



ADVANCES IN BIOTECHNOLOGY



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Published in: June 2018

Online Edition available at: <http://openaccessebooks.com/>

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Advances in Biotechnology

Chapter 1

Adverse Effects of Amphetamines on Cardiovascular System: Review and Analyses of Trends

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Abstract

Background

Amphetamine and amphetamine-type stimulants are powerful physical and psychostimulants; they are Phenethylamine derivatives. The use of amphetamines can be either medicinal or illicit. Several amphetamines have been redesigned into illegal drugs of potent properties. Hence, they are named as designer drugs or novel (new) psychoactive substances.

Materials and Methods

This manuscript is a hybrid study of; data crunching and retrospective analysis of a trends database (1), and a systematic review of literature in relation to the amphetamines-induced adverse effects on the cardiovascular system (2). Google Trends database has been analysed in retrospect (2012-2017) to evaluate the attentiveness of surface web users towards amphetamine and a potent renowned amphetamine derivative known as captagon (fenethylamine).

Results

Amphetamines appear to be highly popular worldwide, particularly in the developed world including North America and European countries, and to a less extent in the developing countries and the Middle East. However, the trends are oscillating with time with significant year-to-year fluctuation. However, there was a steadiness in the temporal patterns (trends), for example in 2013-2014 (p-value=0.258). Variations in the trends were found to be correlated with global events including international terrorism. The adverse effects of amphetamines were found to be highly re-

lated to the cardiovascular system with a high incidence of intoxications and deaths among substance (ab) users.

Conclusion

Several amphetamines are potent and used illicitly beyond their therapeutic potential, as in the case of captagon, culminating in momentous public and economic threats. Legalising bodies should exercise tremendous and systematic efforts to counteract the threats. Database analysis can provide an accurate insight into this phenomenon that has been growing exponentially in the past decade.

Keywords: Phenethylamine drugs; Amphetamine; Amphetamine-type stimulants; Psychostimulant; Sympathomimetics; Novel Psychoactive Substances; Captagon; Fenethylamine; Cardiovascular Diseases; Google Trends; Drug-Related Side Effects; Adverse Reactions.

1. Introduction

Novel or new psychoactive substances (NPS), also known as designer drugs or research chemicals or legal highs, potentially posing health threats similar to the classic (archetypal) illicit substances; these substances (NPS) are not yet fully controlled by the United Nations drug conventions, although they do pose a serious threat to communities of nations around the world [1,2]. The rapid spread of the NPS, represents a major obstacle for the economy, policy makers, legislators, medical and paramedical professionals, and information-communication technology (ICT) personnel.

The United Nations Office on Drugs and Crime (UNODC) has identified six main groups of NPS: synthetic cannabinoids, synthetic cathinones, phenethylamines, ketamine, piperazines and plant-based substances. A seventh miscellaneous group of substances was also added later [3]. Despite the policies and current guidelines against the commerce and the electronic commerce of NPS, they continue to be highly popular and growing at an exponential rate paralleled only by the logarithmic growth in the ICT. In 2014, the UNODC via its World Drug Report indicated that the number of NPS substances have doubled over the period 2009-2013 [2]. Further, in 2013, It is estimated that almost a quarter of a billion people of age between 15-64 years used an illicit drug, which corresponds to an estimated global prevalence of 5.2% [4]. Similarly, the number of NPS of use reported in the European Union is increasing each year exponentially, for the period 2009 to 2014 [5]. In March 2015, the EMCDDA published an update on the NPS in Europe. The report divided the NPS market into several categories: legal highs, research chemicals, food supplements, designer drugs, and medicines. All produced in clandestine laboratories [4]. A tiny amount of data is known about the diffusion of NPS in the developing world, including the Middle East and the Arab world; as the current civil war and terrorism in Syria continue, the demand for illicit drugs, including the renowned substance known as captagon (fenethylamine). Captagon, a psychostimulant and an amphetamine-type substance, is also a diffused substance in; Iraq, Turkey, Iran, Jordan, Kuwait, Oman, UAE and

Qatar [6].

The marketing strategies of illicit drugs have significantly changed over the years. The Internet has become increasingly important as a communication and a distribution modality; this method is also known as the electronic commerce (e-commerce) or electronic trade (e-trade). The e-commerce does also take place on the anonymous deep web and the darknet marketplace. Additionally, potent substances can be easily purchased online, and in uncertain doses, they entail a high risk of serious poisonings, morbidity, and even sudden deaths [7]. In the European Union (EU), 41 novel psychoactive substances were identified for the first time in 2010, 49 in 2011, 73 in 2012, 81 in 2013, and 37 by April 2014 via the European Early Warning System [7,8].

2. Materials and methods

This study is made of two integral components, a review of the literature and an inferential retrospective analysis of Google Trends database. A systematic review of the literature was carried out via medical and paramedical databases including; PubMed/Medline, the Cochrane Library, Embase, Scopus, CINAHL, OpenGrey, and Google Scholar. Other databases were also systematically explored including; Oxford Scholarship Online, the University of Hertfordshire Online Library, Semantic Scholar, and Sci-Hub. Accordingly, the literature review methodology covered a broad range of scholarly written articles exclusively found on the surface web, published and unpublished resources, including the Grey Literature. A list of pre-specified keywords was implemented for the purpose of finding the most appropriate articles that are pertinent to the topic of amphetamine and amphetamine-type stimulants. Furthermore, boolean operators (“AND”, “OR”, “NOT”) were utilised in order to increase the specificity of the search strategy, to either narrow down or expand the number of hits retrieved from each database [9,10].

The literature review aimed at these topics; amphetamines and captagon, the collateral use of the deep web and the darknet, the exploitation of use of Google Trends database for the purpose of epidemiological analyses and geographic mapping, and the adverse reactions of amphetamines in relation to the cardiovascular system, including incidents of intoxications and fatalities. Priorities were given to articles; written in the past five years, systematic reviews and meta-analyses, randomised controlled trials (RCTs) and pragmatic RCTs, rigorous longitudinal analyses, and studies with inferential statistical analyses. The purpose of this filtering of articles is to retrieve studies of the highest attainable level-of-evidence [11,12].

The second component of this study relies on the data derived from Google Trends database, the analyses to be applied are longitudinal and retrospective in nature, and in relation to amphetamines and a particular amphetamine-derived substance known commercially as captagon. The analyses will be based on data extrapolated from millions of users of the surface

web, which is followed by the application of inferential statistical analyses of data science, particularly parametric tests, including; the Analysis of Variance and Covariance (ANOVA), Student's t-test, and z-test. The level of significance of the results was set at an alpha value of 0.05 and 95% confidence interval (95% CI). Additionally, further data were retrieved in relation to the geographic mapping of these substances. To summarise, this chapter is a hybrid analytic study made of a targeted review of literature and extrapolations based on retrospective data from Google Trends. Accordingly, the level-of-evidence is estimated to be of level-3b in accordance with the classification system imposed by the Oxford Center for Evidence-Based Medicine in 2009 [13].

3. Results

Amphetamines are phenethylamine derivatives; these are potent stimulants of the central nervous system (CNS) and psychostimulants [3,14,15]. Amphetamine and amphetamine-type stimulants (ATS) have been used widely for therapeutic purposes. For example, in treating depression, attention deficit disorder (ADD), attention deficit hyperactivity disorder (ADHD), and as a booster of physical performance in athletes [16-18]). The substance can be addictive, leading to dependence, tolerance, and withdrawal syndrome [19,20]. Recently, an NPS substance known as captagon (fenethylamine), became widely popular for its addictive properties and its use as a powerful physiological and psychostimulant effects. Captagon can promote high physical performance and endurance, cognitive enhancement, and reduction of sleep and food requirement [6,21,22]. Therefore, it has been well-known to be used by soldiers, and even terrorist. Recently, it became well known that it was used by terrorist organisations including the Islamic State of Iraq and the Levant (ISIL), captagon was incriminated in Paris terror attacks in 2015 [6,23,24].

3.1 Amphetamines and Cardiovascular Interactions

Stimulants have profound effects on the cerebrovascular systems and cardiovascular system, leading to congestive cardiomegaly, cardiac chambers and valvular fibrosis, and cerebral infarction and haemorrhage [25]. Furthermore, Hennissen and co-authors in their meta-analytic study found that there was statistically significant increments in diastolic blood pressure, systolic blood pressure, and heart rate reported in individuals using amphetamine and amphetamine-type stimulants [26].

Amphetamine and ATS potentiate the release of catecholamines, blocks their reuptake, and interacts directly with catecholamine receptors. Further, some amphetamine and ATS metabolites inhibit monoamine oxidase (MAO) enzyme leading to a secondary increment in the plasma concentration of norepinephrine. Other metabolic derivatives may increase serotonin release. Histologically, the effects of amphetamines are similar to the cocaine-induced changes; those include vascular hypertrophy, interstitial fibrosis, microvascular changes (hypertro-

phy) of the tunica media of the arteries. Accelerated atherosclerosis has also been observed in relation to amphetamines use and misuse. In addition, amphetamines induce calmodulin activation and increase the levels of thermic shock proteins [27]. Amphetamines stimulate the release of norepinephrine affecting both alpha (α) and beta (β) adrenergic receptor sites. Alpha-adrenergic stimulation causes vasoconstriction and an increase in total peripheral resistance, while β -Adrenergic receptor stimulation leads to an increase in heart rate, stroke volume, cardiac ejection fraction, and skeletal muscle blood flow. For instance, Adderall intoxication in humans can manifest with; hyperactivity, hyperthermia, tachycardia, tachypnea, mydriasis, tremors, abnormalities in peripheral white blood cells and thrombocytopenia, hypoglycemia, and seizures [28].

The heart is a target organ of injury for numerous chemicals including prescription and non-prescription chemical compounds. Pathologic mechanisms of chemical-induced cardiomyopathies include; direct toxic effects, neurohormonal activation, alteration of calcium homeostasis, oxidative stress, modulation of cardiac gene expression, and apoptosis [29]. Furthermore, amphetamine and ATS lead to indirect stimulation of the autonomic nervous system through the release of catecholamines, dopamine, and serotonin in nerve terminals of the central and peripheral nervous systems [30]. In addition, serotonergic 5-HT(2A) receptors agonists and to a fewer extent alpha-adrenoceptors agonists, including amphetamines, can cause vasoconstriction and tissue ischemia. Therefore, those drugs can induce fibrosis of the cardiac valves leading to heart failure [31]. Moreover, the pharmacological mechanism for the vasoconstriction and consequent fibrosis was recently found to be partially attributed to amphetamines interaction with a specific subset of receptors known as trace amine-associated receptors (TAARs) which are located in blood vessels. TAARs were found to play a substantial part in mediating the cardiac toxicity [32].

Amphetamines and cocaine are also considered to be risk factors for pulmonary arterial hypertension (PAH). Methamphetamine and amphetamines act more potently on norepinephrine and dopamine transporters and to a less extent on serotonin transporters. Those neurotransmitters have vasoconstrictive and growth modulating effects on smooth muscle cells leading to the development of PAH [33]. Fulceri and colleagues have also found that the combination of MDMA and loud noise, as in recreational mass events, can potentiate the effects of prolonged loud noise exposure which might explain the unexpected fatal events that happen in recreational situations [34].

In relation to captagon, the adverse reactions mimic those of amphetamine and ATS; these include; an increase in heart rate (tachycardia), body temperature, respiration and blood pressure as well as extreme depression, neurological excitation, lethargy, sleep deprivation, heart and blood vessel toxicity, and malnutrition in case of chronic substance misuse [35]. Hazardous side effects included: psychosis, visual distortions and hallucinations, acute heart

failure, acute myocardial infarction (AMI), and epileptic fits [36,37]. High-risk adverse effects that are also incompatible with war (combat) zones requirements include psychosis, visual distortions and hallucinations, acute heart failure, acute myocardial infarction (AMI), and epileptic fits [36]. Acute myocardial infarction has been increasingly reported since the beginning of the civil war in Syria (2011), the Middle East and in Turkey [38]. The first case of AMI in association with captagon was documented in a 21-year old man [37].

3.2 The Deep Web and the Darknet

The deep web, also known as the invisible web, seems to be the most important for the e-commerce activities of illicit substances including NPS. This is due to the anonymity granted in this division of the internet, and the use of anonymous payment system [39,40]. The deep web utilises technologies which provide anonymity for users. These technologies include: the use of specific browsers (Tor Browser, Grams search engine), login credentials specific to each e-market, secure routing protocols, virtual private networks (VPN), Internet Protocol Masking (IP masking), and Bitcoin payment system [41-43].

By the end of 2015, more than 700 NPS had been reported by a large number of countries in the world. Synthetic cathinones; synthetic cannabinoids; phenethylamines; and psychedelics account for the greater number of these substances. This thriving growth was facilitated and promoted by the online drug culture which finds its expression; in chat rooms, drug fora, blogs, and e-markets, on both the surface web and the deep web. The deep web, with high-level of anonymity, has progressively modified the NPS phenomenon into a virtual one. The rapidly evolving changes in the NPS online markets (e-markets) constitutes a major challenge to the provision of detailed knowledge on these substances [44-46].

The deep web represents the online content which is not indexed by the standard search engines (including Google, Yahoo, MSN, etc.). Novel psychoactive substances are promoted on the deep web in a plethora of e-markets. The e-markets of the deep web can also hold several illicit activities in relation to child pornography, human trafficking and slavery, unethical medical experimentations, human organs' trade activities, and other crimes including terrorism [47,48].

The darknet is a vital component of the deep web; its e-marketplace is a huge virtual place where several illicit activities exist including the NPS electronic trade (e-trade) [49-51]. Dozens of e-markets are active on the darknet e-marketplace, including; Hansa, Darknet Hero League, AlphaBay, Agora, Nucleus Market, Majestic Garden, Real Deal Market, Oasis, Abraxas, Outlaw Market, Middle Earth, Silkkitie, Oxygen, Tochka Market, and Arsenal [6,52,53]. Those e-markets can be systematically analysed and thoroughly mapped not only for NPS e-trade activities, but also from a social science perspective, the aim is to analyse and categorise the basis of power for; e-markets, e-vendors, and e-customers [54-56].

3.3 Google Trends Analyses: Amphetamine and Amphetamine-Type Stimulants

The popularity and the epidemiology of (ab)use of amphetamines and ATS can be inferred with high accuracy via the web, specifically the surface web by means of trends databases including *Google Trends* [57,58]. Google trends analyses can be either retrospective or cross-sectional. The analyses are highly accurate, as they are based on extrapolation of data from millions of users of the web (surface web). Furthermore, extrapolations can be geographically mapped (geo-mapped) for a particular region of the world. For instance, geo-mapping of surface web users' interest in relation to amphetamines and ATS [59-61]. The attentiveness of surface web users can be analysed, using Google Trends, in relation to phenethylamine, amphetamine, and ATS, and in retrospect to provide an accurate inference on the *electronic epidemiology* (e-epidemiology) on the Internet [59,61]. This can be inferred by using three specific keywords; *phenethylamine*, *amphetamine*, and *captagon* in the past five years (2012-2017) [62]. It seems that amphetamine was much more popular than both phenethylamine and captagon (Figure 1). The attentiveness was more oscillating with time in case of amphetamine and captagon, while interest in phenethylamine was steadier and almost represent a baseline superimposed on the x-coordinate (Figure 1). Interest in amphetamine reached a maximum in August 2014 and to a lesser extent (smaller peak) in November 2012. On the other hand, interest in captagon reached a maximum in November 2015, while a lower peak can be noticed in July 2016. These events have been correlated with terror attacks around the world, and the substance e-trade by extremist organisations including ISIL [6,22,63]. The relative interest of surface web users was as follows (mean +/- standard deviation); phenethylamine (0.07 +/- 0.25), amphetamine (49.73 +/- 5.25), and captagon (1.68 +/- 6.44). The trends of amphetamine were found to be the highest and the most oscillating (Figure 1). Accordingly, inferential statistics, using student's t-test (Figure 2), confirmed that the trends were significantly variable year-to-year with an exception for; 2012 versus 2015 (p -value=0.743); 2013 versus 2014 (p =0.258), and 2016 versus and 2017 (p =0.888). Further analysis, using regression models (Figure 2), revealed the presence of some degree of linear correlation (R^2 score=0.015) between the trends in 2013 and 2014, it means that the surface web users were attentive around the same time of the year (months) in 2013 when compared to 2014.

The top related queries by surface web users included; *phenethylamine drugs*, *phenethylamine*, *phenegran*, *amfetamina*, *moc energy amfetamin*, *moc energy amfetamina*, *captagon isis*, *isis*, *captagon daesh*, *captagon syria*, *captagon effet*, *captagon efectos*, and, *isis drug*. Geo-mapping (Figure 3). It seems that phenethylamine is geo-mapped primarily in the United States (US), while amphetamine was geo-mapped in 61 countries, including (highest to lowest frequency); Norway, Poland, Ukraine, Moldova, Sweden, Finland, Russia, Estonia, Bulgaria, and Belarus. On the other hand, captagon was geo-mapped (Figure 4) into seven countries only; France (35%), Turkey (16%), Germany (14%), Italy (10%), Spain (10%), Canada (10%),

and US (5%). France represented a statistical outlier possibly due to the correlation with international terrorism and terrorist attacks concentration in the country, while Turkey was the only country from the Middle East representing approximately 1/7th of the entire geo-map. A summative geo-mapping for all three substance categories (Figure 5) has shown that the top contributing countries are Norway (5%), Poland (5%), Ukraine, (5%), Moldova (5%), Finland (4%), Sweden (4%), Russia (4%), Estonia (4%), Bulgaria (3%), and Belarus (3%). Norway, Poland, Ukraine, and Moldova represented statistical outliers of the geo-map. The top countries contributed to 41.1% of the global geo-mapping. Other countries contributed to another 40% these included; Latvia, Australia, Kazakhstan, Denmark, Germany, Bosnia and Herzegovina, United States, Switzerland, Croatia, Serbia, Lithuania, Chile, Canada, United Kingdom, Austria, Netherlands, Ireland, Slovenia, Czechia, New Zealand, and Mexico. On the other hand, countries from the Middle East and the Arab world contributed to 2.3%. It included; Iran (0.8%), Israel (0.5%), Saudi Arabia (0.5%), and Turkey (0.4%). Furthermore, an additional regression analysis (Figure 6) showed the absence of a positive linear correlation in between the geo-mapping of amphetamine and captagon ($R^2=0.017$). To summarise, amphetamines appear to be important for surface web users from the developed world, including some states of the former USSR (Soviet Union), while the developing countries, including the Middle East and Arabic world, contributed minimally. Further analyses are required for the deep web, but these are beyond the scope of this chapter.

