFRA*), resulting

in constitutive activation of the tyrosine kinase and its downstream signaling pathways. Oncogenic *KIT* or *PDGFRA* mutations are compelling therapeutic targets for the treatment of GISTs, and the *KIT*/*PDGFRA* inhibitor imatinib is the standard of care for patients with metastatic GISTs. However, most GIST patients develop clinical resistance to imatinib and other tyrosine kinase inhibitors. Five mechanisms of resistance have been characterized: (1) acquisition of a secondary point mutation in *KIT* or *PDGFRA*; (2) genomic amplification of *KIT*; (3) activation of an alternative receptor tyrosine kinase; (4) loss of *KIT* oncoprotein expression; and (5) wild-type GIST. Currently, sunitinib is used as a second-line treatment for patients after imatinib failure, and regorafenib has been approved for patients whose disease is progressing on both imatinib and sunitinib. Phase II/III trials are currently in progress to evaluate novel inhibitors and immunotherapies targeting *KIT*, its downstream effectors such as phosphatidylinositol 3-kinase, protein kinase B and mammalian target of rapamycin, heat shock protein 90, and histone deacetylase inhibitor. Other candidate targets have been identified, including *ETV1*, *AXL*, insulin-like growth factor 1 receptor, *KRAS*, *FAS* receptor, protein kinase c theta, *ANO1* (*DOG1*), *CDC37*, and aurora kinase A. These candidates warrant clinical evaluation as novel therapeutic targets in GIST.

Key words: Gastrointestinal stromal tumors; Tyrosine kinase inhibitors; *KIT*; Platelet-derived growth factor receptor α ; Targets

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Core tip: Oncogenic *KIT* and platelet-derived growth factor receptor α (*PDGFRA*) mutations are compelling therapeutic targets in gastrointestinal stromal tumors (GISTs), and the *KIT*/*PDGFRA* kinase inhibitors imatinib, sunitinib, and regorafenib are the standards of care for patients with unresectable or metastatic GIST. However, most patients eventually develop resistance to these kinase inhibitors, resulting in an urgent need to identify biologically rational targets for novel therapies.

Herein, we review advances in the research on GIST and the therapies that are used to treat it. Additionally, we discuss novel agents, targets, and strategies for the future treatment of GIST.

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INTRODUCTION

Gastrointestinal stromal tumors (GISTs) were originally described as smooth muscle or neural tumors of the gastrointestinal (GI) tract; however, in 1983, Mazur *et al*^[1] referred to GISTs as "stromal tumors"^[2,3]. Subsequent studies identified the interstitial cells of Cajal as the origin of GISTs. In 1998, activating mutations of the *KIT* receptor tyrosine kinase (RTK) were found in GISTs^[4]. In 2003, platelet-derived growth factor receptor α (*PDGFRA*) mutations, an alternative target, were identified in GISTs that lacked *KIT* mutations^[5].

GISTs are the most common mesenchymal tumors of the GI tract and are frequently seen in the stomach (60%), small intestine (25%), colorectum (5%-10%) and occasionally in the esophagus and appendix^[5]. Histologically, GISTs may be composed of spindle cells (70%), epithelioid cells (20%), or a mixture of these types (10%)^[6]. Morphologically, GISTs may be mistaken for smooth muscle neoplasms, such as leiomyoma and leiomyosarcoma (Figure 1)^[6]. Consensus guidelines for GIST prognosis, accentuate risk stratification based on the tumor volume and mitotic index of the primary tumors (Table 1)^[2].

The majority of GISTs contain oncogenic mutations of *KIT* (approximately 85%) or *PDGFRA* (approximately 5%-10%)^[2,4-6]. The resulting mutant oncoproteins are crucial for GIST oncogenesis, proliferation, and survival, as demonstrated by the clinical successes of small molecule therapeutics targeting *KIT* and *PDGFRA*^[7-9]. Imatinib, sunitinib, and regorafenib are the standard first-, second- and third-line therapies, respectively, in patients with inoperable GISTs^[10-12], and adjuvant imatinib is used in patients with localized GISTs with a high risk of recurrence^[13].

Except from imatinib, sunitinib, and regorafenib, which target the activated oncoproteins *KIT* and *PDGFRA* in inoperable or metastatic GIST, the increasing novel drugs are currently in clinical trials, and additional potential therapeutic targets have been identified. Herein, we summarize these agents, targets, and strategies for the future treatment of GIST.

KIT AND PDGFRA ARE MAJOR THERAPEUTIC TARGETS IN GISTS

Oncogenic mutant *KIT* and *PDGFRA* play a critical function in the initiation of the transformation event that leads to

GIST. Mutations in *KIT* are usually found in the regulatory and dimerization domains, which are located in the extracellular region encoded by exon 9 (approximately 13% of GISTs), the juxtamembrane region encoded by exon 11 (approximately 66% of GISTs), or the tyrosine kinase (TK)[I] [adenosine triphosphate (ATP) binding pocket]; and TK[II] (activation loop) domains encoded by exon 13 (approximately 1% of GISTs) and exon 17 (approximately 0.6% of GISTs), respectively^[2,14,15]. Five percent to ten percent of GISTs contain mutations in *PDGFRA* exon 12 (juxtamembrane region) (1.5%) or exon 18 (activation loop) (5.6%). The remainder (10%-12%) are wild-type for both *KIT* and *PDGFRA*^[2,6]. The percentage of population of *KIT* and *PDGFRA* mutations is shown in Figure 2^[2].

GISTs harboring insertions, deletions, and missense mutations in *KIT* exon 11 can be found throughout the GI tract^[16]. A enhanced metastasis and proliferation has been associated with loss of heterozygosity at the *KIT* locus^[17,18]. The vast majority of GIST cases with alterations of *KIT* in exon 9 involve an insertion of six base pairs, resulting in the duplication of Ala and Tyr residues. These mutations often occur in high-risk primary GISTs of the small intestine^[17,19,20], advanced or relapsed GISTs^[18,21]. A recent study demonstrated that GISTs harboring *KIT* exon 17 and exon 13 mutations show slightly overrun population among a subset of GISTs. Most of single base pair substitution *KIT* mutations in exon 13 and 17 in small intestinal GISTs, have no marked effects on the clinicopathologic characteristics when compared to the "average" small intestinal GIST^[22].