4. Conclusion

The growth of NPS industry, including the trade activities and its links to international terrorism, are reaching unprecedented levels primarily in the region of the Middle East. The e-commerce phenomenon of amphetamines seems to be highly prevalent in the western countries of the developed world, primarily; the US, UK, Italy, Canada, Scandinavia, and western Europe. On the other hand, the contribution of the Middle East and Arabic country to the e-trade phenomenon seems to be minimal; it can be described as infinitesimal especially when juxtaposed to those of the developed countries from the European Union and the United States.

The vast majority of incidents related to intoxications and deaths are also reported from the developed world. Furthermore, a considerable proportion of these events is related to pharmacological adverse effects in relation to the cardiovascular system. Unless some reasonable efforts and ingenious upgrades of the current research methodologies are achieved, the NPS trade and e-trade will ever continue to be on the rise, leading to more incidents of intoxications, morbidities, and mortalities, including cardiovascular-related one.

Research enhancements should aim at; increasing the quality and quantity of studies in the poorly-mapped developing countries including Middle East and Arabic countries, incor-

poration of efficient use of data science and advanced web analytics, compulsory training in relation to the disciplines of data science and basic neuroscience, validation and incorporation of data mining techniques and real-time analyses of databases, inclusion of the rarely-used experimental studies including; quasi-experiments, RCTs, pragmatic RCTs, and animal modeling, enhancement of the internet snapshot techniques, and full exploitation of trends databases of the surface web.

5. Figures

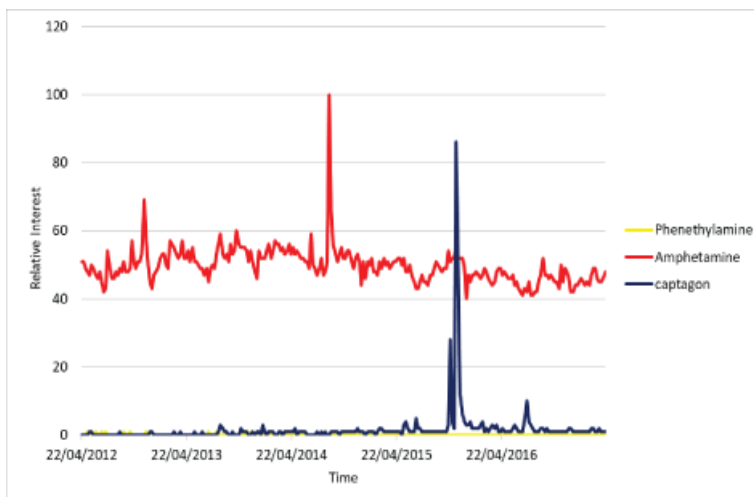


Figure 1. The attentiveness of surface web users in; Phenethylamine, Amphetamine, and Captagon.

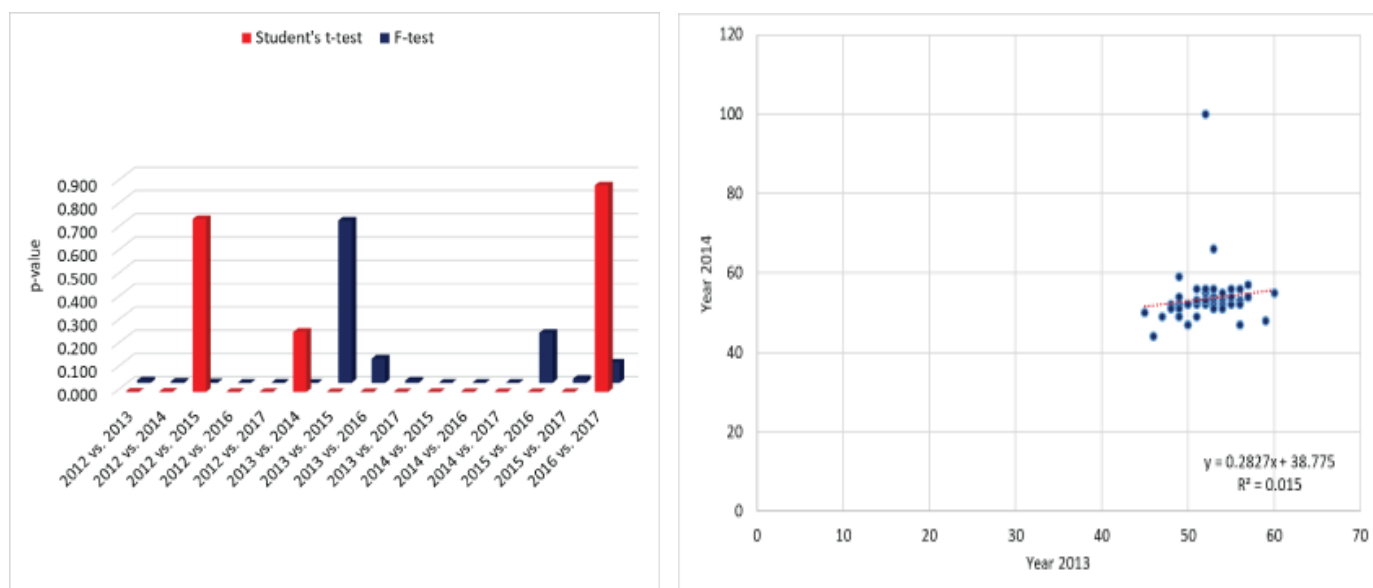


Figure 2. Inferential Statistics: Attentiveness of web users to Amphetamine in 2012-2017 (above), and Linear Regression for 2013 vs. 2014 (below).



Figure 3. Geo-mapping of interest in Phenethylamine (above, blue), Amphetamine (middle, red), and Captagon (below, yellow-brown).

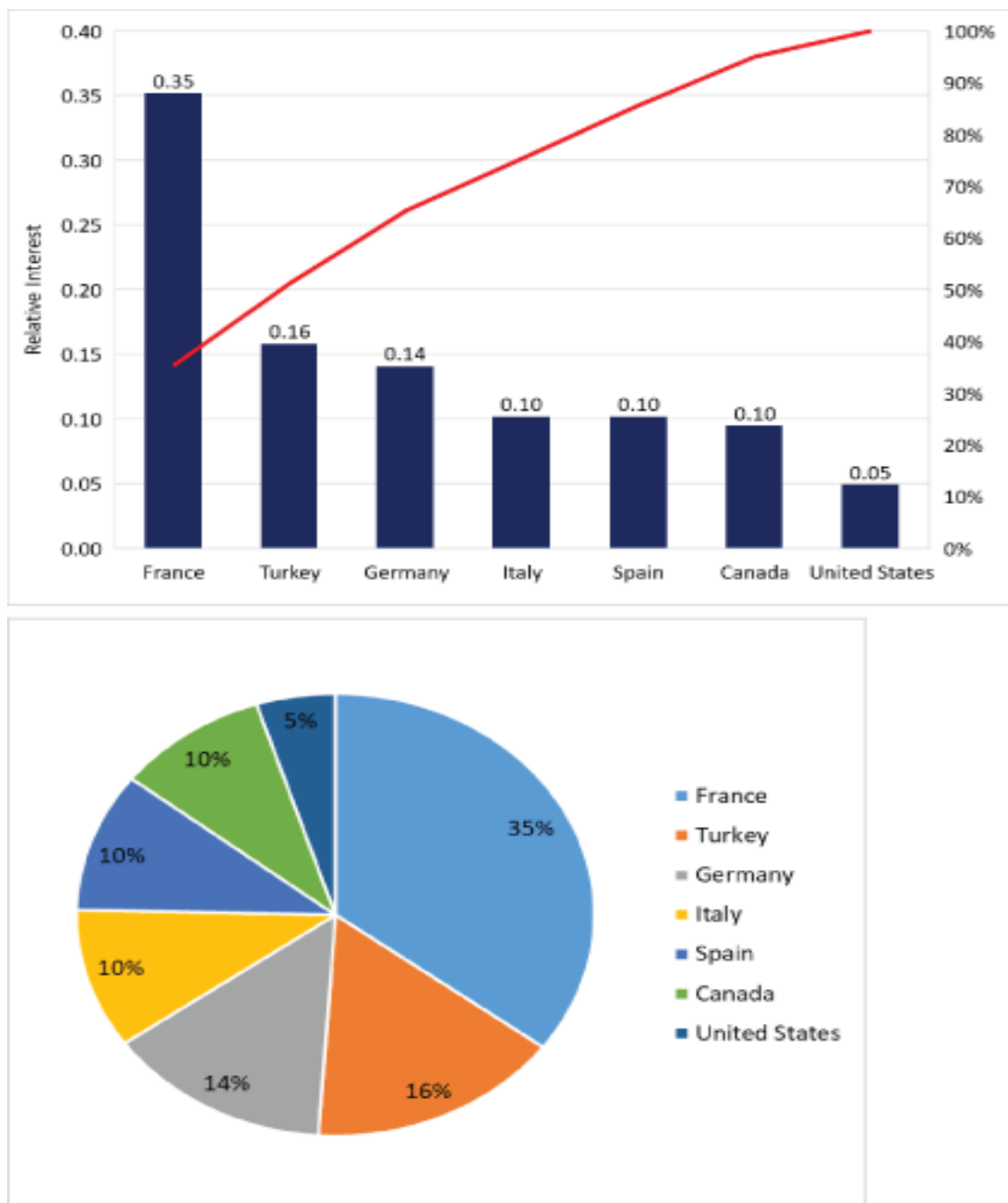


Figure 4. Geo-mapping of Captagon: Pareto Chart (above), and Pie Chart (below).

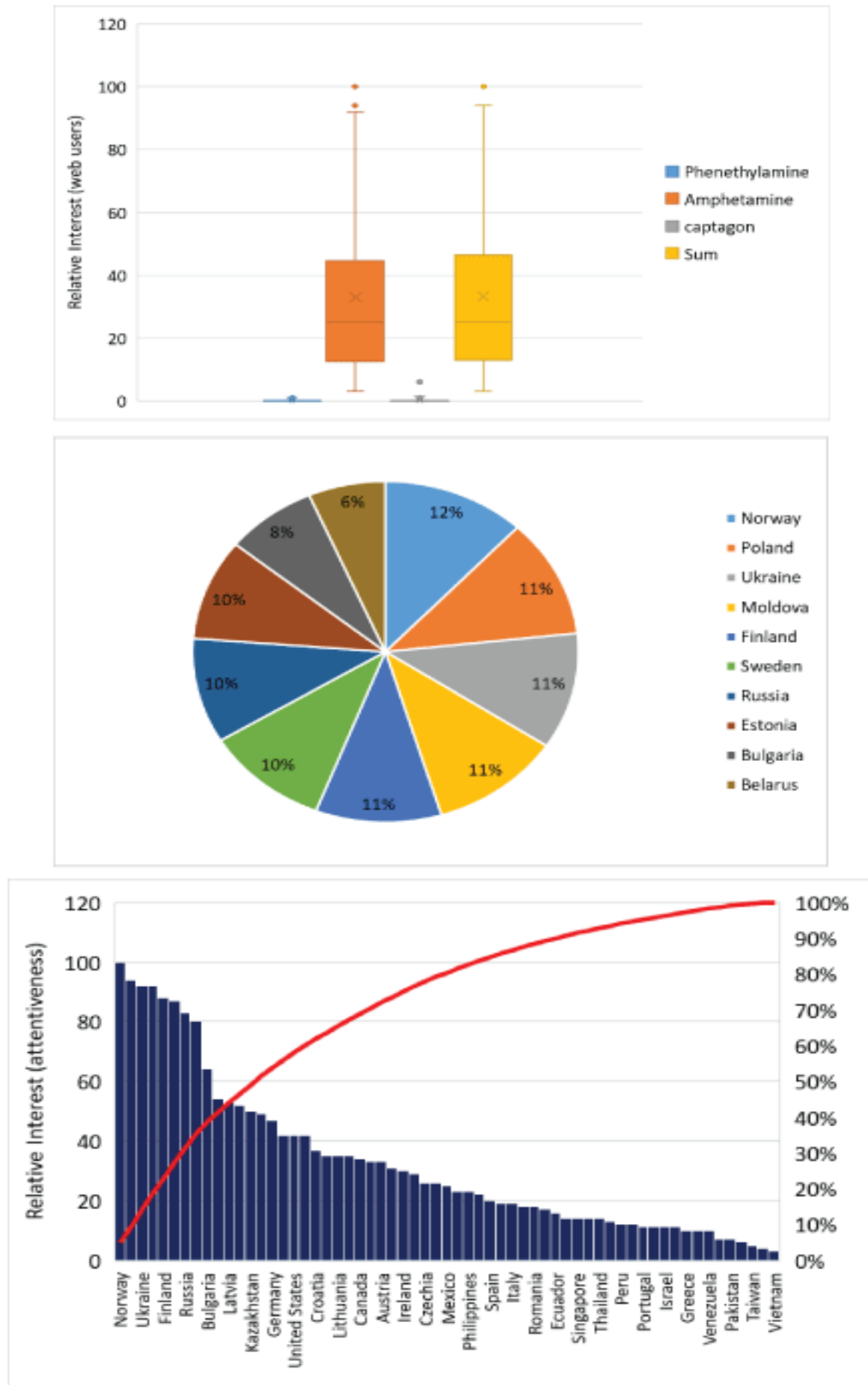


Figure 5. Summative Geo-mapping: Boxplot (above), Pie Chart (middle), and Pareto Chart (below).

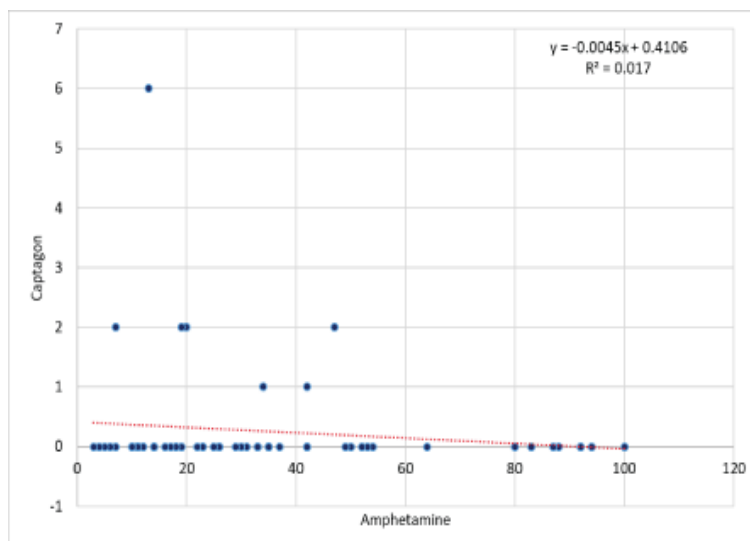


Figure 6. Inferential Statistic via Regression Analysis: Geo-mapping of Amphetamine vs. Captagon.

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Advances in Biotechnology

Chapter 2

Cancer Epigenetics: Role of Epigenetic Events in the Onset and Progression of Cancer

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Abstract

The word “Epigenetics” describes inheritable changes in gene expression that are independent of alterations in DNA sequences. Epigenetics is one of the most rapidly expanding fields in biology and over the past 16 years, the epigenetic regulation of DNA-based processes has been intensely studied. Epigenome is essential for the regulation and in unraveling the stages of normal and abnormal cellular development, including the phases of growth, differentiation, senescence, aging and immortalization during carcinogenesis. The recent characterization of DNA methylome at single nucleotide resolution has allowed the mapping of epigenetic machinery: DNA methylation, post-translational histone and other protein modifications, nucleosome positioning and noncoding RNAs (specifically microRNA [miR] expression) which act in concert to exert their cellular effects. Recent advancements in cancer epigenetics has highlighted the extensive reprogramming of every component of the epigenetic machinery in cancer. Disruption of the epigenome can contribute to cancer via altered gene function and malignant cellular transformation. The reversible nature of gene silencing by epigenetic modifications has facilitated the emergence of the promising field of epigenetic therapy. In contrast to conventional chemotherapy; several epigenetic drugs have been proven to prolong survival and to be less toxic. DNA methylation and histone modifications may serve as a potential targets for the development and implementation of new therapeutic approaches in the clinical settings. Many clinical trials are ongoing with novel classes of agents that target various components of the epigenetic machinery and have already made progress with the recent FDA approval of three epigenetic drugs for cancer treatment. In this book

chapter, we discuss the roles of epigenetic modifications in tumorigenesis; their clinical utility in cancer management as biomarker for detection, diagnosis and prognosis as well as highlight emerging epigenetic therapies being developed for cancer treatment.

Abbreviations

APC: Adenomatosis polyposis coli; CDH13: Cadherin 13; ER- α : Estrogen receptor- α ; MLH1: mutL homolog 1; VHL: von Hippel-Lindau tumor suppressor; RAR-b2: Retinoic acid receptor b2; GSTP1: Glutathione S-Transferase Pi 1; MBD: Methyl-binding domain; HDAC: Histone deacetylase; LOI: Loss of imprinting; CDH1: Cadherin-1; ES: Embryonic stem cells; MeCP: Methyl cytosine binding protein; MAGE: Melanoma-associated gene; DPP6: Dipeptidyl peptidase 6; VIM: Vimentin; HOXA2: Homeobox protein Hox-A2; IAP : Inhibitor of Apoptosis (IAP); DNMT: DNA methyltransferase; Rb: Retinoblastoma; HATs: Histone acetyltransferases; NID2: Nidogen 2; CRBP1: cellular retinol binding protein 1; TP73: Tumor Protein P73; RUNX3: Runt-related transcription factor 3; RAR: Retinoic acid receptor; THBS1: Thrombospondin 1; ER- β : Estrogen receptor- β ; HDACI: Histone deacetylase inhibitors; SirT1: Sirtuin (silent mating type information regulation 2 homolog) 1; BRCA1: Breast Cancer Type 1 Susceptibility Protein; CDKN2B: Cyclin-dependent kinase inhibitor 2B (p15); PRMT5: Protein Arginine Methyltransferase 5; SUV39H1: Suppressor Of Variagation 3-9 Homolog 1; RASSF1A: Ras association domain family 1 A; MGMT: O6-Methylguanine-DNA-Methyltransferase; ER β : Estrogen receptor beta; MECP2: Methyl-CpG-binding 2 protein; HP1 α : Heterochromatin protein1 α ; PRC2: Polycomb repressive complex 2; ZBTB 33: Zinc finger and BTB domain containing protein 33; MSP: Methylation specific PCR; SEPT9: Septin 9; DAPK1: Death-Associated Protein Kinase 1; IGF2: Insulin-like growth factor 2; S100P: S100 calcium binding protein P; GATA2: GATA-Binding Protein 2; CDKN1A: Cyclin Dependent Kinase Inhibitor 1A; G9a: Histone-lysine N-methyltransferase 2 (known asEHMT2); SFRP1: Secreted Frizzled Related Protein 1; TMS1: Target Of Methylation-Induced Silencing 1; MBD1: Methyl-CpG binding domain protein 1; PCDH10: Protocadherin 10; ER α : Estrogen receptor alpha; 5-FC: 5-fluoro-2-deoxycytidine; SHOX2: Short stature homeobox 2; TWIST1: Twist Family BHLH Transcription Factor 1; SAHA: Suberoylanilide hydroxamic acid; CDKN2A: Cyclin dependent kinase inhibitor 2A (p16); SAT2: Spermidine/spermine N1-acetyltransferase family member 2.

1. Introduction

Carcinogenesis is driven by the accumulation and interplay of genetic and epigenetic abnormalities that affect the structure and function of the genome [1-3] and result in dysregulated gene expression and function. The term “Epigenetics” coined by C.H.Waddington refers to the study of heritable changes that are independent of alterations in the primary DNA sequence. The epigenetic alternations implicated in the initiation and progression of cancer are DNA methylation, post-translational histone and other protein modifications, nucleosome positioning and noncoding RNAs (specifically microRNA [miR] expression) which act in concert to exert their cellular effects (Fig. 1). These modifications jointly constitute the “epigenome” to modulate the regulation of many cellular processes, including gene and microRNA expression, DNA-protein interactions, suppression of transposable element mobility, cellular differentiation, embryogenesis, X-chromosome inactivation and genomic imprinting [4]. Epigenome is essential for the regulation and in unraveling the stages of normal and abnormal cellular development, including the phases of growth, differentiation, senescence, aging and immortalization during carcinogenesis [5].

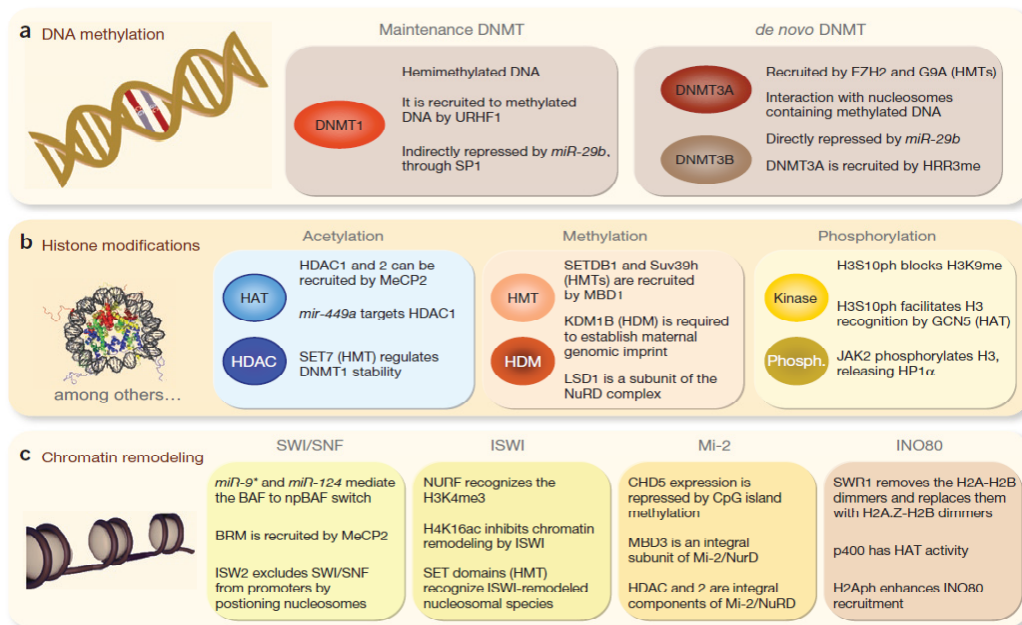


Figure 2 Epigenetic machinery and interplay among epigenetic factors. Epigenetic marks are catalyzed by different epigenetic complexes, whose principal families are illustrated here. (a-c) Epigenetic regulation depends on the interplay among the different players: DNA methylation (a), histone marks (b) and nucleosome positioning (c). The interaction among the different factors brings about the final outcome. This figure illustrates selected examples of the possible interrelations among the various epigenetic players.

Figure1: Epigenetic machinery and interplay among epigenetic factors (Adapted from A. Portela and M. Esteller, 2010 [97].)

The emergence of epigenetic machinery as key regulators of gene regulation and expression has provided significant insights into oncogenesis. Driven by aberrant DNA methylation and histone modifications, epigenetic aberrations are critically responsible for the disruption of cellular machinery and homeostasis. Failure of the proper maintenance of the epigenetic machinery results in altered gene function and malignant cellular transformation. Aberrant epigenetic modifications occur at an early stage of neoplastic development and serve as an essential player in cancer progression [6].

DNA methylation is characterized by the chemical modification of cytosine with the transfer of a methyl moiety at the 5- carbon of the cytosine base in CG dinucleotides by DNA methyltransferases (DNMTs). DNA methylation play vital role in the regulation of gene transcription and chromatin status. In contrast to normal cell, cancer cell show global hypermethylation mainly of repetitive elements and localized hypermethylation leading to silencing of genes (e.g., tumor suppressor) with associated loss of expression [7]. Nucleosomes the basic unit of chromatin, basically consist of 146bps of DNA wrapped around an octomer of Histone complex (two subunits each of H2A, H2B, H3 and H4 histones). The H1 linker histone binds to the outside of nucleosome and seals two turns of DNA. The less structured N-terminal domains of all core histones protrude from the core histone and are subjected to modifications [8-10]. The epigenetic cross-talk between histone modifications and DNA methylation influences chromatin condensation, stability and nuclear architecture, primarily regulating its accessibility and compactness.

The most common epigenetic modifications observed during malignancies are increased

methylation of CpG islands within gene promoter regions and deacetylation and or methylation of histone proteins which results in aberrant gene expression and altered epigenomic pattern [7,9]. In recent years, tremendous pace of research on epigenetics provides insights into the significant role altered epigenetic alterations plays in mediating tumor onset and progression, their utility as candidate targets being explored for risk assessment, early detection, prognosis, prediction of response to therapy and on the development of compounds that target enzymes which regulate the epigenome as anticancer agents, thereby outlining the great promise this field holds to advance our understanding of oncogenesis and help in the development of strategies for cancer management [11-14].

In the present book chapter, we discuss the current understanding of epigenetic modifications associated with tumorigenesis with focus on histone modification and DNA methylation and provide an overview of the potential utility of methylation markers for cancer detection, diagnosis and prognosis. We also highlight the prospect of epigenetic therapies in designing effective strategies for cancer treatment and prevention.

2. DNA methylation in gene regulation

One of the best characterized epigenetic modifications is DNA methylation which is involved in various biological processes such as the silencing of transposable elements, regulation of gene expression, genomic imprinting, and X-chromosome inactivation [15-17]. (Table 1) Various reports implicate the significant role of DNA methylation in carcinogenesis, right from the silencing of tumor suppressors to the activation of oncogenes and the promoting metastasis [18]. DNA methylation serves as a key element in tissue differentiation during early embryonic development.

Aberrant DNA methylation being recognized as the most common molecular abnormalities during tumorigenesis, are frequently associated with drug resistance [19]. Most CpG sites which are outside the CpG islands are methylated, thereby suggesting its role in the global maintenance of the genome. However, most CpG islands in gene promoters are generally unmethylated, allowing active gene transcription. When a given stretch of cytosine of CG dinucleotide in the CpG island located in the promoter of a given gene is not methylated, the gene is not silenced through methylation. Such CpG island is termed as “hypomethylated”. Contrary, methylation of cytosine of CG dinucleotide in the CpG island located in the promoter of a given gene results in methylation induced gene silencing and such CpG island is termed as “hypermethylated” [20]. Furthermore, methylated cytosines preferentially bind to a protein known as methyl cytosine binding protein, or MeCP, which inhibits the recognition of methylated promoter by transcription factors and RNA polymerase [21].

In normal cells, CpG islands in active promoters are not methylated in order to maintain euchromatin structure, thus allowing active gene expression. However, the CpG islands within

coding regions are often methylated. Reverse patterns are observed in cancer cells, where hypermethylation at CpG island containing gene promoter results in their transcriptional inactivation by changing the open euchromatin structure to compact heterochromatic structure [22].

3. Interrelation between DNA methylation and histone modifications

As mentioned before, all the epigenetic players act in concert to exert their cellular effect. Apart from performing their individual roles, histone modification and DNA methylation machinery interact with each other to determine gene transcription status, chromatin organization and cellular identity. The relationship between DNMT3L and H3K4 is a striking example which reflects the interplay between histone modifications and DNA methylation. The specific interaction of DNMT3L with histone H3 tails induces *de novo* DNA methylation by recruiting DNMT3A. Conversely, this interaction is strongly inhibited by H3K4me [23].

Several histone methyltransferases including G9a, SUV39H1 and PRMT5 have been reported to direct DNA methylation to specific genomic targets by directly recruiting DNA methyltransferases (DNMTs) which in conjugation with repressive histone marks further enhances the suppression of gene expression [24,25]. In addition to direct recruitment of DNMTs, histone methyltransferases and demethylases influence DNA methylation level by modulating the stability of DNMT proteins [26,27]. Early studies have shown that histone H3K9 methyltransferase controls DNA methylation in fungi (*Neurospora crassa*). Mutation of histone H3K9 methyltransferase resulted in reduced methylation thereby signifying H3K9 methylation acts as an upstream epigenetic mark which controls DNA methylation [28].

For the repression of gene expression and chromatin condensation, DNMTs can recruit HDACs and methyl binding protein. DNA methylation can also direct histone modifications. The strongest link between DNA methylation and histone modification is served by Methyl binding proteins which includes methyl CpG binding protein 2 (MeCP2), Methyl-CpG binding domain protein 1 (MBD1), and Kaiso [also known as ZBTB 33 (Zinc finger and BTB domain containing protein 33)]. However, their confinement to methylated promoter mediates the recruitment of histone deacetylases (HDACs) and histone methyltransferases, which suggests that DNA methylation, induces chromatin structural changes via alternation of histone modification. For instance, methylated DNA mediates H3K9methylation through recruitment of effector protein MeCP2, thereby maintaining a repressive chromatin state [29]. (Fig. 2)

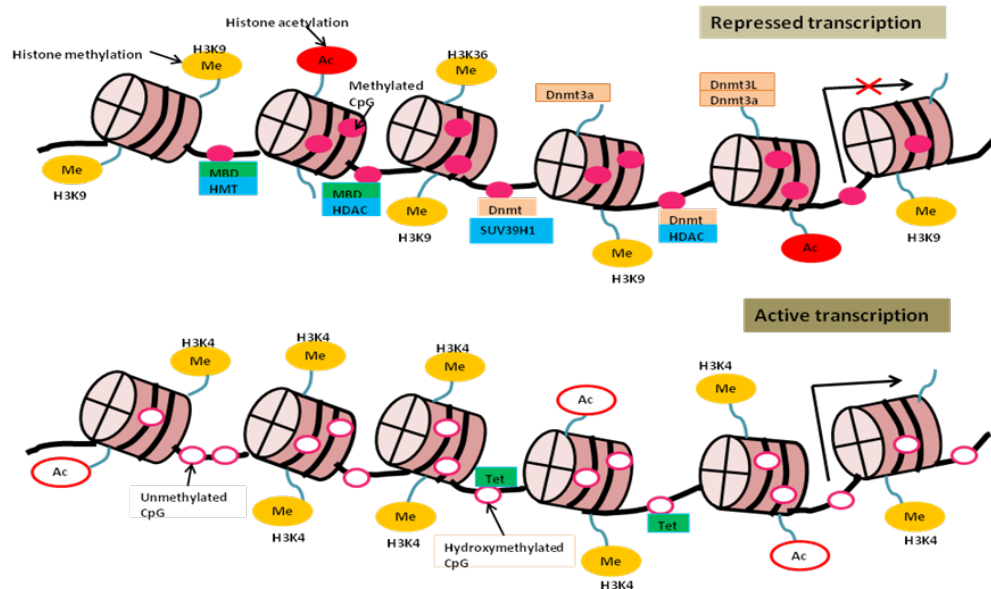


Figure 2: Epigenetic cross talk between DNA methylation and histone modification (Adapted from:K. Grønbaek et.al. 2007 [12].)

During development, both DNA methylation and histone modification are involved to establish patterns of gene repression. Certain forms of histone methylation results in generation of local heterochromatin, that is readily reversible. In contrast, a highly stable long term repression is maintained by DNA methylation. Recently several studies provide insight that DNA methylation and histone modification pathways can be dependent on each other and this cross talk can be achieved through biochemical interactions between SET domain histone methyltransferases and DNA methyltransferases [30].

For instance, in embryonic stem cells (ES), the pluripotency genes such as Oct3/4 and Nanog are inactivated after lineage commitment. This silencing process involves the recruitment of repressor complex: the SET domain containing histone methyltransferases G9a together with histone deacetylase. Subsequently methyltransferases DNMT3A and DNMT3B, which mediate *de novo* methylation, are recruited by G9a through its ankyrin (ANK) domain, at the promoter [31,32]. In context to G9a, it seems that the different protein domains are responsible to carry out the histone methyltransferases activity and the link with DNA methyltransferases activity. Therefore, mutation of the SET domain disrupts H3K9 methylation without affecting DNA methylation thereby suggesting that DNA methylation is not dependent on histone modification; instead on the recruitment of G9a (in particular, ankyrin motif) and the interrelation between histone modification and DNA methylation is generated through enzyme interactions [24,33].

Cooperation between histone modifications and DNA methylation in order to achieve silencing is reflected by the Polycomb targeted genes. (Fig. 3) In normal cells, repression involves formation of local heterochromatin – the SET domain histone methyltransferase (EZH2), as a part of Polycomb repressive complex 2 (PRC2) mediates the histone H3 lysine 27 trimethylation leading to heterochromatinization through the PRC1 complex, that consist

of chromodomain protein PC, thereby blocking the recruitment of transcriptional

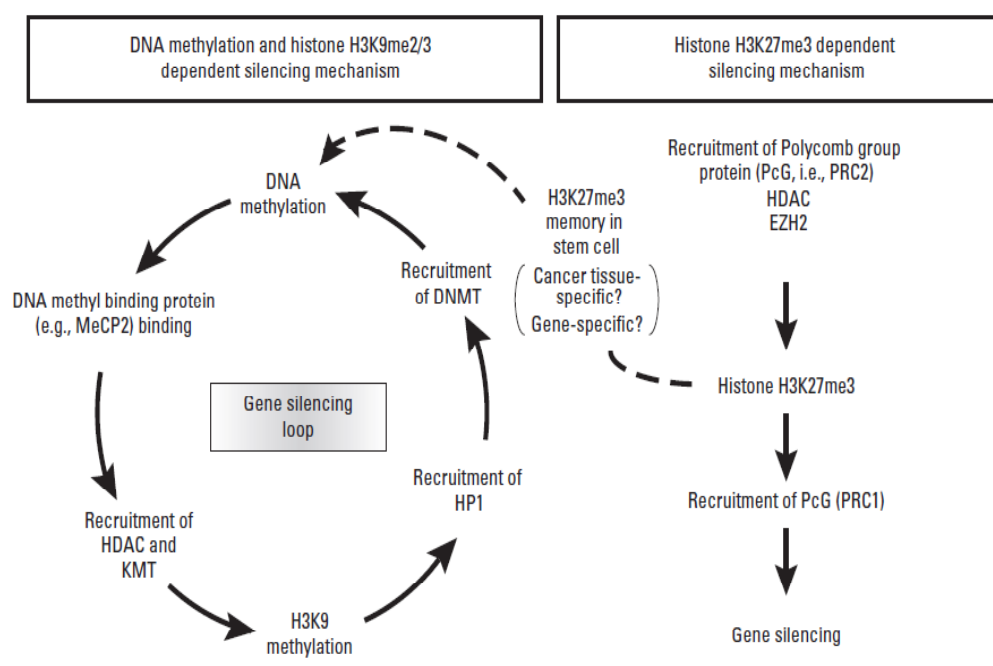


Figure 3: Two distinct histone modifications for gene silencing in human cancers (Adapted from Y. Kondo, 2009 [40].)

activation factors [34,35]. Interestingly, polycomb induced repression are easily reversible and in ES cells, almost all polycomb targeted genes are marked by both the repressive H3K27me3 modification as well as activating modification H3K4me3. This bivalent modification pattern confers the potential of a gene to be driven either to its active or inactive state. Those genes which were silenced by this mechanism might readily get activated during differentiation. On contrary, genes in their active conformation might revert to the repressed state [36,37].

Most of the genes repressed by polycomb complexes are generally associated with unmethylated CpG islands. However under certain circumstances (such as cancer), a number of these genes might become targets of *de novo* methylation, possible through the interaction between EZH2 and the methyltransferases DNMT3A and DNMT3B [38, 39]. Upon methylation, some of these genes lose their polycomb repressive proteins, but still remain inactive due to the DNA methylation, as an alternate silencing mechanism. This epigenetic switch reduces epigenetic plasticity, locking the silencing of key regulators and contributing to carcinogenesis. However, in some genes H3K27me3 and DNA methylation co-exist on the same promoter, in such cases PcG-mediated H3K27me3 is the dominant silencing machinery [40]. (Fig. 4)

4. Epigenetic modifications in cancer

Recent studies indicate that tumorigenesis cannot be accounted by genetic alternations alone but also involve epigenetic modifications. Thus, tumour cells are activated by both genetic and epigenetic alterations. The interplay among the different players such as DNA methylation, histone modifications and nucleosome positioning is critical for the regulation of gene and noncoding RNA expression. During carcinogenesis, these epigenetic marks play an

important role in tumor development and progression by modulating the chromatin structure, gene and miRNA expression. (Fig. 5) Additionally, tumor cells reflect a profoundly distorted epigenetic landscape. The epigenetic alternations, their possible mechanisms and associated biological consequences by which they promote tumorigenesis have been discussed in Table 1.