The majority of *PDGFRA* exon 14 and 18 alterations are missense mutations. GISTs harboring *PDGFRA* mutations are confined to the stomach and omentum. These tumors are shortage of *KIT* expression, they typically present an epithelioid morphology, and they are commonly associated with a benign prognosis^[23,24]. GISTs harboring a D842V *PDGFRA* exon 18 mutation are resistant to imatinib and other RTK inhibitors^[25-28].

Inhibition of *KIT* or *PDGFRA* kinase activity by imatinib results in an objective response in approximately 80% of metastatic GIST patients (approximately 50% partial response, approximately 30% stable) with a 3-year survival rate of 69%-74%^[8]. However, the median survival of metastatic GIST patients was 19 mo in the pre-imatinib period^[10,15]. Constitutive activation of *KIT* or *PDGFRA* results in the activation of downstream signaling intermediates necessary for proliferation, survival, adhesion, and blockage of differentiation, including the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) and RAF/mitogen-activated protein kinase (MAPK) pathways. Targeting *KIT*/*PDGFRA* and its downstream intermediates has proven to be an effective strategy in the treatment of GISTs^[29-32].

MECHANISMS OF IMATINIB RESISTANCE

Imatinib, an ATP-competitive inhibitor of *KIT* and *PDGFRA*, is the first-line therapy for patients with

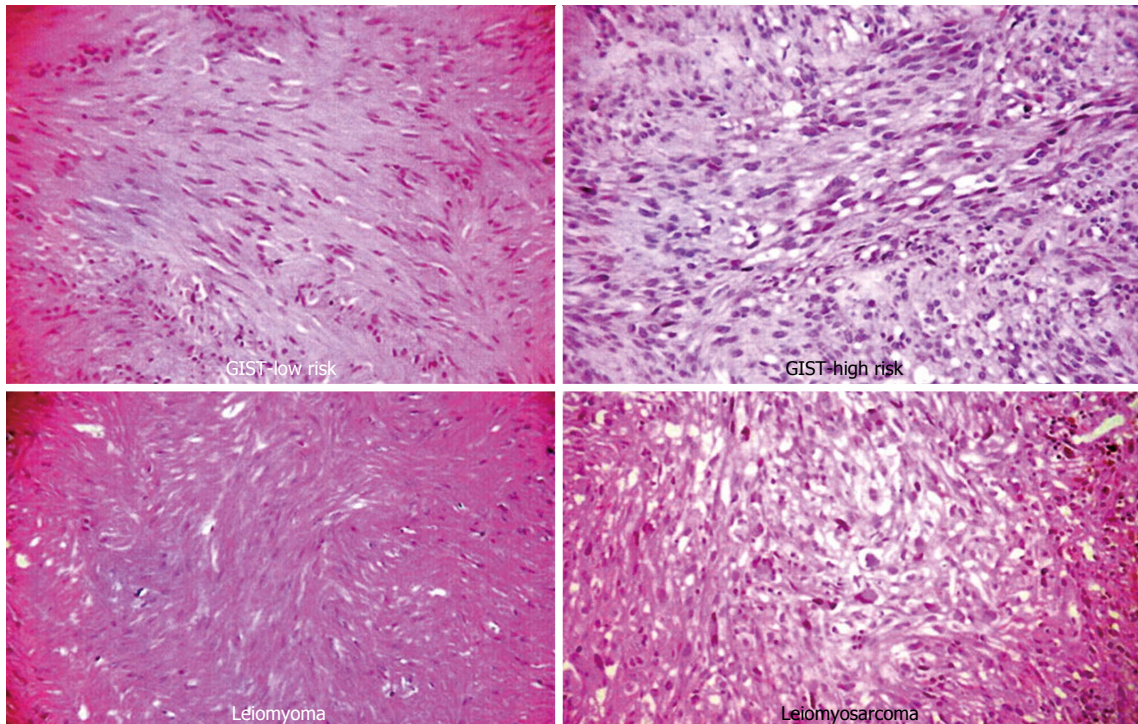


Figure 1 Morphologic similarities of low-risk gastrointestinal stromal tumor and leiomyoma and of a high-risk gastrointestinal stromal tumor and leiomyosarcoma. GIST cells can be divided into 3 types: spindle cell (70% of cases), epithelioid cell (20% of cases), and mixed cell (containing a mixture of spindle and epithelioid cells). GIST: Gastrointestinal stromal tumor.

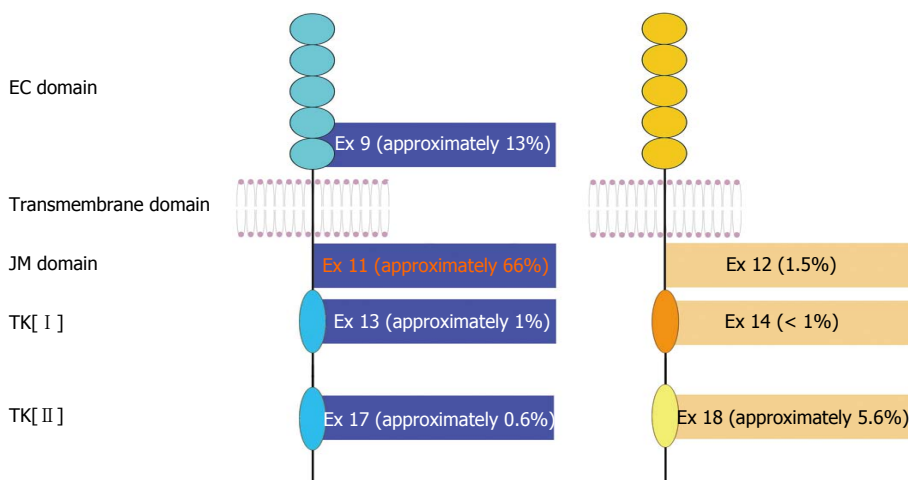


Figure 2 Schematic structure of *KIT* and platelet-derived growth factor receptor α receptor tyrosine kinases and distribution of *KIT* mutations in gastrointestinal stromal tumor. EC: Extracellular; JM: Juxtamembrane; TK[I]: Tyrosine kinase domain I; EX: Exon.

advanced GIST or primary GIST with a significant risk of recurrence after surgery^[28,33-35]. Among patients with advanced GIST, 75% to 90% will show a response to imatinib^[15]. Analysis of the crystal structure of the KIT-imatinib complex reveals that the drug fills a hydrophobic region of the ATP binding pocket, effectively blocking ATP binding and inactivating KIT and its downstream signaling^[36,37].

Despite the dramatic clinical success of imatinib, most inoperable GIST patients eventually develop resistant to imatinib. Imatinib resistance in GIST is classified as either primary or secondary imatinib

resistance. Approximately 10% of GISTs demonstrate primary imatinib resistance of clinical progression within 3 to 6 mo of the start of treatment^[28,38]. Primary imatinib resistance is usually observed in tumors that lack *KIT* or *PDGFRA* mutations (wild-type GISTs), but it is also common in tumors harboring *KIT* exon 9 mutations^[28,38]. Approximately 40% to 50% of GIST patients experience secondary imatinib resistance of clinical progression after 12-36 mo of response or disease stabilization. Molecular studies showed that activated KIT expression in imatinib-resistant tumors was similar to or greater than those typically found in

Table 1 Risk stratification of primary gastrointestinal stromal tumor by mitotic index, size and anatomic location^[2]

Prognosis of primary GIST		
Risk	Size (cm)	Mitotic count (per 50 HPF)
Very low risk	< 2	< 5
Low risk	2-5	< 5
Intermediate risk	< 5	6-10
	5-10	< 5
High risk	> 5	> 5
	> 10	> Any mitotic rate
	Any tumor	> 10

HPF: High power fields; GIST: Gastrointestinal stromal tumor.

untreated GISTs^[15]. Secondary *KIT* mutations were rare in GISTs with primary resistance but often found in GISTs with secondary resistance (10% vs 67%; $P = 0.002$). Polyclonal secondary kinase mutation was detected in 18.8% patients. The secondary kinase mutations were nonrandomly distributed and were associated with attenuated imatinib sensitivity compared with *KIT* exon 9 and exon 11^[15]. Mechanisms of acquired resistance include secondary mutations in *KIT* or *PDGFRA*, genomic amplification of *KIT*, or activation of an alternative RTK^[6,14,39-46]. An even more challenging resistance mechanism, seen in approximately 5%-10% of clinically progressing *KIT*-mutant GISTs involves a transition from dependence on oncogenic *KIT* to a new imatinib-insensitive oncogenic driver, accompanied by the loss of former *KIT* expression^[39,40].

NOVEL INHIBITORS IN PRE-CLINICAL MODELS AND CLINICAL TRIALS

Tumorigenesis is a complex, multi-step process, and oncogenic RTK proteins frequently play key roles^[47] (Table 2). Oncogenic RTK mutations can lead to constitutive kinase activation and thereby enhance growth and survival in cancer cells^[48,49]. Tyrosine kinases can be divided into two categories: receptor tyrosine kinases and non-receptor tyrosine kinases. At present, approximately 90 types of TK members have been identified, including 58 RTKs, such as PDGFR, epidermal growth factor receptor (EGFR), fibroblast growth factor receptor, and 32 non-RTKs^[50]. Oncogenic RTK mutants are useful therapeutic targets, as shown by the clinical benefit of small molecular inhibitor therapies in chronic myeloid leukemia (BCR-ABL)^[51], metastatic breast cancer [human epidermal growth factor receptor 2 (HER2)]^[52], GIST (*KIT*/*PDGFRA*)^[8], and non-small-cell lung cancer [EGFR, hepatocyte growth factor receptor (MET), anaplastic lymphoma kinase, HER2]^[47,53-62].

Sunitinib is an oral multi-target tyrosine kinase inhibitor (TKI) with activity against *KIT*, *PDGFRA*, FMS-Like Tyrosine Kinase 3, Vascular endothelial growth factor receptor (VEGFR), and orphan receptor tyrosine

kinase^[63]. Sunitinib is approved for use as a second-line therapy for patients with imatinib-resistant GIST^[9,64,65]. A clinical benefit of sunitinib was seen in common primary GIST with *KIT* exon 9 (58%), *KIT* exon 11 (34%), and wild-type *KIT*/*PDGFRA* (56%)^[9]. Progression-free survival (PFS) was greater improvement for patients with a wild-type genotype ($P = 0.0356$) or with primary *KIT* exon 9 mutations ($P = 0.0005$) than for those with *KIT* exon 11 mutations. Overall survival (OS) showed the similar pattern. The PFS and OS were greater improvement for patients with secondary *KIT* exon 13 or 14 mutations than for those with exon 17 or 18 mutations^[9]. The safety and efficacy of regorafenib in metastatic or unresectable GIST patients after failure of imatinib and sunitinib were evaluated in phase III, and the results showed that regorafenib can markedly improve PFS compared with control in metastatic GIST patients with progression after standard treatments^[12,66]. Currently, regorafenib has been approved for patients whose tumors are progressing on both imatinib and sunitinib. A large number of therapies are in various stages of pre-clinical and clinical trial development and are summarized in Table 2^[10,13,14,21,30,64,67-84]. These therapies can be divided into four groups: TKIs, PI3K/mTOR inhibitors, heat shock protein 90 (HSP90) inhibitors, and others.

Multiple TKIs, including nilotinib, sorafenib, dasatinib, vatalanib, and motesanib, are being investigated as potential therapies for GIST. Nilotinib, an inhibitor of *KIT*, *PDGFRA* and BCR-ABL, has been shown to be active in a small series of imatinib-resistant and sunitinib-resistant GIST patients in a phase I study^[67,71,74,85]. Sorafenib, an inhibitor of RAF kinase, VEGFR, PDGFR, and *KIT*, inhibited *KIT* activity in some *KIT* primary and secondary mutations in a phase II trial in imatinib- and sunitinib-resistant GIST^[69,80,86,87]. Dasatinib, a dual SRC/ABL kinase inhibitor, binds and inactivates wild-type and mutant *KIT* regardless of the conformation of the *KIT* activation loop^[42,43]. Linsitinib (OSI-906) is a selective inhibitor of insulin-like growth factor receptor (IGFR)/insulin receptor. The combination of imatinib and linsitinib has been shown to be effective in wild-type GIST with insulin-like growth factor 1 receptor (IGF1R) overexpression or amplification^[88]. Vatalanib (PTK787) and motesanib (AMG706), multi-kinase inhibitors, have been evaluated in phase II trials for patients who are resistant to both imatinib and sunitinib^[89,90]. Vatalanib has shown activity in patients with imatinib-resistant or both imatinib- and sunitinib-resistant GIST^[89,90]. Motesanib treatment was shown to have acceptable toxicity, and it resulted in disease stabilization in GIST patients^[82].