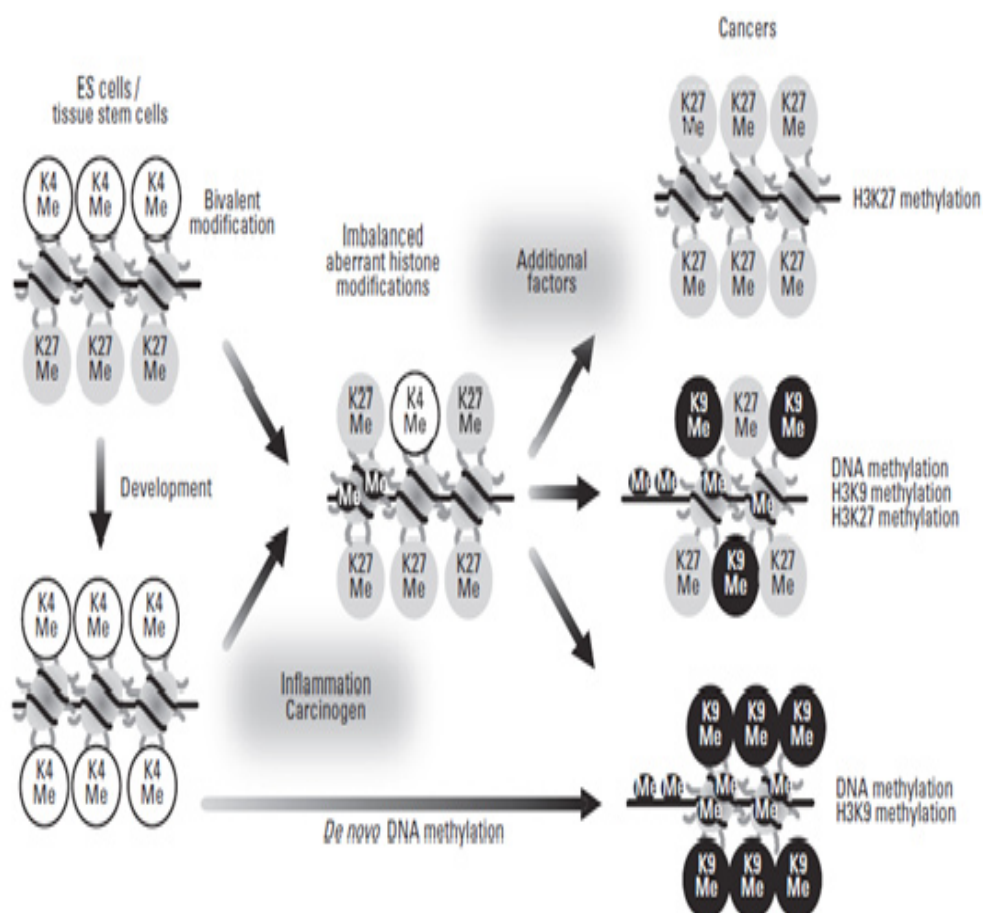


Figure 4: A model representing *de novo* methylation and *de novo* histone modifications in human cancer (Adapted from Y. Kondo, 2009 [40].)

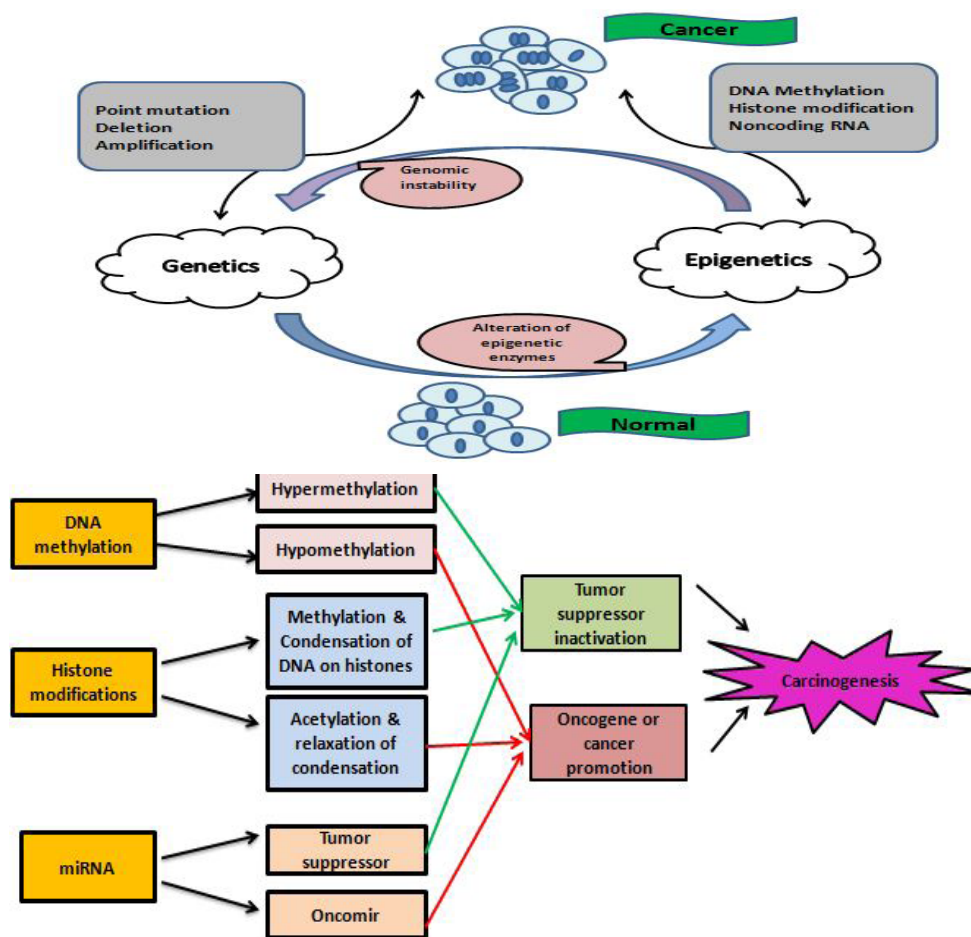


Figure 5: Epigenetic alterations that contribute to carcinogenesis

Table 1: Summarized outline of the epigenetic changes and possible mechanisms by which they promote tumorigenesis

Epigenetic Alterations	Mechanism	Consequences
DNA hypermethylation	<i>De novo</i> hypermethylation at promoter CpG islands leads to silencing of tumor suppressor genes and cancer-associated genes	Genomic and chromosomal instability, growth advantage, increased proliferation
DNA hypomethylation	Activation of cellular oncogenes Activation of transposable element	Increased proliferation, growth advantage, Genomic instability, transcriptional noise
Loss of imprinting (LOI)	Reactivation of silent alleles, biallelic expression of imprinted genes	Expansion of precursor cell population
Relaxation of X-chromosome inactivation	Mechanism is still unknown, but appears to be age related	Altered gene dosage, growth advantage
Histone acetylation	Gain-of-function Loss-of-function	Activation of tumor promoting genes Defects in DNA repair and checkpoints
Histone deacetylation	Silencing of tumor suppressor genes	Genomic instability, increased proliferation
Histone methylation	Loss of heritable patterns of gene expression (cellular memory)	Genomic instability, growth advantage

4.1 DNA methylation in cancer

Cytosine methylation is the most extensively studied epigenetic modification in humans, which primarily occurs by the covalent modification of cytosine bases in the CpG dinucleotide. These CpG dinucleotides are not evenly distributed across the human genome, but tend to cluster in short stretches called “CpG islands” [7] which is defined as regions of more than 200 bases with a G+ C content of at least 50% and a ratio of observed to statistically expected CpG frequencies of at least 0.6 as well as regions of large repetitive sequences (e.g. centromeric repeats, retrotransposon elements, rDNA etc.) [41,42]. In mammalian genomes, CpG dinucleotides are usually quite rare (~1%). CpG islands occupy about (~60%) at the promoter of human genes, which are normally unmethylated, thereby allowing transcription. However, during early development or in differentiated tissues some of them (~6%) become methylated in a tissue-specific manner [43].

CpG-island methylation is associated with gene silencing and transcription regulation. Aberrant hypermethylation leads to transcriptional inactivation [44]. DNA methylation plays a key role in X chromosome inactivation, imprinting, embryonic development, silencing of repetitive elements and germ cell-specific genes, differentiation, and maintenance of pluripotency [45-47]. DNA methylation is vital for the regulation of non-CpG islands, CpG island promoters, and repetitive sequences to maintain genome stability [44,45]. Repetitive sequences appear to be hypermethylated which prevents chromosomal instability, translocations and gene disruption by the reactivation of endoparasitic sequences [48]. The DNA methylation at CpG island shores, which are located up to 2 kb upstream of the CpG island, is closely associated with transcriptional inactivation. Most of the tissue-specific DNA methylation seems to occur at CpG island shores and are conserved between human and mouse [49,50].

DNA methylation regulates gene silencing by different mechanisms. Methylated DNA can promote the recruitment of methyl-CpG-binding domain (MBD) proteins, such as MeCP2, MBD1, MBD2, and MBD4, which in turn recruit histone modifying and chromatin-remodeling complexes to the methylated sites, leading to transcriptional repression [48,51,52] or by precluding the recruitment of DNA binding proteins from their target site (e.g., c-myc and MLTF), which directly inhibits transcription [53]. Long-term repression of active genes through DNA methylation is performed by DNA methyltransferases (DNMTs). However an active gene with unmethylated CpG islands generates an open chromatin structure favorable for gene expression by the recruitment of Cfp1 and its association with histone methyltransferases Setd1, thereby creating domains enriched with histone marks such as acetylation and H3K4 trimethylation [54].

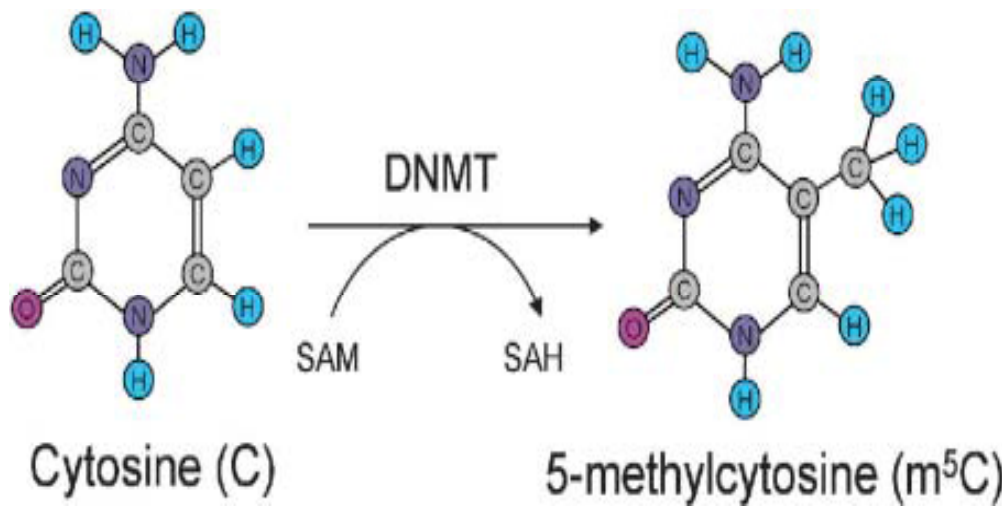


Figure 6: Methylation of cytosine

DNA methylation is mediated by enzymes DNA methyltransferases (DNMTs) that catalyze the transfer of a methyl group from *S*-adenosyl methionine to DNA. (**Fig. 6**) Though, five members of the DNMT family have been reported in mammals: DNMT1, DNMT2, DNMT3a, DNMT3b and DNMT3L, only DNMT1, DNMT3a and DNMT3b possess methyltransferase activity. The maintenance DNMT, DNMT1 has a 30- to 40-fold preference for hemimethylated DNA and is the most abundant DNMT in the cell, transcribed mostly during the S phase of the cell cycle. DNMT1 also has *de novo* DNMT activity and is responsible for post- replicative methylation i.e., to methylate hemimethylated sites generated during semi-conservative DNA replication. The *de novo* DNMTs (DNMT3A and DNMT3B), highly expressed in embryonic stem (ES) cells and downregulated in differentiated cells are responsible for establishing the pattern of methylation during embryonic development [55-57](Fig. 7)

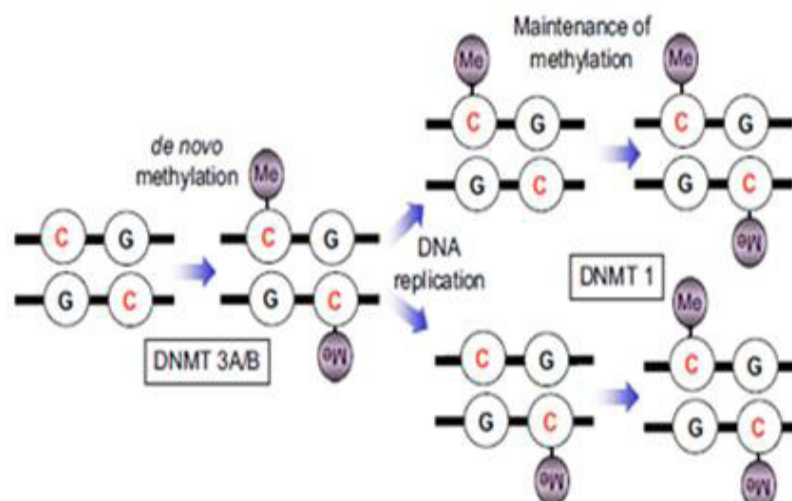


Figure 7: Establishment and propagation of methylation patterns. Cellular DNA methylation patterns seem to be established by a complex interplay of at least three independent DNA methyltransferases: *de novo* (by DNA methyltransferases DNMT3A and DNMT3B) and maintained (by DNMT1).

Epigenetic dysregulation in malignant cells is characterized by global hypomethylation and focal hypermethylation. During tumor initiation and progression, the epigenome undergoes

massive global loss of DNA methylation (20–60% less overall 5-methyl-cytosine) and acquisition of specific patterns of hypermethylation at the CpG islands of certain promoters resulting in their transcriptional inactivation [5,58]. In normal cell, CpG island-containing gene promoters are usually unmethylated, thereby maintaining euchromatic structure, which is the transcriptional active conformation allowing gene expression. However, during cancer development, DNA hypermethylation of several tumor suppressor genes at their CpG island-containing promoters has been shown to result in their abnormal silencing by changing open euchromatic structure to compact heterochromatic structure. DNA methylation mediated epigenetic silencing results in gene inactivation and promotes carcinogenesis, thus signifying that DNA methylation impinges on carcinogenesis [59].

Thus, DNA methylation plays a vital role in promoting tumorigenesis by local hypermethylation associated with the promoter of tumor suppressor genes resulting in their silencing and in parallel by global hypomethylation triggering the reactivation of several cellular protooncogenes. (Table 1).

4.2 Hypermethylation in cancer

DNA methylation is the first epigenetic alterations which were identified in cancer. Aberrant DNA methylation is deeply associated with cancer initiation and progression. The cancer epigenome typically reflects genome-wide hypomethylation and site-specific CpG island promoter hypermethylation [60,61] The underlying mechanism for these global changes initiation is still under investigation. However, recent studies have shown that some changes occur very early in cancer development.

Hypermethylation, typically observed at specific CpG islands, is a significant mechanism of tumor suppressor genes silencing that contributes to tumor initiation and progression [21], [62]. The transcriptional inactivation which is caused by promoter hypermethylation, typically affect various genes that are involved in the main cellular pathways such as DNA repair (hMLH1, MGMT, WRN, BRCA1), Ras signaling (RASSF1A, NORE1A), cell cycle control (p16INK4a, p15INK4b, RB), apoptosis (TMS1, DAPK1, WIF-1, SFRP1) vitamin response (RARβ2, CRBP1) p53 network (p14ARF, p73 (also known as TP73), HIC-1) metastasis (CDH1, CDH13, PCDH10) detoxification (GSTP1) [63,64] (Table 2). Several other tumor suppressor genes have also been reported to undergo tumor silencing by hypermethylation [48,65].

Furthermore, promoter DNA hypermethylation can indirectly inactivate additional classes of genes by silencing transcription factors and DNA repair genes. For instance, promoter hypermethylation-induced silencing of transcription factors, such as RUNX3 in esophageal cancer [66] and GATA-4 and GATA-5 in colorectal and gastric cancers [67] which further contributes to the inactivation of their downstream targets has been reported. Silencing of

DNA repair genes such as MLH1, BRCA1 facilitates cells to accumulate further genetic lesions resulting in the rapid progression of cancer. Thus promoter hypermethylation provides tumor cell with growth advantage, increase in their genetic instability and aggressiveness. It has been proposed that the hypermethylated promoters are associated with molecular, clinical and pathological features of cancer and can serve as potential biomarkers, holding great diagnostic and prognostic promise for clinicians [60].

Table 2: Epigenetically regulated genes in cancer

Cancer- associated Pathway	Gene
Cell cycle	RB, p16 ^{INK4a} , p15 ^{INK4b} , cyclin D2, cyclin E
Signal transduction	RASSF1, APC, ErbB2
Apoptosis	DAPK, Caspase-8 gene
DNA repair	MGMT, MLH1, BRAC1
Carcinogen metabolism	GSTP1
Hormonal response	Oestrogen receptor gene, retinoic acid receptor b2 (RAR-b2)
Senescence	TERT, TERC
Invasion/ metastasis	E-cadherin gene, VHL, TIMP-3
Transcription	Runx3, Twist, ER α , ER β , RAR, vitamin D receptor
Drug responsiveness	Gluthionefy S-transferase, thymidylate synthase
Angiogenesis	THBS1

Characterization of human cancer has been reported to be associated with an overall miRNA downregulation [68] as a result of hypermethylation at miRNA promoter [69]. Repression of miR-124a by hypermethylation mediates CDK6 activation and Rb phosphorylation [70]. Hypermethylation induced inactivation of miRNA expression is not only associated with cancer but also to metastasis development. For example, promoter hypermethylation induced silencing of miR-148, miR-34b/c and miR-9 facilitates tumor metastasis [71].