The PI3K/AKT/mTOR pathway is crucial for proliferation and survival in GIST^[29,30,68,91-93]. Preclinical experiments have confirmed that targeting the PI3K/AKT/mTOR pathway is a rational therapeutic strategy. Early studies with mTOR inhibitors have shown limited success, possibly due to feedback activation of AKT

Table 2 Novel agents are being developed for gastrointestinal stromal tumor therapy^[10,13,14,21,30,64,67-84]

Agent	Molecular target	Phase
Kinase inhibitors		
Nilotinib	KIT, PDGFRs, BCR-ABL	I
Sorafenib	Raf, KIT, PDGFRB, VEGFR, FLT3, RET	71%
Dasatinib	Src, ABL, KIT, PDGFRs	Phase II ongoing in advanced sarcomas and accepting patients
Cediranib (AZD2171)	VEGFR, KIT, PDGFRs	Phase II ongoing
OSI-930	VEGFR, KIT	Phase II ongoing, not recruiting
Linsitinib (OSI-906)	IGF1R	Phase III
Vatalanib (PTK787)	VEGFR, KIT, PDGFRs	67%
Motesanib (AMG706)	VEGFR, KIT, PDGFRs, RET	24%-27%
XL820	KIT, PDGFRB, VEGFR	Phase II ongoing, not recruiting
mTOR and AKT inhibitors		
Perifosine	AKT	Phase II ongoing in combination with imatinib
Everolimus	mTOR	26%
Temsirolimus	mTOR	Phase II ongoing, closed recruitment
Hsp90 inhibitors		
17-AAG	Hsp90	Phase II / III
Ganetespib (STA-9090)	Hsp90	Phase II
AUY922	Hsp90	Phase II
AT13387	Hsp90	Phase II ongoing in combination with imatinib
IPI-504	Hsp90	78%, phase III ended due to safety concerns
Others		
Flavopiridol	Transcription inhibitor	Phase I ongoing in combination with doxorubicin
Clinical benefit is defined as complete or partial response or stable disease		

PDGFRs: Platelet-derived growth factor receptors; PDGFRA: Platelet-derived growth factor receptor α ; PDGFRB: Platelet-derived growth factor receptor β ; VEGFR: Vascular endothelial growth factor receptor; FLT3: FMS-Like Tyrosine Kinase 3; IGF1R: Insulin-like growth factor 1 receptor; AKT: Protein kinase B; mTOR: Mammalian target of rapamycin; Hsp90: Heat shock protein 90; RET: Orphan receptor tyrosine kinase.

after mTORC1 inhibition. Simultaneous targeting of multiple nodes in the PI3K/AKT/mTOR pathway prevents feedback activation and may translate into more complete pathway inhibition. A few therapies targeting this pathway are currently being evaluated in phase I and II clinical trials^[94]. A number of drugs currently in development include inhibitors of pan-Class I PI3K (BKM120 and GDC0941), PI3K/mTOR (BEZ235, SF1126 and GDC0980), AKT (Perifosine), and mTOR (Everolimus/RAD001 and Temsirolimus). Additionally, combined inhibition of KIT and PI3K/AKT/mTOR results in a greater response compared to either intervention alone^[73,94-97].

Heat shock proteins control the proper folding, function, and stabilization of various client proteins. HSP90 optimizes and maintains the folding and localization of many activated tyrosine kinases and also prevents proteasomal degradation^[98]. HSP90 is abundant in eukaryotic cells, comprising up to 1%-2% of total cellular protein, and it plays key roles in regulating cell proliferation, differentiation, and apoptosis^[99,100]. The HSP90 inhibitor 17-allylamino-17-demethoxygeldanamycin (17-AAG), a geldanamycin derivative^[101], binds a ATP-interaction pocket in the HSP90 NH₂-terminal domain^[102] and shows anti-proliferative effects in various human cancers, where it can degrade HSP90-client oncoproteins with high selectivity^[103,104]. Whereas the clinical application of 17-AAG has been hampered by its low water solubility, IPI-504, a 17-AAG derivative, exhibits improved aqueous solubility while maintaining the biological

HSP90-inhibitory properties of 17-AAG^[105]. Furthermore, clinical trials with new-generation synthetic HSP90 inhibitors are ongoing in various cancer types. HSP90 is an attractive target in GIST as it is a key chaperone for KIT and PDGFRA^[79,106]. Targeting HSP90 results in pro-apoptotic and anti-proliferative effects in GIST and is associated with the inhibition of KIT and PDGFRA signaling^[72,79,107,108]. Other HSP90 inhibitors are in development (NVP-AUY922, AT-13387, KW-2478, and SNX-5422) and show promise for GIST treatment, particularly in combination with TKI^[109].

Other drugs are in various stages of development for the treatment of GIST. Flavopiridol, a transcription inhibitor, has been evaluated in an ongoing phase I trial in combination with doxorubicin^[110]. Histone deacetylase inhibitors (HDACIs) alone or in combination with imatinib have shown pro-apoptotic and anti-proliferative effects in GIST and are associated with inhibition of KIT and a reduction in the expression and activities of downstream pathways^[111].

NOVEL CANDIDATE THERAPEUTIC TARGETS

Other therapeutic targets have been identified for the treatment of GIST, including Ets Variant 1 (ETV1), AXL, FAS, IGF1R, protein kinase c theta (PKC θ), RAS, CDC37, cyclin D1, Dog1, and aurora kinase A. Inhibitors targeting these candidates are being developed, and some are being evaluated in clinical trials.

The E26 transformation-specific family member ETV1 is overexpressed in the GIST and is required in the development of both imatinib-sensitive and imatinib-resistant GIST^[112-114]. ETV1 enhancer binding is a master regulator of an ICC-GIST-specific transcription network. Activated KIT cooperates with ETV1 to induce development of GIST, regulating the ETV1 transcriptional program by prolonging ETV1 protein stability through MAPK signaling^[112,114]. Inhibition of ETV1 reduces the expression of KIT, reduces mutagenesis, and stabilizes the GIST genome, thereby inhibiting GIST growth and progression and inducing apoptosis.