4.3 Hypomethylation in cancer

Global DNA hypomethylation which can occur at various genomic sequences including repetitive elements, retrotransposons, CpG poor promoters, introns and gene deserts, plays a significant role in tumorigenesis [72]. Furthermore, the DNA hypomethylation at repetitive sequences promotes chromosomal instability, translocations, gene disruption and reactivation of endoparasitic sequences [73,74]. Genomic instability established as an outcome of DNA hypomethylation in cancer cells are primarily caused by the loss of methylation from repetitive regions and are characterized as a hallmark of tumor cells. For example, the LINE family member L1, has been reported to be hypomethylated in a wide range of cancers, including breast, lung, bladder and liver tumors.

The association of hypomethylation with oncogenes has been reported in cancers. A striking example is served by c-Myc, a transcription factor that acts as an oncogene. In cancers, it has been widely reported as hypomethylated genes [65]. Hypomethylation at specific promoters activates the aberrant expression of oncogenes and induces loss of imprinting (LOI) in some loci. MASPIN (also known as SERPINB5), a tumor suppressor gene hypermethylated in breast and prostate epithelial cells [75], has been reported to be hypomethylated in other tumor types. On account of hypomethylation, the expression of MASPIN increases with the degree of dedifferentiation of certain cancer cell types [76,77].

Other well-studied examples of hypomethylated genes in cancer include S100P (pancreatic cancer), S-100 (colon cancer) SNCG (breast and ovarian cancers) and melanoma-associated gene (MAGE) and dipeptidyl peptidase 6 (DPP6) (melanomas) [50,78]. (Table 3) The most common LOI event induced by hypomethylation is IGF2 (insulin-like growth factor 2) and has been widely reported in various tumor types such as breast, liver, lung and colon cancer [79]. LOI of IGF2 has been also linked with an increase risk of colorectal cancer [80]. Thus, DNA hypomethylation induced aberrant activation of genes and non coding regions contributes to cancer development and progression.

4.4 Histone modification in cancer

Nucleosome is the fundamental repeating unit of chromatin, which consists of 147-bp segment of DNA wrapped in 1.65 turns around the histone octamer of following core histone proteins: : H2A, H2B, H3, and H4 and neighboring nucleosomes are separated by, on average, ~50 bp of free DNA. The core histones are predominately globular except for their amino-terminal tails that protrude from the nucleosome, which are less structured [81]. All histones are subject to post- transcriptional modifications. Several posttranscriptional modifications that histone tail domain is subjected to includes: acetylation, methylation, phosphorylation, ubiquitination, SUMOylation and ADPriboseylation [40,82].(Fig. 8)

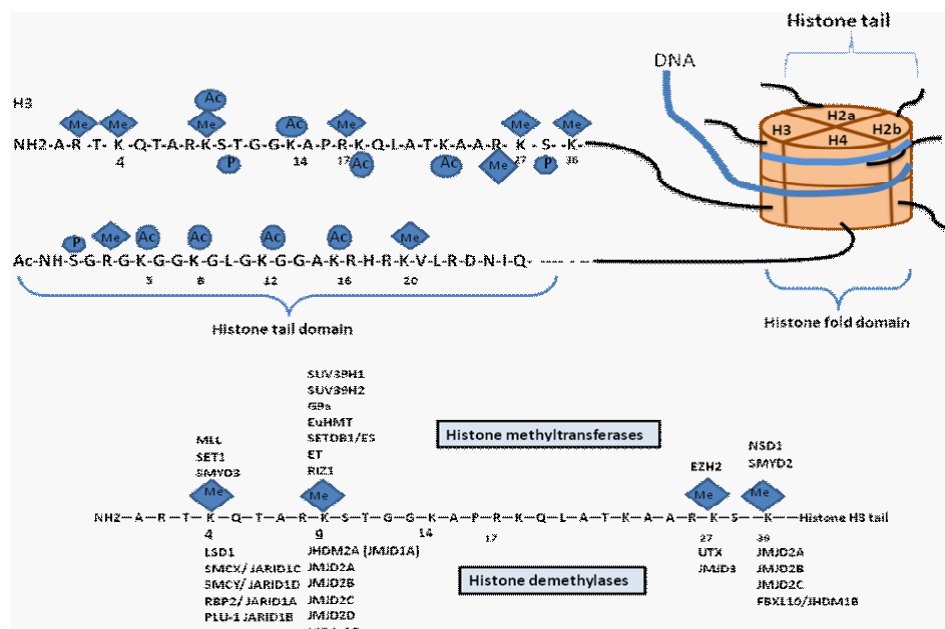
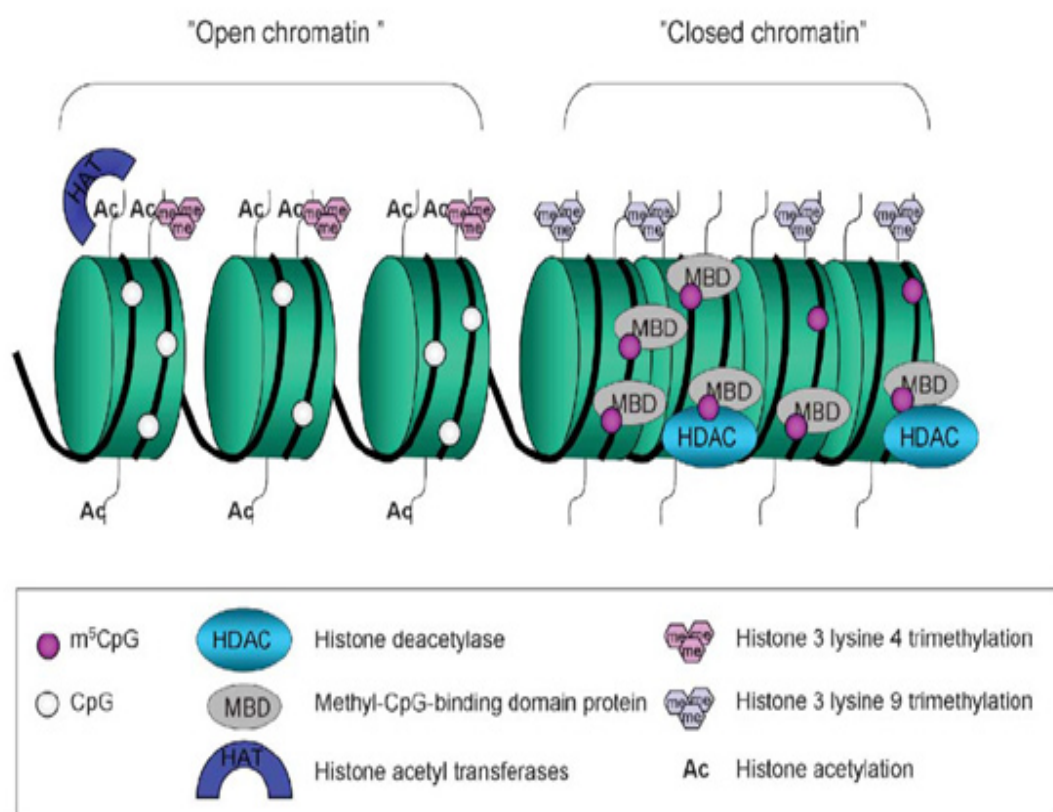


Figure 8: Schematic representation of Histone modifications and modifiers

The complexity of post translation modifications is attributed to histone modifying enzymes which can either activate or repress transcription, on the basis of the type of chemical modification and its location in the histone protein [83]. Recruitment of activating or repressive complexes to DNA can reshape chromatin into relaxed or a tightly packed structure on the basis of the modification pattern of histone and is associated with gene function during development as well as tumorigenesis.(Table1) With respect to its transcriptional state, the human genome can be roughly divided into euchromatin and heterochromatin. Actively transcribed euchromatin is characterized by high levels of acetylation and trimethylated H3K4, H3K36 and H3K79 whereas transcriptionally inactive heterochromatin is characterized by low levels of acetylation and high levels of H3K9, H3K27 and H4K20 methylation.(Fig. 9) Recent studies have revealed that histone modification levels are predictive for gene expression [84].

**Figure 9:** Chromatin structure of active and inactive promoters. (Adapted from: K. Grønbaek et.al. 2007 [12].)

Post translational modifications patterns dynamically regulated by enzymes which either catalyze or remove the covalent modifications to histone proteins, have been described [85,86]. Histone modifying enzymes such as Methyltransferases, histone demethylases and kinases have been reported to be the most specific to individual histone subunits and residues [8]. On contrary, most of the histone acetyltransferases (HATs) and histone deacetylases (HDACs) modify more than one residue, so are not highly specific.

Many transcriptional co-activators (e.g., GCN5, PCAF, CBP, p300, Tip60 and MOF) with intrinsic HAT activity as well as several transcriptional co-repressor complexes (e.g., mSin3a, NCoR/SMRT and Mi-2/NuRD) with HDAC activity have been reported to play important part in chromatin remodeling and gene transcription [87]. It has been reported that phosphorylated RNA polymerase II targets both HDACs and HATs to transcribed regions of active genes, where most HDACs function to reset chromatin by removing acetylation at active genes inhibiting transcription. On other hand HATs are mainly associated with transcriptional activation [27]. It is now evident that the interaction between these histone-modifying enzymes as well as other DNA regulatory mechanisms is essential to tightly link chromatin state and gene transcription.

Histone modifications play important roles in various cellular processes such as transcriptional regulation, DNA repair [88], DNA replication, alternative splicing [89] and chromosome condensation [81]; however their deregulation is implicated in human malignancies [90,91].

In various cancers, the global reduction of monoacetylated H4K16 has been reported as the most prominent alternations in histone modification [92]. HDACs are found to be overexpressed or mutated in different cancer, mediate the loss of acetylation [93]. The Sirtuin family of proteins is the main class of HDACs which are involved in this process. Upregulation in gene expression and deacetylase activity of SirT1 is observed in various cancers. Interaction of SirT1 with DNMT1 affects DNA methylation patterns [94]. The expression of HDAC is also regulated by miRNAs, such as *miR-449a*, induces growth arrest in prostate cancer cells by repressing in the expression of HDAC-1 [95]. Additionally, mutations or deletions as well as translocations in HATs and HAT-related genes has been observed in several cancer such as colon, uterus, lung and leukemia, which contributes to the global imbalance of histone acetylation [96].

Additionally, a global loss of active mark H3K4me3 and repressive mark H4K20me3 as well as a gain in the repressive marks H3K9me and H3K27me3 has been described during carcinogenesis [97]. Aberrant expression of histone methyltransferases and histone demethylases results in altered distribution of histone methyl marks in cancer cells. **(Table 3)** Inactivation of histone modifying genes - histone methyltransferase SETD2 and histone demethylases UTX and JARID1C has been revealed in renal carcinomas [98]. The histone methyltransferase EZH2, overexpressed in various cancers, is a subunit of PRC2/3 complexes which enhances proliferation and malignant transformation [39]. In breast cancer, overexpression of the lincRNA *HOTAIR* reprograms chromatin state to promote cancer metastasis [99]. Histone methyltransferases such as NSD1 undergoes promoter DNA methylation dependent silencing in neuroblastomas [100], while DOT1L, essential for the establishment of euchromatic state allows the expression of tumor suppressor genes [101]. Upregulation of several histone

demethylases such as GASC1, LSD1, JmjC and UTRX have been reported in prostate cancer and squamous cell carcinomas [102]. Histone phosphorylations are key players in DNA damage – repair response, chromosomal stability and apoptosis. JAK2, a nonreceptor tyrosine kinase phosphorylate H3Y41, which in turn prevents the binding of heterochromatin protein 1 α (HP1 α) to this region of H3 resulting in an increase in the expression of the genes located there. In hematological malignancies, chromosomal translocations or point mutations are responsible for JAK2 activation [103].

Table 3: Consequences of DNA methylation and histone modifications in cancer

Aberrant epigenetic modification		Consequences	Genes affected and resulting disease
Cancer			
DNA methylation	CpG island hypermethylation	Transcription repression	MLH1 (colon, endometrium) BRAC1 (breast, ovary), MGMT (several tumor types) p16 ^{INK4a} (colon) [55]
	CpG island hypomethylation	Transcription activation	MASPIN (pancreas), S100P (pancreas), MAGE (melanomas) [104]
	CpG island shore hypermethylation	Transcription repression	HOXA2 (colon), GATA2 (colon) [49]
	Repetitive sequence hypomethylation	Transposition, recombination genomic instability	L1 [55], IAP[55], SAT2[92]
Histone modification	Loss of H3 and H4 acetylation	Transcription repression	CDKN1A[55]
	Loss of H3K4me3	Transcription repression	Hox genes
	Loss of H4K20me3	Loss of heterochromatic structure	Sat2, D4Z4[92]
	Gain of H3K9me and H3K27me3	Transcription repression	CDKN2A, RASSF1[39], [105]

5. DNA methylation as a marker for tumor diagnosis and prognosis

The most well defined epigenetic change in tumors is the aberrant DNA hypermethylation in the promoter regions of genes which is associated with inappropriate gene silencing. This feature can be utilized to explore tumor- specific DNA methylation biomarkers as well as in examining potential candidate DNA biomarker for clinical use as diagnostic, prognostic, or predictive marker [1,106]. DNA methylation biomarkers are molecular target that undergo DNA methylation changes during carcinogenesis. Such a biomarker is essential for early diagnosis of cancer, detection of recurrence as well as for predicting and monitoring therapeutic responses.

DNA methylation biomarkers offer several advantages over genetic and serum markers [107] such as higher incidences of aberrant DNA methylation of specific CGIs, their selective detection in cancer cells, even when it is embedded in substantial amount of contaminating normal DNA, technically simple detection (for instance., can be detected using MSP) and their occurrence at early stage of tumor development, causing gain or loss of function of key processes implicate its potential as early indicator of existing cancer and for evaluation of risk assessment for future development of cancer [108]. Though DNA methylation biomarker has several advantages over genetic markers, it has been reported that combination of the two might serve better outcome. For instance, combination of both markers in stool DNA facilitated the detection of curable stage colorectal cancer and large adenomas with higher accuracy [109].

Moreover, DNA methylation has been recognized as a potential ideal biomarker (diagnostic/ prognostic) due to its methylation stability, amplification ability, high sensitivity, the possibility of localization to a specific gene region, relatively low cost and potential of development as a high-throughput screening method specific for cancer detection [107,110, 111]. Furthermore, the diagnostic and prognostic use of DNA methylation has been reported in various types of cancer, particularly in glioma [7].

A large number of potential DNA methylation marker genes and their role in carcinogenesis have rapidly increased due to the development of recent genome wide techniques for their identification and functional analyses [7,112]. The detection of methylation signatures in virtually any body fluid such as serum/plasma, smears, nipple fluid aspirate and vaginal fluid, among others has been highlighted in numerous reports [113,114]. As blood samples which can be obtained through minimal invasive procedure, serves as ideal substrate for DNA methylation analysis. On other hand, analysing DNA methylation in body fluids remains challenging because of relatively low mount of cell free DNA (cfDNA) compared with cell- derived DNA and for the fact that cfDNA is highly fragmented. DNA methylation markers which are detected in urine or sputum are site directed; however those markers which are detected in serum, plasma or saliva can originate from anywhere in the body. So the methylation markers identified in these substrate should hold specificity for a particular disease or small group of disease thereby enhancing their diagnostic utility [115].

Regarding the clinical implementation of DNA methylation biomarkers, we briefly discuss the established markers as well as the current methylation marker validation studies. Currently, several ongoing studies have focused on testing the utility and clinical implementations of DNA methylation biomarkers as early diagnostic biomarker and disease progression and predictive biomarkers in various malignancies [116,117].

For the early detection of lung, colon and prostate cancer, DNA methylation marker based kits are already available in market. Methylation of septin 9 (SEPT9) and vimentin

(VIM) is used for early detection of colon cancer by analyzing blood (SEPT9) or stool (VIM) samples of patients [118,119]. Improved sensitivity and specificity was exhibited by both markers upon comparison with the fecal occult blood test. Similarly, methylation of SHOX2 is used as a biomarker for distinguishing malignant and benign lung diseases. A sensitivity of 78% and a specificity of 96% have been reported when SHOX2 methylation was analyzed in bronchial aspirates [120]. The methylation of TWIST-1 and NID-2 along with other biomarkers is used to detect bladder cancer [121]. Methylation of Vimentin and NID-2 is associated with assessment of recurrence of bladder cancer [122]. MGMT gene encodes a DNA repair protein, O6-methylguanine DNA methyltransferases. Its methylation has been reported to be associated with survival benefit of glioblastoma patients after treatment with the temozolomide, which is an alkylating drug thus highlighting its predictive potential in clinical settings [123,124].

(Table 4)

Table 4: Commercially available tests based on DNA methylation biomarkers

Biomarker	Application	Disease	Material	Sensitivity/ Specificity (%)	Commercial test	References
SEPT9 + VIM	Early detection	Colorectal Cancer	Blood	80-82/ 89-99	EpiproColon® 2.0 (Epigenomics), ColoVant age™ (Quest Diagnostics), Real-Time mS9 (Abbott)	deVos et al. (2009)
SHOX2	Early detection	Lung Cancer	Sputum	81/95	EpiproLung® BL 1.0 (Epigenomics)	Kneip et al. (2011)
MGMT	Predictive	Brain Cancer	Tumor	-	PredictMDx™ Brain Cancer (MDxHealth)	Hegi et al. (2005)
TWIST2 + NID2	Predictive	Bladder Cancer	Urine	87.9/99.9	CertNDx™ Bladder Cancer Assay Hematuria Assessment (Predictive Biosciences)	Renard et al. (2010)
VIM + NID2	VIM + NID2	Bladder cancer	Urine	90.5/95.5	CertNDx™ Bladder Cancer Assay Hematuria Assessment (Predictive Biosciences)	Reinert et al. (2012)

6. Prospective on Epigenetic therapy

The reversible nature of gene silencing by epigenetic modifications has facilitated the emergence of the promising field of epigenetic therapy as a treatment option. The aim of epigenetic therapy is to restore gene function which is silenced by epigenetic changes during tumorigenesis. The three critical components of epigenetic regulation which have been targeted for development of epigenetic therapies for cancer prevention and treatment include: DNA methylation, post-translational histone and protein modification (e.g., acetylation, methylation)

and more recently, post-transcriptional gene regulation by miR [125,126].

Many epigenetics drugs which can effectively reverse DNA methylation and histone modification alternations have been discovered in the recent past. Currently, several agents that target DNA methylation (DNMT inhibitors) and protein acetylation (histone deacetylase inhibitors [HDACIs]) are in clinical development (ClinicalTrials.gov; www.clinicaltrials.gov.) So far, three epigenetic drugs have been approved by The US FDA which includes: decitabine and Vidaza® for myelodysplastic syndromes [127] and vorinostat for cutaneous T-cell lymphoma [128,129](**Table 5**).As such no compound that specifically targets miR activity is in clinical development, however chromatin modifying agents hold the potential to re- activate miR expression thereby resulting in target protein modulation [130].

Table 5: Examples of approved agents in epigenetic therapy for cancer management

Agent	Class	Disease indications	FDA approval data	Main study institution	Number of patients	Basis of approval
5-azacitidine	DNMT inhibitor	Myelodysplastic syndrome	2004	Memorial Sloan-Kettering; Mount Sinai	191	Phase III trial; 23% response rate; significantly improved median survival compared to supportive care (18 months vs 11 months)
Decitabine	DNMT inhibitor	Myelodysplastic syndrome	2006	MD Anderson	170	Phase III trial; 17% response rate; trend toward improved median survival compared to supportive care (12 months vs 8 months)
Vorinostat	HDAC inhibitor	Cutaneous T-cell lymphoma	2006	Duke	74	Phase IIB trial; 30% response rate; median time to progression was 5 months
Romidepsin	HDAC inhibitor	Cutaneous T-cell lymphoma	2009	National Institute of Health; King's College London	167 (96+71)	Phase II trial; 34% - 38% response rate; median response duration was 11 -15 months

DNA demethylating compounds are the first epigenetic drugs approved for use as cancer therapeutics, can be categorized into two distinct mechanistic groups: “**nucleoside analogs**” that are incorporated into the DNA of rapidly growing tumor cells during replication, covalently bind and trap the DNA methyltransferases (DNMTs) blocking their activity, followed by their proteosomal degradation(e.g. Vidaza (5-azacytidine)) [131]and the “**non-nucleoside inhibitors**” which effectively inhibit DNA methylation without being incorporated into the DNA (e.g. quinolone-based small molecule, SGI-1027) [132].

These DNMT inhibitors tend to induce the de-repression of hypermethylation-induced gene silencing thereby reactivating tumor suppressors and other cancer related genes [133]. They have been also demonstrated to reverse resistance to chemotherapy in vivo [134].

The most clinically advanced nucleoside DNMT inhibitors are the azanucleoside prodrugs, decitabine (5-aza-2-deoxycytidine) and Vidaza (5-azacytidine). Originally being developed as cytotoxic agents, these compounds were subsequently reported to have demethylating properties at lower concentration [135]. Their mode of action is yet not well defined. In addition, they are chemically unstable [136]. Cytidine deaminase metabolizes Vidaza and decitabine to inactive forms [135]. SGI-110, a novel DNMT inhibitor is protected from enzymatic degradation by Cytidine deaminase is progressing through preclinical trials [137]. 5-fluoro-2-deoxycytidine (5-FC) is the most recent agent of this class to enter clinical trial [138]. However, there are drawbacks such as the chemical instability and S-phase specificity has resulted in poor efficiency against cancer stem cells and tumors with low proliferation index, thereby limiting the clinical application of nucleoside DNMT inhibitors. The formation of bulky DNA adducts results in cytotoxicity, which is dose limiting and is manifested as bone marrow suppression and neutropenia [135], [139].

In contrast non-nucleoside DNMT inhibitors are less toxic and potentially more chemically stable [140]. MG98 is an antisense oligonucleotide to DNMT1, with antitumor activity and has completed phase I trials [141]. Quinolone based small molecules such as SGI-1027 and RG108, are inhibitor of DNMT1 which do not bind to DNA or RNA. Being comparatively less Cytotoxic, they might serve as promising clinical candidate [142].

Treatment with HDAC inhibitors, in order to re-establish normal histone acetylation patterns, has been reported to exhibit antitumorigenic effects which are mediated by their ability to reactivate silenced tumor suppressor genes [143]. HDAC inhibitor, such as Suberoylanilide hydroxamic acid (SAHA) has been clinically approved for T cell cutaneous lymphoma treatment. Furthermore, other HDAC inhibitors for instance, depsipeptide and phenylbutyrate are under clinical trials [144].

Recently various combinatorial cancer treatment strategies that involves both DNA methylation and HDAC inhibitors together has been explored and have proved out to be more effective than the individual treatment approaches. Combined treatment with 5-Aza-CdR and trichostatin A exhibited the de-repression of certain putative tumors suppressor genes [145]. Enhanced antitumorigenic effects of depsipeptide were observed upon simultaneous treatment of leukemic cells with 5-Aza-CdR [146]. Combined treatment with phenylbutyrate and 5-Aza-CdR demonstrated greater reduction of lung tumor formation in mice, thus implicating the synergistic activities of DNA methylation and HDAC inhibitors [147].

Recently, the role of HMT inhibitors has also been explored. DZNep, a HMT inhibitor

has been reported to induce apoptosis in cancer cells, preferentially targeting PRC2 proteins, generally overexpressed during carcinogenesis [148]. However, its specificity still remains contradictory [149]. Further development of specific HMT inhibitors is critically needed.

For epigenetic therapy, miRNAs may also serve as promising targets. It was demonstrated that the treatment with 5-Aza-CdR and 4-phenylbutyric acid downregulates the oncogene BCL6 via the reactivation of miR-127, which strongly highlights the potential of a miRNA-based treatment strategy [69]. Synthetic miRNAs that mimic tumor suppressor miRNAs can also be used to selectively repress oncogenes [150]. For the targeted delivery of synthetic miRNAs to tumor cells, development of efficient vehicle molecules is highly essential.

The development of several drugs which can potentially modulate the epigenome to restart transcription of epigenetically silenced genes, thereby augmenting the action of conventional cancer treatment methods, offers an entirely new approach to cancer therapy. On the same note, better understanding of the pharmacokinetics of epigenetic drugs is critically required to identify clinically beneficial properties as well as to develop newer and more efficacious treatments.

7. Key Highlights

- Epigenetic machineries are essential for normal mammalian development and regulation of gene expression.
- Hypermethylation of CpG islands is known to be common event during carcinogenesis.
- Aberrant promoter methylation leads to epigenetic gene silencing leading to loss of gene function in cancer.
- Hypermethylation of tumor suppressor genes is associated with their transcriptional silencing thereby contributing to oncogenesis.
- Methylation analysis of CF-DNA in preferentially any body fluid serves a novel approach for non invasive cancer detection
- Epigenetic drugs targeting the epigenome to induce functional re-expression of aberrantly silenced genes, offers new approach to cancer therapy.

8. Conclusion

An unanticipated progress in unrevealing the molecular mechanisms associated with the epigenetic regulation of normal development and its far implication in treatment of human diseases has been explored over the past 20 years. The deregulation of epigenetic mechanisms

which is responsible for tumorigenesis also augments the effect of oncogenic mutations. Targeting early tumor development and its progression serves as a logical therapeutic approach for the management of aberrant epigenetic alternations. Therefore, epigenetic alternations which are associated with the onset and progression of cancer, serves as potential clinically useful targets. Extensive research testing the utility and clinical implementation of DNA methylation based markers for early detection, diagnosis, prognosis or prediction of cancer cases is in progress. However, DNA methylation marker kits for the early detection of various cancers (such as lung, colon and prostate cancer) are already commercialized. Exploration of the molecular events that initiates and maintains epigenetic gene silencing has facilitated the discovery of epigenetic drugs targeting the epigenome, including DNA methylation and histone modifications. Several epigenetic agents have mapped their way in clinical utility upon approval by US Food and Drug Administration (FDA). The future will see the utility and success of combination of epigenetic drugs along with other therapy for the management of cancer significantly and with efficacy.

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Advances in Biotechnology

Chapter 3

Magnesium as a Novel Regulator of Human Health and Diseases

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Abstract

Magnesium (Mg) plays an important role in various cellular processes such as DNA repair and replication, transporting potassium and calcium ions and signaling transduction. Dietary source which are rich in Mg includes seeds, cocoa, nuts, green leafs and almonds. The daily dietary intake of Mg is frequently found to be below than the recommended in western country. Certainly it is recognized that Mg deficiency may lead to many disorders of the human being for example diabetes, cardiovascular disease and TB. Moreover, Mg deficit also leads to inflammation and amplifies the level of free radicals where it causes oxidative DNA damage and tumor formation. The presented book chapter provides a summary of low Mg impact on human health and development of various diseases.

1. Introduction

Mg is the second most abundant divalent cation in living cell which is commonly found in the earth crust and other planets. Out of the eight main elements of earth crust, Mg is one of the four major elements to form the whole mass of earth. Mg is naturally obtained from the diet source such as cereal, wheat, nuts, green vegetables, soya, fish, chocolate, legumes, nuts and dairy products [1]. The maximum percentage of Mg in diet is lost during cooking or purification. It means that processed food contain less amount of Mg as a compared to raw food. For instance, Mg is exhausted by 82% in the conversion from wheat to flour [2]. This probably explains lower than recommended daily allotment (RDA) of Mg intake by the large population all over the world. In USA, Food and Nutrition Board Commission represents a data for RDA of Mg intake per day [3]. They recommended that Mg intake is 4.5 mg/kg/day as RDA of

Mg on the basis of balanced studies [4]. The average content of Mg for adult RDA in western societies is about 350 mg [5]. The actual amount of Mg requirement depends on the levels of metabolic activity and type of work, life style and disease [6]. It is intracellular element and more than half of its total body's content is incorporated in bone. Beside bones, the maximum concentration of Mg is available in muscles (27%) and soft tissue (19.3%), whereas serum has only 0.3% [7]. Some Mg is also bound in the form of ions with protein while only one third Mg is available in free form as protein bounded form is not accessible for biochemical process, only ionized Mg form is active for biological activity. The concentration found in the protein-bound Mg is 25% impelled with albumin and 8% to globulin [8]. The association of Mg with globulin may be important because globulin is key of minerals in metabolism and cofactor for hundreds of enzyme activity. Since Mg is a mineral which is not synthesized in our body it must be obtained through dietary foods or through supplements. Mg shortage can cause low serum potassium and calcium levels, retention of sodium, and low circulating levels of regulatory hormones. Mg metabolism is closely regulated by hormones but it appears that there is no particular hormone responsible to control Mg homeostasis [9]. These changes in Mg concentration cause neurological and muscular symptoms such as tremor and muscle spasms. Further Mg deficiency causes loss of appetite, nausea, vomiting, personality changes and death from heart failure. Some of the factors which can lead to Mg deficiency include the alcohol abuse, inadequately controlled diabetes, excessive or chronic vomiting or diarrhea. Thus the effect of inadequate and deficient intake levels of Mg is critical to human health [10].

2. Mg in cellular physiology

Mg play consequential role in many physiology activity including cell signaling, energy production, protein synthesis, oxidative phosphorylation, glycolysis and nucleic acid. Some cations may replace Mg for these function but other are strictly dependent upon the Mg, which indicates that cell must have minimum Mg to maintain their physiology conditions. Mg has an essential role in the active transport of calcium and potassium ions across cell membranes, this function is significant to nerve impulse conduction, muscle contraction, and normal heart rhythm [11]. High amounts of Mg are especially needed for cells to work in different organ actively from metabolically intensive such as heart, brain and muscle [6]. Slight changes in Mg concentration in body lead to major consequences. The deficiency of Mg is a manifestation of various pathologies. The deficiency of Mg play crucial roles in different types of disorder such as cardiovascular disease (cardiac death, atherosclerosis, heart failure), heart failure, thyroid and parathyroid, liver cirrhosis and gastrointestinal tract disease [12].

Mg ions play important role in cellular activity. By macromolecular surface binding they stabilize structures of proteins, nucleic acids, and cell membranes [13]. Mg activate many enzymes, which are important for those that perform hydrolysis and phosphate group transfer [12]. ATP hydrolysis to ADP is the most important stimulatory role of Mg in cell energy

metabolism [14]. Mg is combined with ATP, ADP and GTP, necessary for the many activities of enzymes that is involved in phosphate group transfer such as glucokinase, phosphofructokinase, phosphoglycerate kinase, pyruvate kinase [15]. In fact, all reactions involving ATP require the presence of Mg ions [16]. This ion also play important role in the nucleic acid and protein [14]. and maintaining genomic stability, through ensuring the fidelity of DNA replication and repair process. Almost more than half Mg is found in the nucleus is associated with nucleic acid and free nucleotides. In addition, Mg has a critical roles in modulating cell cycle progression, cell proliferation, differential and apoptosis [17].

The transporters which are involved in Mg homeostasis have been the main focus of research due to its importance in human health. Genetic screenings on human diseases and microarray-based expression studies have resulted in the identification of numerous Mg transporter proteins which can be ubiquitous or tissue specific. The ubiquitous transporter transient receptor potential melastatin type 7 (TRPM7), Mg transporter 1 (MagT1), and solute carrier family 41 member 1 (SLC41A1) [18,19]. The tissue specific Mg transporters such as transient receptor potential melastatin type 6 (TRPM6; kidney, colon), cyclin M2 (CNNM2; kidney) and cyclin M4 (CNNM4; colon) [20,21]. It has been reported that any conformational changes in these transporters can lead to deadly diseases like TB, cancer, diabetes etc.

3. Ubiquitous Nature of Mg

Mg is present in every organism like bacteria, fungi, virus and human being. Its importance and function is evolutionary conserved in all organisms as discussed in following sections.

3.1. Mg acquisition in bacteria

There are three classes of Mg transporters present in bacteria. CorA, MgtE and MgtA [22,23,24]. Most of bacteria contain multiple type of transporter either belonging to same or different classes. Whereas all these transporters can import Mg but they vary in the energy requirements for moving Mg, their ability to export Mg, the conditions under which the proteins are made, and their phylogenetic transportation within bacteria as well as in archaea and eukarya. Putative Mg binding sites present in the monomer merge in the extracellular cytoplasmic domain are thought to be control channel opening and closing in response to intracellular Mg level [25]. Mg Structurally MgtA is different from Cor A. As with Cor A, intracellular Mg is thought to be regulated by MgtA. P-type ATPases are Mg transporter family belong to MgtA class. These families of proteins need energy from the decomposition of adenosine triphosphate (ATP) to transport a variation of charged molecules. To relay of the phosphate group from ATP to the protein results in a symmetry change in the protein that stimulate Mg transport [26]. In addition to the regulation of transcription beginning by extra cytoplasmic Mg levels, transcription elongation into the protein-coding regions through Mg transporter

genes can respond to the concentration of Mg in the cytoplasm [27,28].

3.2. Mg acquisition in fungi

Mg is found abundantly in many fungi. However, their amount may vary between species. Mg is necessary for multiple fundamental biological activity, of yeast. For example in *Saccharomyces cerevisiae* importance of Mg for survival has been already proved. So far, there are three type of Mg transporters that has been identified in yeast. The Alr Mg transporter present in the cell membrane, the Mnr2 Mg transporter system present in the vacuolar membrane [29]. There are two Alr proteins (Alr1 and Alr2) which are orthologues of the bacterial CorA transporter, allocate with the latter a highly conserved GMN motif [6]. Both Alr1 and Alr2 located in the plasma membrane and perform the function of Mg importers [30]. The storage site of Mg in vacuole present within the yeast cell. The Mg^{2+}/H^{+} exchange mechanism was discovered to drive Mg entry into the organelles [31]. The vacuolar membrane protein that functions as putative Mg transporter exhibit similarities with Alr1 and Alr2 is responsible for Mg efflux from vacuoles. Genetic confirmation shows that this protein functions by releasing Mg into the cytosol under Mg-deficient conditions

3.3. Mg in virus

There is considerable amount of Mg present in DNA and RNA. Most of the virus that infect eukaryotes has Mg in their intracellular membrane that activate the specific transporter. For Example, XMEN diseases are very rare genetic disease mostly appearing in men that have mutation in the MAGT1 Mg transporter gene. MAG1 bring the Mg inside from the immune cells to support their function [32].

4. Mg and human health disorders

4.1. Mg in non infectious disease

Mg is an essential element that acts as an enzymatic catalyst and electrolyte. Because it is required for several biological processes, it has an extremely important role to play in health and disease (Fig. 1). The investigations has proved that the alteration in the physiology and metabolism of Mg induce the threat of developing metabolic diseases, viz. obesity, /type II diabetes obesity.

4.1.1. Mg and diabetes

Mg deficiency leads to diabetes mellitus, both type 1 and 2, with 25-39% of patients being affected [33]. There are several factors that affect Mg during diabetes as many of the enzymes involved in glycolysis are Mg dependent. Increased insulin resistance has been found in patients with reduced free Mg levels, and animal studies have shown proliferating glucagon

stimulation decreased insulin secretion and reduced insulin uptake with Mg deficiency. The past studies show that, hypomagnesemia is strongly associated with type 2 diabetes patient with hypomagnesemia show instantly decline in progression and have increased risk of diabetes complications. Experimental studies showed that patients along with hypomagnesemia have lower pancreatic beta-cell activity and are more insulin resistant [34]. Furthermore insulin receptor autophosphorylation is reliant on intracellular Mg concentrations, making Mg a straight role in the progress of insulin resistance. So Mg supplementation improved insulin sensitivity and metabolic control in a double-blind randomized trial, suggesting that Mg is an important factor in the etiology and management of diabetes mellitus [35,36].