AXL (UFO/ARK/Tyro), an RTK stimulated by its ligand growth arrest-specific 6, shows potent oncogenic and transforming activity in normal and cancer cells^[115-117]. AXL also plays a role in tumor cell invasion, metastasis, and survival^[141,118,119]. AXL is active in GIST metastases that lose KIT expression at the time of clinical progression on imatinib^[41,120]. In KIT-independent GISTs, AXL knockdown results in upregulation of p21, p27 and p53 protein expression and shows anti-proliferative effects^[120]. MP470, a KIT/AXL inhibitor, shows a synergistic cytotoxic effect in GIST cells when combined with docetaxel (taxotere)^[41].

Fas and its ligand FasL belong to the tumor necrosis factor family of death receptors. Activation of Fas by FasL induces cell apoptosis through caspase 8 signaling. Down-regulation of Fas is associated with tumorigenesis^[121,122]. Fas and FasL expression were positively correlated in primary GISTs, but there was no association KIT mutation status^[123]. MegaFasL, a hexameric form of soluble FasL, is an active apoptosis-inducing agent and potentiated the apoptotic effects of imatinib in GIST cell lines^[123].

The IGF/IGF1R signaling system has been implicated as a relevant therapeutic target in a variety of cancers. When IGF1 binds with IGF1R, it activates downstream signaling cascades, such as the PI3K/AKT/mTOR and RAF/MEK/MAPK pathways, to trigger protein synthesis, and it also activates anti-apoptotic and proliferative pathways^[124-126]. Recent reports have shown that *IGF1R* is amplified in a subset of GISTs^[127] and over-expressed in wild-type and pediatric GIST^[88,128,129]. Recent studies have shown that the IGF/IGF1R pathway may be a promising therapeutic target for GIST^[127,130-135].

PKC θ , a member of the protein kinase C family commonly expressed in T cells and myogenic cells^[136,137], is expressed at high levels and activated in GIST irrespective of the *KIT* or *PDGFRA* status. Therefore, PKC θ serves as a diagnostic marker of GIST^[138-141]. PKC θ knockdown is accompanied by inactivation of KIT in KIT+/PKC θ + GIST cell lines. PKC θ knockdown resulted in inhibition of PI3K/AKT signaling, upregulation of pro-apoptotic proteins p21 and p27, cell cycle arrest, and apoptosis, recapitulating the effect of direct KIT targeting^[142]. PKC θ is a compelling therapeutic target in GISTs, including those with mutations that confer resistance to KIT/PDGFR α inhibitors.

Wild-type GISTs often demonstrate primary imatinib

resistance. In some cases, these tumors are succinate dehydrogenase (SDH)-deficient GISTs with mutations in *SDHA*, *SDHB*, or *SDHC*^[143,144], while others have no known genetic mutations. A recent report suggested that *KRAS* mutations might confer imatinib resistance in GIST, and although rare, *KRAS* gain-of-function mutations contribute to clinical imatinib resistance^[145,146]. Serrano *et al.*^[145] used a Sequenom panel to screen for *RAS*, *BRAF*, and *PI3KCA* mutations in 27 wild-type GIST patients. Only one of these 27 GISTs contained a mutation in this pathway, harboring concomitant *HRAS* G12V and *PIK3CA* H1047R mutations^[145]. *KRAS* and *HRAS* can contribute to GIST oncogenesis and indicate the importance of the PI3K/AKT and RAS/RAF pathways in GIST tumorigenesis.

As discussed previously, HSP90 inhibitors strongly inactive KIT kinase activity, but clinical applications in GIST patients have been prevented due to the toxicity resulting from inactivation of HSP90 client proteins beyond KIT and PDGFRA. Genome-scale short-hairpin RNA (shRNA) screening identified CDC37, an HSP90 cofactor, as an essential GIST-specific gene^[147]. Validation studies in treatment-naïve and imatinib-resistant GIST cell lines demonstrated that CDC37 is a viable therapeutic target in GIST, recapitulating the effect of HSP90 inhibition while remaining selective for KIT/PDGFR α and a limited number of other HSP90 clients^[147]. CDC37 inhibition represents a potential HSP90 targeting strategy that limits toxicity for GIST patients.

The strongly expressed DOG1 (ANO1/TMEM16A) has been used as a diagnostic marker to differentiate GIST from other sarcomas^[148-151]. Loss of DOG1 expression occurs together with loss of KIT expression in a subset of GISTs that are resistant to imatinib. Although DOG1 inhibition do not inhibit cell growth *in vitro*, DOG1 knockdown delays the growth of xenograft models of GIST and is associated with the up-regulation of insulin-like growth factor binding protein 5, a potent antiangiogenic factor implicated in tumor suppression^[152]. These findings suggest that DOG1 is a potential target in GIST through its role in IGFR signaling.

A recent analysis of the prognostic significance of aurora kinase A (AURKA) in imatinib-treated patients with advanced GIST suggested that the expression of AURKA may predict recurrence in patients with primary, surgically resected GISTs^[153,154]. AURKA overexpression is a prognostic factor of poor PFS and OS. Inhibition of AURKA suppresses the growth of both imatinib-sensitive and imatinib-resistant GIST cells in a concentration-dependent manner, and it results in a synergistic cytotoxicity with imatinib^[154].

CONCLUSION

Oncogenic KIT or PDGFRA receptor tyrosine kinase mutations are compelling therapeutic targets in GISTs, and the KIT/PDGFR α kinase inhibitors imatinib,

sunitinib, and regorafenib are standards of care for patients with unresectable or metastatic GIST. However, most patients eventually develop resistance to KIT/PDGFR kinase inhibitors, indicating that there is an urgent need to identify novel therapeutic strategies. A number of novel drugs are undergoing clinical trials, and several novel therapeutic targets have been identified, showing promise for the future treatment of GIST.

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Prospective Study

Modern advances in reducing anxiety and pain associated with cystoscopy: Systematic review

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Data sharing: Technical appendix, statistical code, and dataset available from the corresponding author at email address: kyoko.sakamoto@va.gov. All participants gave informed consent for data sharing.

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Abstract

AIM: To investigate if music reduces anxiety and pain in the Veterans Affairs population undergoing flexible cystoscopy.