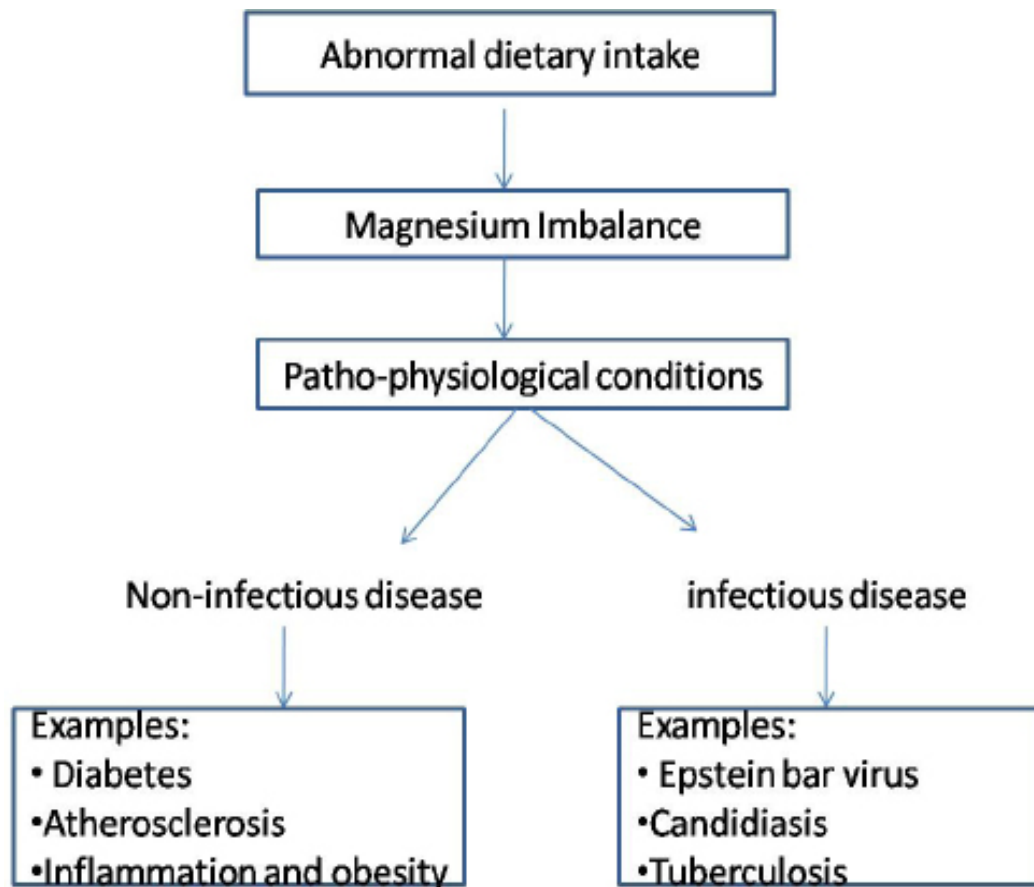


Figure 1: Mg imbalance leading to various health disorders

4.1.2. Mg and atherosclerosis

The epidemiological and experimental evidence links Mg deficiency and atherosclerotic cardiovascular disease. Mg deficiency contributes to atherosclerosis by effecting their lipid metabolism, blood pressure and platelet aggregation. Experimental evidence recommends that Mg deficiency characterized by increased VLDL, LDL, triglycerides, cholesterol and triglyceride-rich lipoproteins may play a role in the pathogenesis of atherosclerosis. In contrast to this, others recommend on experimental basis that a low concentration of high density lipoprotein (HDL) and apoprotein A1 may be important for Mg deficiency which causes atherosclerosis [37]. The biochemical mechanism of Mg deficiency is a factor for accelerating atherosclerosis

through HDL and/or LDL. The mechanism of atherosclerosis may be through the huge production of oxygen derived free radical caused by the chronic condition with in artery, occur due to Mg deficiency. It suggests that Mg deficiency favor the free radical production and oxidation of lipid moieties [38]. Further studies suggest the involvement of Mg with HDL and LDL that is responsible in the contribution for atherosclerosis.

4.1.3. Mg in inflammation and obesity

The low grade inflammation may lead to obesity. Because low Mg more often occur in obese than non-obese individuals [39,40,41], one of the stressor causing the activation of inflammatory pathways is Mg deficiency. The severe Mg deprivation, which instantly decrease extracellular Mg, results in inflammatory response in animals. Mostly inflammatory response is caused by an increase in intracellular calcium and the priming of phagocytic cells, which results in the release of inflammatory cytokines [42].

However, dietary Mg deficiency severe enough to cause a marked drop in extracellular Mg in some days is doubtful in humans. Through, animal deficiency findings support the suggestion that subclinical Mg deficiency can cause, or come up with, chronic inflammatory stress in humans through an effect on the cellular entry of calcium and its signaling that results in the release of inflammatory neuro-peptides, cytokines, prostaglandins, and leukotrienes [43,44].

4.1.4. Mg in cancer

Tumor cells restrain high concentrations of intracellular Mg. In tumor cell line, Mg can be transported into the cell even when extracellular Mg concentrations were low [45]. Mg uptake through divalent cation channel TRPM7 has been optional to stimulate tumor cell proliferation. TRPM7 expression is upregulated in hepatoma, pancreatic adenocarcinoma, gastric cancer, and breast cancer tissue [46,47]. Although TRPM7 has been primarily described as a Mg channel, it is also permeable for other divalent cations [48]. Given the involvement of Mg in cell proliferation, the influx of Mg through TRPM7 has been proposed as the main regulator of tumor growth. However, recent studies using prostate cancer cells suggest that TRPM7-mediated Ca uptake may also play an important role in tumor growth. The appearance of Mg transporter CNNM3 is increased in human breast cancer tissue [49]. CNNM3 binds oncogene PRL2 and facilitates the entry of Mg in the tumor cell to drive cell proliferation.

4.1.5. Mg in neurodegenerative diseases

Low serum Mg concentration is linked with a broad range of neurological diseases such as migraine and depression. Neuronal Mg concentrations are of main significance in the regulation of N-methyl-D-aspartate (NMDA) receptor. NMDA receptors are vital for excitatory synaptic transmission and neuronal plasticity, therefore play an important role in developmen-

tal smoothness, learning, and memory [50]. Examples of neurodegenerative diseases comprise Parkinson's, Alzheimer's, and Huntington's disease. In case of Parkinson disease, there are low levels Mg concentrations in cortex, white matter, basal ganglia, and brain stem. In addition, Mg transporter SLC41A1 is located on the PARK16 locus that is linked with Parkinson's disease [51]. Recent characterization of the SLC41A1-pA350V single nucleotide polymorphism (SNP) linked to Parkinson's disease evidenced a gain-of-function effect.

4.2. Mg in infectious disease

Over past decades, emergence in the cases of infectious disease caused by fungi, bacteria and technology of Mg in ionized form used in the treatment in following sections. Virus have been on the climb worldwide. Due to their wide spread and continue use of antimicrobial drugs in treating infection has led to emergence of resistance among the various strains of microorganism that's lead to multiple drug resistance. The sufficient amount of Mg helps in the treatment of diseases such as hypertension, acute myocardial infarction and colorectal cancer [5]. So Mg has critical role in health and disease and ionized Mg may provide better insight about the Mg and its metabolism (Fig. 1). As our knowledge progress the advanced technology of Mg in ionized form used in the treatment in following sections.

4.2.1. Mg and epstein bar virus

It is most common virus in human and best known as the cause of infectious mononucleosis. It is particular associated with form of cancer, such as Hodgkin lymphoma, gastric cancer and condition which is linked to human immunodeficiency virus and CD8 T lymphocytes (CTLs). In recent year many primary immunodeficiency has been associated with abnormalities in ions channels and transporter, including those involved in permeability is calcium and Mg [54-57]. XMEN disease suggests that Mg is important for intracellular regulation of immune system.

4.2.2. Mg and candidiasis

The most common fungal pathogen of humans is *Candida albicans*. This is fourth most common cause of hospital acquired infectious disease and is the first cause of systemic candidiasis, with mortality rates approaching 50% [58]. *C. albicans* is a commensal fungus opportunistic human fungal pathogen that causes candidiasis in immuno-compromised condition such as in AIDS, organ transplant, diabetes, or in cancer patients, it results in mucosal, cutaneous or invasive mycoses [59]. Mg in fungi play diverse role as a counter ion for solutes, specially ATP and other nucleotides, DNA and RNA. By binding to RNAs and many proteins, Mg is also necessary to initiate and sustain physiological structures and acts as an important cofactor in catalytic processes. Mg also maintains membranes and dynamic conformations of macromolecules. In yeast, vacuolar Ca accumulation is blocked by increased Mg in the medium,

and *alr1*_ mutants having lower Mg exhibit elevated Ca [60]. Gooday (1978) has previously suggested that Mg may play an important role in regulating this key enzyme in *C. albicans*. Moreover, *C. albicans* need Mg for germ-tube formation [61]. Mg-lacking media, metal ion chelators and the ionophore A23187 repressed germ-tube formation. Yeast-phase cells, which did not form germ-tubes, had a lower Mg content and failed to gather Mg when kept under conditions for germ-tube formation [62]. It suggests that Mg have a central role in regulating virulence of *C. albicans*.

4.2.3. Mg and tuberculosis

Mycobacterium tuberculosis, causing Tuberculosis (TB) remains a major health concern into the 21st century. It has been evaluate that up to one-third of the global population harbors the bacteria, with approximately 0.17 crores deaths due to TB yearly [63]. Further, the recent emergence of multi- and especially drug resistance strains highlights the continued relevance of this pernicious human pathogen [64]. The entire mechanism of pathogenesis is unknown, but likely involves a multi-factorial attack of the immune system [65]. The beginning biosynthetic enzyme (Rv3377c/MtHPS) involved in isoTb biosynthesis release noticeable inhibition by its Mg co-factor, key to the hypothesis that the depletion of Mg observed upon phagosomal absorb may act to trigger isoTb biosynthesis. While MTB is typically grown in relatively high levels of Mg (0.43 mM), transfer MTB to media with phagosomal levels (0.1 mM) led to a significant (~10-fold) increase in accumulation of the MtHPS outcome, halimadienyl diphosphate, as well as easily detectable amounts of the derived bioactive isoTb [66]. Moreover, the integral membrane protein PerM is important for MTB determination during chronic infection in mouse. Consequently PerM mutant need additional Mg as compared to wild type MTB for replication and survival in media with reduced Mg [67]. The survival defect of PerM mutant in low Mg and chronic infection are stable with Mg deprivation constitutes an IFN- γ dependent host defense strategy. It suggests that Mg is essential for bacterial growth and it serve as wide role like function as cofactor with ATP in several enzymatic processes.

5. Conclusion

Mg is crucial micronutrient whose bioavailability essentially impacts on several enzymatic processes in the cell hence further attention should be paid for having an enough content of this element in diet. Disorders of Mg metabolism are ordinary in hospital patients and are frequently unrecognized. Low Mg intake may be a contributor to many diseases including diabetes, cardiovascular disease and TB.

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Advances in Biotechnology

Chapter 4

Response of Cultivars and Rooting Media on Root Behavior and Bud establishment in Rose (*Rosa Hybrida*) under Cuttage-Buddage System

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Abstract

An investigation was designed to comprehend and evaluate the vegetative response of rose cuttings to three cultivars and in four different rooting media. Experiment was conducted on three cultivars namely; Happiness, Avon and Kiss of Fire using four rooting media viz; soil (control), vermiculite, perlite and sand:soil:FYM (1:1:1 v/v). It was conducted as a factorial experiment on the basis of Completely Randomized Design with three replications. There were five budded cuttings in each replication. The findings revealed that cuttings of *Rosa indica* var. *odorata* budded with buds of cv. Happiness (V1) recorded maximum bud take percentage for all the four rooting media. Cultivar Happiness performed better than cv. Avon in terms of days for bud sprouting, diameter of primary and secondary branches, while, length of primary and secondary branches was better in cv. Avon. The highest number of roots (33.16) and longest root (13.84 cm) were recorded for Happiness + vermiculite. However, better survival percentage under field condition was recorded in Avon and Kiss of Fire than Happiness. Among the rooting media, vermiculite appeared to be superior due to its positive effect on root growth and development, followed by sand:soil:FYM (1:1:1 v/v), while perlite was least effective. During the investigation period the two factors, cultivars and rooting

media interacted significantly with each other in most of the cases. In general, cv. Happiness and Avon performed better than Kiss of Fire. As far as rooting media is concerned, vermiculite is recommended as an appropriate rooting media for optimum rooting and growth of rose cuttings.

Key words: Rose; cultivars; rooting media; bud establishment

1. Introduction

Rose is a leading cut flower grown commercially all over the world. It ranks first in global cut flower trade. This flower has a worldwide consumption of more than 40 billion [1]. The major rose growing countries are the Netherlands, USA, Columbia and Israel. In India, commercial cultivation of roses for export market has picked up recently. Roses are mostly cultivated in Maharashtra, Karnataka, Tamil Nadu, Punjab and Delhi. Out of 320 EOU (Export Oriented Units) registered on flowers, rose occupies a major area under protected environment [2]. At present in India, about 8 million new bushes of roses are made each year, mostly for garden display purposes.

Roses are known for their exquisite flowers, bewitching colours and most delightful fragrance. Apart from making garlands, bouquets, buttonholes, preserves, they are also used for worship in temples. Rose water is an important commercial product obtained from rose petals. It has medicinal property and is often used in Ayurvedic system of medicine [3]. A limited quantity of rose oil extracted from flower petals is used in flavouring soft drinks and alcoholic liquors. Rose petals are also used for making *gulkand*, *pankhuri*, *gul-roghan*. Roses are also used for making pot-pouri, conserves, rose vinegar and rose petal wine. It is now a well known fact that rose hips are a very good source of ascorbic acid, the life giving vitamin C [4,3].

“Cutting-graft” was first proposed by McFadden in 1963 by combining cutting and grafting in one action. Dutch persons named this technique as “Stenting” (to stent), which is contraction of Dutch words “*Stekken*” (to strike a cutting) and “*enten*” (to graft). The stented plant is to be called as stenting [5]. The stenting system of grafting of roses, a popular method of propagation of roses in Europe, was developed by Dr. Peter van der Pol of Wageningen Agricultural University, the Netherlands. It requires mature wood in both the root stock and in the selected scion (the variety to be propagated). In the method of stenting, the joining of the root stock with the scion and the rooting of the root stock takes place simultaneously and as a result considerable time is saved. When the plants raised from stenting, are well established with proper shoots and foliage, the thick branches should be pruned to a height of 20-30 cm from the ground and the sprouted shoots may be allowed to develop for production of cut flowers [6].

Budding is the most common method adopted for the multiplication of majority of rose cultivars for obtaining better quality flowers. Generally 16-18 months are taken from rootstock

planting to final budded plant for plantation. This method is labour intensive, time consuming and uneasy to bud. The new method of cuttage- budding is followed to reduce this long period [7]. Therefore, the present investigation was planned with three varieties of rose viz. Happiness, Avon and Kiss of Fire as scion/bud and *Rosa indica* var. *odorata* as rootstock, with the following objectives to assess the influence of cultivars and rooting media on various vegetative characters under shade net, to study the percentage of survival of different cultivars in different rooting media and to screen out the most appropriate treatment/combination for growth parameters.

2. Materials and Methods

The present investigation was conducted during 2009-2010 under shade net at the Model Floriculture Centre of Department of Horticulture, Govind Ballabh Pant University of Agriculture and Technology, Pantnagar, Distt. Udham Singh Nagar. The experiment materials which were used for the study consisted of three Hybrid Tea cultivars namely, Happiness, Avon and Kiss of Fire, a rootstock *Rosa indica* var. *odorata* and for evaluating the effect of different growing media, four types of media were used viz. soil (Control), vermiculite, perlite and sand:soil:FYM (Farm Yard Manure).

The details of treatments and symbols allotted to them in experiment are given in Table 1.

S.No.	Treatment	Symbol
1.	Happiness + Sand:Soil:FYM (V_1+M_4)	T ₁
2.	Happiness + Vermiculite (V_1+M_2)	T ₂
3.	Happiness + Perlite (V_1+M_3)	T ₃
4.	Happiness + Soil (V_1+M_1)	T ₄
5.	Avon + Sand:Soil:FYM (V_2+M_4)	T ₅
6.	Avon + Vermiculite (V_2+M_2)	T ₆
7.	Avon + Perlite (V_2+M_3)	T ₇
8.	Avon + Soil (V_2+M_1)	T ₈
9.	Kiss of Fire + Sand:Soil:FYM (V_3+M_4)	T ₉
10.	Kiss of Fire + Vermiculite (V_3+M_2)	T ₁₀
11.	Kiss of Fire + Perlite (V_3+M_3)	T ₁₁
12.	Kiss of Fire + Soil (V_3+M_1)	T ₁₂

The experiment was laid out in 2 factors Completely Randomized Design. Thus there were 12 treatment combinations which were replicated three times and each treatment consisted of a 5 plants in a replication. The recorded data were statistically analysed using RBD (Randomized Block Design) and 2 factors CRD (Completely Randomized Design). The critical difference (CD) at 5% level of significance was worked out for detecting significant differ-

ences between treatment means.

3. Result and Discussion

The treatment combination V_1+M_2 recorded highest bud take percentage (86.66%) whereas, V_3+M_3 recorded lowest per cent bud take (40.00%) among all the treatment combinations. Treatment T_2 (Happiness + vermiculite) recorded minimum days to bud sprouting (20.06 days), the results were statistically at par with other treatments (Table 2). The maximum days (23.26 days) to bud sprouting were taken by treatment T_{11} (Kiss of Fire + perlite). This may be due to incompatibility between rootstock and cultivars (used as scion). This result is in close conformity with the findings of [8,9,10]. Cultivars and rooting media did not affect percent bud take and days to bud sprouting significantly.