METHODS: This study was reviewed and approved by the University of California, San Diego Human Research Protections Program Institutional Review Board. Patients were prospectively randomized to undergo flexible cystoscopy with or without music. Thirty-eight patients were randomized into either the No Music group ($n = 24$) or the Music group ($n = 14$). We used the state-trait anxiety inventory and the visual analog pain scale, respectively. Statistics were generated and compared using an independent t -test and chi-squared tests. P values < 0.05 were considered statistically significant. Outpatient cystoscopy is a safe and useful procedure employed frequently in Urology for diagnosis and evaluation of genitourinary pathologies. However, cystoscopy-related distress cannot be ignored. Three components of outpatient cystoscopy have been evaluated to improve the cystoscopic experience: local anesthetic control, cystoscopic equipment redesign and environmental modification. We reviewed the literature pertaining to these modifications.

RESULTS: The mean age was 65.3 and 67.1 years for men in the No Music and Music groups, respectively.

Although, the majority of patients in each group self-identified as Caucasians (66%), African American, Hispanic and other ethnicities represented 13%, 8% and 13% respectively. The majority of patients (68%) reported experiencing hematuria. Thirty-four percent had a history of bladder cancer, and eighteen percent had a history of prostate cancer. Ten patients (26%) admitted to taking antidepressants. Physiologic parameters that correlated to pain and anxiety (systolic blood pressure, diastolic blood pressure, and heart rate) were statistically similar in both groups prior to and after flexible cystoscopy. The median delta anxiety between the No Music and Music groups were not significantly different (0.78 *vs* -1.46), and the pain scores between the No Music and Music groups (1.5 *vs* 1.6) were not statistically different ($P = 0.28$ and $P = 0.92$, respectively).

CONCLUSION: Preliminary results demonstrate that music does not reduce anxiety or pain associated with flexible cystoscopy.

Key words: Flexible cystoscopy; Veterans; Anxiety; Music

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Core tip: Flexible cystoscopy is a very common in-office procedure performed in urology. There have been several technological advances made in the instrumentation of flexible cystoscopies, however, there have also been advances made in reducing patient pain and anxiety associated with this procedure such as viscous lidocaine jelly and music. We reviewed the literature on effects of modifiable factors on patient pain and anxiety associated with flexible cystoscopy, and also includes preliminary data on a Veterans Affairs randomized prospective trial evaluating the effect of classical music on pain and anxiety associated with flexible cystoscopy.

Mirheydar HS, Raheem OA, Elkhoury FF, Jabaji R, Palazzi KL, Patel N, Du R, Maroney S, Sakamoto K. Modern advances in reducing anxiety and pain associated with cystoscopy: Systematic review. *World J Transl Med* 2015; 4(1): 38-43 Available from: URL: <http://www.wjgnet.com/2220-6132/full/v4/i1/38.htm> DOI: <http://dx.doi.org/10.5528/wjtm.v4.i1.38>

INTRODUCTION

Cystoscopy is a common urologic examination indicated for a wide variety of genitourinary conditions^[1-4]. Cystoscopy was first conceptualized over two centuries ago^[5] and underwent multiple advancements in technology to allow patients to undergo the procedure with relative comfort. Regardless of the reason for the inspection, however, cystoscopy is invasive and can be

a distressing experience for patients.

Since patient distress negatively impacts adherence to cystoscopy guidelines, many studies have investigated techniques to reduce cystoscopy-associated anxiety and pain^[1]. Three general methods to alleviate pain and anxiety are local anesthetic control, cystoscopic equipment redesign, and environmental modification. Lidocaine lubricants, inhaled nitrous oxide, and anxiolytic medication have been proposed to ameliorate flexible cystoscopy-associated distress, though none sufficiently relieves patient fear, pain, and anxiety^[6-14]. The cystoscopic instrument has evolved from the rigid cystoscope to the flexible cystoscope, changing from analog visualization to digital and more recently digital high definition visualization.

For environmental modification, music is gaining increasing recognition as an effective tool to alleviate perceived pain and has been shown to be beneficial in a variety of clinical settings^[15-23]. Herein, we report the result of a prospective randomized trial of the effect of music on pain and anxiety in the Veterans Affairs patient population during flexible cystoscopy. Furthermore, we reviewed the above-mentioned three factors (local pain control, equipment redesign and environmental modification) incorporated to lessen the anxiety and pain levels during cystoscopy.

MATERIALS AND METHODS

This study was reviewed and approved by the University of California, San Diego Human Research Protections Program Institutional Review Board (IRB). Patient privacy and confidentiality are protected according to HIPAA guidelines. Following IRB approval, male patients at the Veterans Affairs San Diego Medical Center were prospectively randomized to undergo flexible cystoscopy with or without music. Thirty-eight patients were randomly assigned into one of two groups using an adaptive biased-coin randomization method: (1) the No Music group (patients did not hear music during preparation and draping of patient and anesthetization of urethra); or (2) the Music group (patients listened to the same excerpt of classical music). Inclusion criteria were age > 18 years old. There were no women in this study, although that was not an exclusion criterion. Exclusion criteria were current urinary tract infection, anatomic urethral abnormalities, and inability to complete the surveys. Patients' past medical history, including history of bladder cancer, prostate cancer, hematuria, and current or past use of anti-depressants and anti-anxiety medication, was recorded. All patients with a prior history of bladder cancer were confirmed to have had prior cystoscopy and were currently undergoing cystoscopy for surveillance, while those without bladder cancer were undergoing cystoscopy for diagnostic purposes (e.g., microhematuria). Each patient was consented for the study on the day of the procedure.

	Least anxious ←				→ Most anxious
I feel pleasant	[1]	[2]	[3]	[4]	
I feel nervous and restless	[1]	[2]	[3]	[4]	
I feel satisfied with myself	[1]	[2]	[3]	[4]	
I feel I could be as happy as others seen to be	[1]	[2]	[3]	[4]	
I feel like a failure	[1]	[2]	[3]	[4]	
I feel rested	[1]	[2]	[3]	[4]	
I am calm cool and collected	[1]	[2]	[3]	[4]	
I feel that difficulties are piling up so that I cannot overcome them	[1]	[2]	[3]	[4]	
I worry too much over something that really doesn't matter	[1]	[2]	[3]	[4]	
I am happy	[1]	[2]	[3]	[4]	
I have disturbing thoughts	[1]	[2]	[3]	[4]	
I lack self-confidence	[1]	[2]	[3]	[4]	
I feel secure	[1]	[2]	[3]	[4]	
I make decisions easily	[1]	[2]	[3]	[4]	
I feel inadequate	[1]	[2]	[3]	[4]	
I am content	[1]	[2]	[3]	[4]	
Some unimportant thought runs through my mind and bothers me	[1]	[2]	[3]	[4]	
I take disappointments so keenly that I cannot put them out of my mind	[1]	[2]	[3]	[4]	
I am a steady person	[1]	[2]	[3]	[4]	
I get in a state of tension or turmoil over my recent concerns and interests	[1]	[2]	[3]	[4]	

Figure 1 State-trait anxiety inventory.

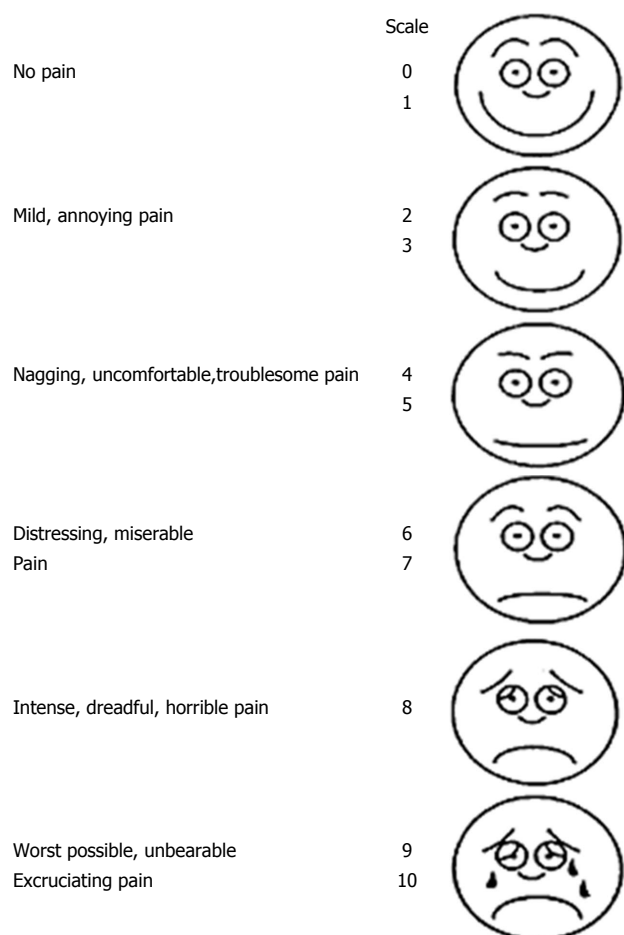


Figure 2 Visual analog pain scale.

Pre-operative and post-operative systolic and diastolic blood pressure, heart rate, and respiratory rate were measured for each patient. The pre-procedure vital signs, including blood pressure, heart rate,

and respiratory rate, were all obtained prior to the cystoscopy and prior to playing music. Post-procedure vital signs were obtained after the cystoscopy was completed. During the 10-min dwell time of the 2% intraurethral lidocaine jelly, the patient either listened to classical music for 10 min or did not hear any music at all. A 15 French Olympus® digital flexible cystoscope was connected to a digital video monitor that was used for all procedures. The music was played prior to the cystoscopy.

To measure anxiety level and pain level, we used the State-Trait Anxiety Inventory (STAI) and the Visual Analog Pain Scale, respectively (Figures 1 and 2)^[24,25]. Both are validated surveys. The State-Trait Anxiety Inventory (range 20-80) measures the transitional emotional status evoked by a stressful situation, such as a medical procedure or surgery. The Visual Analog Pain Scale (range 0-10) quantifies pain level using verbal and visual descriptors. Higher scores indicate higher anxiety and pain for both tests. The patients were asked to complete two anxiety surveys, one pre-procedure and one post-procedure. Patients were also asked to complete a visual analog pain scale survey post-procedure.

Statistics were generated and compared using an independent *t*-test and chi-squared tests. *P* values < 0.05 were considered statistically significant. Delta anxiety score is defined as pre-cystoscopy anxiety score - post-cystoscopy anxiety score.

RESULTS

Thirty-eight patients were randomized into either the No Music group (*n* = 24) or the Music group (*n* = 14). The subjects were all male as noted above. Table 1 outlines the demographics and clinical characteristics of these subjects. The mean age was 65.3 and 67.1

Table 1 Summary of patients' demographics and clinical characteristics of the music and number of music groups *n* (%)

Variables	Groups		<i>P</i> value
	Music group (<i>n</i> = 14)	No music group (<i>n</i> = 24)	
Age, mean + SD	67.1 + 9.9	65.3 + 10.4	0.9541
Race			0.698
Caucasian	10 (71)	15 (63)	
African American	1 (7)	4 (17)	
Hispanics	1 (7)	2 (8)	
Others	2 (15)	3 (12)	
History of bladder carcinoma	10 (71)	3 (1)	0.1488
History of prostate carcinoma	2 (14)	5 (21)	0.3039
History of hematuria	12 (87)	14 (58)	0.2049
Antidepressant or antianxiety medications	4 (29)	6 (25)	0.7092

Table 2 Summary of patients' peri-procedural vital signs between the music and number of music groups

Variables	Groups		<i>P</i> value
	Music group (<i>n</i> = 14)	No music group (<i>n</i> = 24)	
Pre Cystoscopy			
Systolic blood pressure (mmHg), mean + SD	135.5 + 17.7	135.5 + 17.9	0.9834
Diastolic blood pressure (mmHg), mean + SD	79.4 + 12.9	80.0 + 11.5	0.7908
Heart rate (beat/min), mean + SD	73.5 + 13.3	75.3 + 14.3	0.4405
Post Cystoscopy			
Systolic blood pressure (mmHg), mean + SD	139.1 + 22.8	137.2 + 17.3	0.5837
Diastolic blood pressure (mmHg), mean + SD	81.8 + 13.0	83.0 + 11.6	0.5717
Heart rate (beat/min), mean + SD	71.3 + 15.6	74.9 + 14.2	0.16

years for men in the No Music and Music groups, respectively. The majority of patients in each group self-identified as Caucasians. A majority of patients (68%) reported experiencing hematuria. Thirty-four percent had a history of bladder cancer, and 26% admitted to taking antidepressants. Physiologic parameters that correlated to pain and anxiety (systolic blood pressure, diastolic blood pressure, and heart rate) were statistically similar in both groups pre- and post-flexible cystoscopy. Patients' peri-procedural vital signs between the Music and No Music groups are summarized in Table 2. The median delta anxiety between the No Music and Music groups were not significantly different (0.78 vs -1.46), and the pain scores between the No Music and Music groups (1.5 vs 1.6) were not statistically different ($P = 0.28$ and $P = 0.92$, respectively).

DISCUSSION

Outpatient cystoscopy is a safe and useful procedure

employed frequently in Urology for diagnosis and evaluation of genitourinary pathologies. However, cystoscopy-related distress cannot be ignored^[1]. Three components of outpatient cystoscopy have been evaluated to improve the cystoscopic experience: local anesthetic control, cystoscopic equipment redesign and environmental modification. We reviewed the literature pertaining to these modifications. In addition, since flexible cystoscopy is a more tolerable procedure more commonly utilized in the United States, we performed this study to investigate if music reduces anxiety and pain in American veterans undergoing flexible cystoscopy.

A recent study suggests that listening to classical music during rigid cystoscopy enhances patient comfort and decreases post-procedure pain and anxiety^[23]. In addition to this report, prior studies have demonstrated music's ameliorating effect on pain and anxiety in patients undergoing a variety of procedures, including rigid cystoscopy^[16-18,20].

Contrary to these studies, our study did not show reduction of cystoscopy-related anxiety by listening to classical music during flexible cystoscopy, regardless of its indication. The lack of the effect of music in our group may be due to the small number of subjects. In addition, due to the patient demographics of the Veterans Affairs hospitals, the majority of our patients have been Caucasian males. Subsets of patients treated at the Veterans Affairs Hospitals respond to painful stimuli differently than their civilian counterparts, possibly due to the increased prevalence of PTSD and generalized anxiety disorder^[24,25]. A subset of patients with history of bladder carcinoma that required repeated surveillance using office-based flexible cystoscopies were also included in this cohort. We observed that listening to music at time of respective cystoscopy did not influence their peri-procedural STAI anxiety scores when adjusting for other variables. We included blood pressure, heart rate, and respiratory rate as objective representations of the emotional state of the patient in addition to validated questionnaires. These parameters are directly influenced by sympathetic nervous system activation due to emotional distress or anxiety through the release of catecholamines, specifically norepinephrine and epinephrine^[26,27]. Higher catecholamine levels from anxiety and fear result in elevated blood pressure, heart rate, and respiratory rate. Thus, the combination of these objective physiologic indicators with the subjective patient-provided information on anxiety and fear provided a more holistic assessment of the impact of music on how a patient experiences flexible cystoscopy.

Several randomized studies have shown the benefit of intraurethral lidocaine gel in reducing the pain associated with flexible cystoscopy and others have shown no improvement^[6,8-13]. However, a recent meta-analysis^[28] evaluating the effect of lidocaine gel on pain during flexible cystoscopy concluded that intraurethral instillation of lidocaine gel vs plain lubricating gel

reduces the likelihood of moderate to severe pain during flexible cystoscopy. Although fiber optic technology is still utilized for many of the flexible cystoscopes in the country, digital technologies are available. In other endoscopic devices, visualization using digital technology has higher resolution, decreased distortion, improved color representation, and larger image size compared with the standard fiber optic visualization with a narrower field of view^[29]. More recently, digital technology with distal sensor high definition images has been compared to standard digital visualization^[30]. These reductions will likely result in improved patient comfort during outpatient flexible cystoscopy.

Modern advances in flexible cystoscopic instrumentation and peri-procedural instillation of intraurethral lidocaine have both decreased pain associated with flexible cystoscopy. Listening to music during rigid cystoscopy has been shown to reduce pain and discomfort. Our prospective, randomized study explored the effect of classical music on pain and anxiety associated with flexible cystoscopy, and preliminarily demonstrates that music does not reduce anxiety or pain associated with flexible cystoscopy in the Veterans Affairs population.

COMMENTS

Background

Flexible cystoscopy is a very common in-office procedure performed in Urology. Several technological advances have been made in flexible cystoscopy instrumentation, however, there have also been advances in reducing patient pain and anxiety associated with this procedure, such as viscous lidocaine jelly and music. The authors reviewed the literature on effects of modifiable factors on patient pain and anxiety associated with flexible cystoscopy, and also include preliminary data on a Veterans Affairs randomized prospective trial evaluating the effect of classical music on pain and anxiety associated with flexible cystoscopy. This study explored the effect of classical music on pain and anxiety associated with flexible cystoscopy, and preliminarily demonstrates that music does not reduce anxiety or pain associated with flexible cystoscopy in the Veterans Affairs population.

Research frontiers

Flexible cystoscopy is a very common in-office procedure performed in Urology. Several technological advances have been made in flexible cystoscopy instrumentation, however, there have also been advances in reducing patient pain and anxiety associated with this procedure, such as the use of viscous lidocaine jelly and music.

Innovations and breakthroughs

In this systematic review, the authors attempted to evaluate whether listening to music at time of office-based flexible cystoscopy can alleviate pain and anxiety associated with this procedure. Previous studies have highlighted that listening to music during rigid cystoscopy reduces pain and discomfort. In contrast, they study did not show reduction of cystoscopy-related anxiety by listening to classical music during flexible cystoscopy, regardless of its indication. The lack of effect of music in their group may be due to the small number of subjects recruited in this study. Additionally, owing to the patient demographics of Veterans Affairs hospitals, the majority of our patients have been Caucasian males. Subsets of patients treated at Veterans Affairs hospitals respond to painful stimuli differently than their civilian counterparts, possibly due to the increased prevalence of PTSD and generalized anxiety disorder.

Applications

Although listening to music at time of office-based flexible cystoscopy may play a limited role in reducing pain and anxiety in the Veteran population, these findings cannot be generalized to the general patient population, particularly civilian, as responses to pain and/or anxiety associated with flexible cystoscopy

can manifest differently among heterogeneous patient populations.

Terminology

Well described medical and technological terminology commonly known to the general audience and the wider medical community was used. State-trait anxiety inventory; visual analog pain scale.

Peer-review

This study investigated cystoscopy-related distress, reviewing benefits of intraurethral lidocaine use as well as of digital cystoscopes for reducing procedural anxiety and pain. Indeed, the authors performed a study to investigate if listening to classical music reduces anxiety and pain in patients undergoing flexible cystoscopy who were treated at a Veterans Affairs hospital in the United States.

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