Among the different rooting media tried, vermiculite (M_2) was found to be the best rooting media regarding maximum diameter of primary branches (0.68 cm) with cv (Table 2). Happiness (V_1) at 120 days after planting, while it was registered minimum (0.59 cm) in Kiss of Fire (V_3) with perlite medium. This might be due to optimum conditions of aeration, water holding capacity and nutrient present in vermiculite. Vermiculite is a suitable rooting medium with distinct properties. Similar findings on aforesaid vegetative characters have also been reported by [11]. The effect of cultivar, rooting media and their interaction was found non significant, however, maximum number of secondary branches (1.94) were registered in Happiness (V_1) with vermiculite (M_2), while it was recorded minimum (1.11 each) in treatment T_1 [Happiness + sand:soil:FYM (1:1:1 v/v)] and T_5 [Avon + sand:soil:FYM (1:1:1 v/v)]. Similar results were also reported by [12,13,14]. The results reveal that diameters of secondary branches were in general better (0.22 cm each) in Happiness + vermiculite (T_2), Happiness + soil (T_4) and Avon + soil (T_8) which was at par statistically. Treatments T_3 (Happiness + perlite), T_5 (Avon + soil) and T_7 (Avon + perlite) exhibited minimum diameter of secondary branches 0.18 cm each at 120 days after planting.

Avon (V_2) performed better (20.33 cm) in sand:soil:FYM (1:1:1 v/v), while Avon + perlite (T_7) recorded minimum length of primary branches (11.48 cm) at 120 days after planting. Looking upon the effect of rooting media in general, sand:soil:FYM (1:1:1, v/v) was found best with respect to length of primary branches. The results are in line with those of obtained by [15,16]. Among the three cultivars tested, Avon (V_2) recorded the longest secondary branch (15.71 cm), while shortest secondary branch was recorded in cv. Happiness (V_1) at 120 days after planting. In case of different rooting media, control (soil) was found superior, very closely followed by vermiculite. Perlite was the least responsive. These results are in collaboration with the findings of [17].

The number of leaves per plant was also significantly affected by cultivar, rooting media and their interaction. Happiness (V_1) produced more number of leaves (40.21) when planted

in vermiculite medium (M_2). Minimum number of leaves per plant (24.88) were recorded in Happiness (V_1) with perlite medium (M_3) at 120 days after planting.

Out of the three cultivars and four rooting media, the best performance in terms of maximum number of roots, length of roots, fresh and dry weight of roots was observed in treatment combination of Happiness (V_1) + vermiculite (M_2) and it was minimum in treatment combination of Kiss of Fire (V_3) with perlite medium (M_3) in most of the rooting parameters (Table 3). This could be due to difference in medium because vermiculite provides improved aeration, drainage and optimum moisture retention and nutrient availability. The results discussed are in accordance with the earlier findings of [18] who reported that rooting of rose plants was better in vermiculite than in river sand or perlite. Variation in percentage of rooting may be due to genetic factor and low rooting capacity of rootstocks [19].

Table 2: Effect of cultivars, rooting media and two factor interaction on vegetative parameters of Rose (*Rosa hybrida*).

Treatment	Per cent bud take	Days for bud sprouting	Dia of primary branches (cm) at 120 DAP.	No. of secondary branches	Dia of secondary branches (cm) at 120 DAP.	Length of primary branches (cm) at 120 DAP.	Length of secondary branches (cm) at 120 DAP.	No. of leaves/plant after 120 days of planting.
Cultivars								
Happiness (V_1)	68.83	20.84	0.66	1.37	0.21	13.36	12.09	31.19
Avon (V_2)	58.83	21.87	0.63	1.24	0.19	17.16	12.80	34.85
Kiss of Fire(V_3)	53.33	22.44	0.61	1.34	0.20	14.72	10.46	30.32
S.Em±	4.1943	0.4170	0.0063	0.1127	0.0030	0.2254	0.2354	0.2652
C.D. at 5%	NS	1.2172	0.0184	NS	0.0089	0.6581	0.6873	0.7741
Rooting media								
Soil (Control) (M_1)	57.77	21.59	0.64	1.33	0.21	15.47	12.61	30.96
Vermiculite (M_2)	73.33	21.45	0.65	1.48	0.21	16.11	13.60	38.14
Perlite (M_3)	46.66	22.41	0.62	1.35	0.18	11.83	9.26	26.25
Sand:Soil:FYM (1:1:1 v/v) (M_4)	62.22	21.42	0.62	1.12	0.20	16.91	11.67	33.14
S.Em±	4.8432	0.4815	0.0072	0.1302	0.0035	0.2603	0.2719	0.3062
C.D. at 5%	14.1362	NS	0.0212	NS	0.0103	0.7599	0.7936	0.8939
Interaction effects (cultivars and rooting media)								
$V_1 M_1$	60.00	20.49	0.68	1.27	0.22	13.97	12.23	29.66
$V_1 M_2$	86.66	20.06	0.68	1.94	0.22	14.23	15.05	40.21
$V_1 M_3$	53.33	22.00	0.65	1.16	0.18	12.00	9.16	24.88
$V_1 M_4$	73.33	20.79	0.64	1.11	0.21	13.26	11.93	29.99

V ₂ M ₁	60.00	22.14	0.64	1.44	0.22	17.88	15.71	35.22
V ₂ M ₂	73.33	22.21	0.66	1.27	0.20	18.96	14.23	39.99
V ₂ M ₃	46.66	21.98	0.61	1.16	0.18	11.48	9.38	27.22
V ₂ M ₄	53.33	21.16	0.61	1.11	0.18	20.33	11.88	36.99
V ₃ M ₁	53.33	22.14	0.60	1.27	0.19	14.56	9.88	27.99
V ₃ M ₂	60.00	22.09	0.62	1.22	0.21	15.15	11.53	34.21
V ₃ M ₃	40.00	23.26	0.59	1.72	0.19	12.01	9.24	26.66
V ₃ M ₄	60.00	22.30	0.62	1.16	0.20	17.15	11.21	32.44
S.Em±	8.3887	0.8340	0.0126	0.2255	0.0061	0.4509	0.4709	0.5304
C.D. at 5%	NS	NS	NS	NS	0.0179	1.3163	1.3746	1.5483

Table 3: Effect of cultivars, rooting media and their interaction on rooting behaviour of Rose (*Rosa hybrida*).

Treatment	Number of roots per plant	Length of the longest root (cm)	Fresh weight of roots (g)	Dry weight of roots (g)
Cultivars				
Happiness (V ₁)	20.75	10.72	3.83	2.82
Avon (V ₂)	19.62	9.51	4.04	2.98
Kiss of Fire(V ₃)	14.79	7.09	3.78	2.66
S.Em±	0.2678	0.0942	0.0899	0.0904
C.D. at 5%	0.7818	0.2750	0.2625	0.2638
Rooting media				
Soil(Control) (M ₁)	16.22	8.92	3.83	2.67
Vermiculite (M ₂)	24.77	11.44	4.62	3.42
Perlite (M ₃)	13.27	7.24	3.18	2.34
Sand:Soil:FYM (1:1:1 v/v)(M ₄)	19.27	8.83	3.90	2.83
S.Em±	0.3093	0.1087	0.1038	0.1043
C.D. at 5%	0.9028	0.3175	0.3031	0.3046
Interaction effects (cultivars and rooting media)				
V ₁ M ₁	16.83	11.15	3.31	2.29
V ₁ M ₂	33.16	13.84	5.45	4.02
V ₁ M ₃	17.66	8.65	3.14	2.48
V ₁ M ₄	15.33	9.25	3.43	2.48
V ₂ M ₁	18.83	8.21	3.79	2.79
V ₂ M ₂	19.83	12.81	4.24	3.27
V ₂ M ₃	11.50	6.71	3.29	2.26
V ₂ M ₄	28.33	10.33	4.86	3.59
V ₃ M ₁	13.00	7.40	4.40	2.93

V ₃ M ₂	21.33	7.68	4.17	2.99
V ₃ M ₃	10.66	6.36	3.12	2.28
V ₃ M ₄	14.16	6.92	3.42	2.43
S.Em±	0.5357	0.1884	0.1799	0.1808
C.D. at 5%	1.5637	0.5500	0.5251	0.5277

5. Acknowledgement

Arora PK acknowledgements to the Science and Engineering Research Board (SERB), India to support his work under the scheme of Young Scientist.

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Advances in Biotechnology

Chapter 5

Development of Antibody Reagents against Stem Cell Markers: Expression Pattern of Human Pluripotent Stem Cell Marker Nanog and Germ Cell Marker Fragilis by Rat Testis

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Abstract

The relative rarity of stem cells in embryonic or circulating bloods of the adult has human prompted the scientific community to search for markers that are biologically unique to stem cells and could be used for identification of cell type. Stem cell markers can be either on the cell surface or part of an intracellular signaling pathway associated with cell proliferation, differentiation or cell-cell interaction. Further a combination of markers facilitates identifying a particular cell type with consistency. Keeping this in view, attempts were made to develop antibodies against Germ cell marker Fragilis and embryonic stem cell marker Nanog. Antibodies were developed against synthetic peptides made from carefully chosen epitopic region from sequences available in NCBI. Antibodies were validated by Western Blot using various cell lines and tissues. Immuno histochemical staining of adult rat testis with anti-Nanog showed the presence of pluripotent stem cell factor in the tails of the spermatozoa and suggests that NANOG expression was markedly up regulated in the gonocytes shows stem cell potential in adult testis. This confirms that adult rat testis has a population of stem cells that can be act as biomarkers.

Also the strong expression of NANOG protein in adult rat gonocytes raises

the possibility of using the gonocytes to establish a cell line that could have germline characteristics. Together, these results suggest that PGCs maintain expression of pluripotent stem cell markers during and after sexual differentiation of the gonad.

Keywords: stem cell marker; testis; fragilis; nanog

1. Introduction

In the face of extraordinary advances in the prevention, diagnosis and treatment of human diseases, devastating illness such as heart disease, diabetes, cancer and disease of the nervous system, such as Parkinson's disease and Alzheimer's disease, continue to deprive people to health, independence and well-being. Research in human development biology has led to the discovery of human stem cells including embryonic stem (ES) cells, embryonic germ (EG) cells and adult stem cells. The stem cells need to be identified by the researchers to explore its significance and potential use in the field of medical research, and antibodies reagents are used to categorize the stem cells by identifying the marker proteins expressed by the stem cells.

Stem cells are originated from the inner cell mass of the embryo and are present in all stages of development from pre-implantation embryos through adulthood. They are characterized by the ability to renew themselves into a diverse range of specialized cell types. Pluripotent stem cells have the potential to develop into many different cell types in an animal which can only be isolated from the inner cell mass of embryos or from the gonadal ridge [7]. In contrast, multipotent stem cells have the ability to self-renew and to differentiate into two germ layers and can be isolated from adult animals [4]. Human stem cells hold out the potential to almost unimaginable medical breakthroughs and are prospective source of differentiated cells for a variety of therapeutic uses [15]. Thus they serve as a sort of repair system for the body. Transcription factors of embryonic stem cells play a central role in the regulation of pluripotency and self-renewal. These factors include the homeoprotein Nanog [6,16], Sox-2 [1] and the POU domain-containing protein Oct-4 [21-23], and are expressed in high levels in pluripotent cells and are considered markers of primitive stem cells. They regulate many genes in a cooperative fashion [3].

Nanog is a homeodomain-bearing transcription factor, which is reported to be transcribed in pluripotent stem cells in mouse preimplantation embryos, ES, embryonic germ and embryonal carcinoma cells [6,16,24] and in monkey and human ES cells [10]. Nanog is essential to maintain the pluripotency of cells as shown by the loss of pluripotency in Nanog-deficient ES cells and by the loss of epiblast cells in Nanog-null E5.5 embryos [16]. NANOG expression begins in the compacted morula [11] and quickly down regulated thereafter and only remains in the primordial germ cells (PGC) [11,25].

In recent years the male germ line stem cells have received a great deal of attention in

recent years, as it is possible to isolate and culture them in vitro [18,20]. These cells can be genetically modified and they further differentiate to spermatozoa following transplantation into a recipient testis, eventually producing transgenic offspring [17,19]. From all these reports it can be hypothesized that if expression of Nanog remains in PGCs then there must be some expression in the adult male germ cells which can be used to produce efficient transgenic breeds. There are no reports available showing the expression of Nanog in adult testes. The present study was carried out with the objective to check the expression pattern of Nanog in adult rat testis.

2. Material and Methods

2.1. Isolation of immunogens

The protein sequences deduced from NCBI database were analyzed using Clone Manager, a software program for selecting probable immunogenic peptide sequences. The peptides chosen were ES cell marker Nanog and Fragilis a germ cell marker. For synthesis of peptide; portion of amino acids 1-50 of human Nanog and portion of amino acids 1-50 of mouse Fragilis were used as the immunogen. Peptides were synthesized at Imgenex Sandiego, USA.

2.2. Immunization and collection of immune sera

New Zealand white rabbits were immunized with synthetic peptide conjugated to KLH. In general 200 µg of primary dose followed by three boosters containing half of the primary dose were given. Rabbits were bled; serum was separated by centrifugation at 5000 rpm for 10 min. Aliquots were stored at -20°C until purification.

2.3. Screening of sera by ELISA

Indirect ELISA was performed to check antibody titer in the sera. Free immunogens were coated in 96 well flat bottom plates with 100 ng /well, incubated for 2 hour at room temperature and kept at 4°C overnight. Rabbit serum was added at 1:1000 dilutions and anti-rabbit IgG conjugated to alkaline phosphatase (Jackson) were used at 1:5000 dilution. pNPP was used to develop the color. Absorbance was measured at 405 nm.

2.4. Purification of sera by affinity chromatography

For purification, 10ml of Western positive sera was taken and diluted with TBS buffer (100 mM Tris, 150 mM NaCl) at 1:1 before purification. Protein G column matrix was used for affinity purification. Bound antibodies were stripped off from the column by high Ph diethanolamine buffer (pH 11.5). The eluted sample was neutralized by 1M Tris pH 7. Finally a low pH Glycine buffer (100 mM Glycine, pH 2.7) was used to strip remaining antibodies followed by neutralization with 1M Tris pH 7.5. In both cases protein in the sample was measured by

Bio-Rad protein assay kit. All the fractions containing antibodies were dialyzed overnight with TBS containing 0.05% sodium azide. The samples were concentrated and stored at -70°C for longer duration.

2.5. Assessment of sera by Western Blot analysis

Mouse embryonic cell line (3T3) was obtained from ATCC and maintained by using standard protocol. Cell extracts of 3T3 were prepared by lysing the cell using Radioimmuno precipitation assay (RIPA) buffer and 200 μg of extracted protein mixed with SDS sample buffer (1:1) loaded onto a single well and resolved in SDS PAGE under reducing conditions [13]. Separated proteins were electro blotted onto a PVDF membrane by Bio-Rad electroblot apparatus. Electroblotting was performed for 90 min at a constant voltage of 50V. The membrane was stained with amidoblack (Sigma) and destained with 10% methanol and 10% acetic acid. The detection of immunogens in cell line was made by immunostaining using the antibody raised against that immunogen served as primary antibody at 1:5000 dilution and HRPO conjugated Goat anti-rabbit IgG (Jackson) at 1:10,000 dilution served as secondary antibody. Visualization of the antigen-antibody binding was made by using chemiluminiscencent solution (Pierce). Bands were depicted on the Hyper-Film (Kodak) by developing in the dark room.

2.6. Collection of rat testis and slide preparation

Testis tissues were collected from 2 months old rat in 4% Para-formaldehyde and preserved for 24-48 hrs. Then the tissues were washed with PBS with three changes of 5 minutes each and dehydrated by keeping in increasing percentages of alcohol (30% for 1 hour, 50% for 2 hours, 70% for 2 hours, 95% for 1 hour and 100% 2 hours) and Xylene with 2 changes 30 minutes each. After dehydration, infiltration was done by incubating the tissues in molten paraffin (Fisher Scientific) at $58-60^{\circ}\text{C}$, with 3 changes each for 30 min. Tissue embedded paraffin blocks were prepared by using molten paraffin in L shaped metal frames and left over night. The paraffin blocks were then fixed to fine plastic cassettes (Sakura). Tissue section ribbons (5 μm thickness) are cut using a microtome, kept in warm water maintained at $42-47^{\circ}\text{C}$ for proper stretching and lifted with poly-L-lysine coated slides (Fisher Scientific) and dried overnight.

2.7. Peptide blocking

Antibody dilution conventionally taken as 1:2000 diluted the required amount of antibody (2.3 μl for 5ml) in 100 μl TBST. This served as positive control. Antibody: Peptide: 1:25 or 1:40, taken depending on the observed band in the test bleed experiment. On an average our sera has a maximum of 10 mg/ml of antibody (data taken from Western Blotting and ELISA). If blocking is performed on purified protein then its concentration was estimated at 280 nm. For antibody concentration 2mg/ml, 50 μg of peptide Mixed antibody and peptide and kept

on a horizontal shaker for 2 hour at room temperature. Tubes containing antibody and peptide solution were incubated at 37°C for 2 hour. After incubation the tubes were centrifuged at 4°C for 15 minute at 8000 rpm. Supernatant was carefully removed leaving at least 20 µl at the bottom to avoid disturbing invisible immune complexes. Then routine Western Blotting was performed.

2.8. Immunohistochemistry

Slides were deparaffinized by immersing in Xylene with two changes for 5 min each and rehydrated by immersing in decreasing grades of ethanol and distilled water for 1 min each. Antigen retrieval was done by autoclave method and washed with Tris buffer saline having 0.1% Triton X100 three times for 15 minutes. Slides are kept in 1% H₂O₂ to quench the peroxidase. Blocking was done with 5% BSA (Sigma) and normal Goat serum in tris buffer saline with 0.1% Triton X100 (Sigma). Normal rabbit serum as negative control and experiment rabbit serum were diluted (1:1000) using the blocking solution, added to the tissue sections on the slides and incubated overnight at 4°C. Next day the slides were washed with TBST and Goat anti-rabbit IgG biotin conjugate (1:1000) was added and incubated for 30 minutes at room temperature. Avidin-Biotin complex (Vector Labs) was prepared as per the instructions of the manufacturer, added to the slides after washing and incubated for 30 minutes at room temperature. Then color development was done by using Nova red substrate (Vector Labs) and the slides were counterstained with hematoxylin for 5-10 seconds. Then slides were dehydrated with increasing grades of alcohol and Xylene and mounted with cover slip by use of DPX.

3. Results and Discussion

3.1. Results

ELISA results showed seropositivity against the synthetic peptides when incubated with the immunized rabbit sera. The expression level of anti-Nanog serum was more than anti-Fragilis serum (Fig.1). ELISA results gave preliminary confirmation of the presence of the antibody against the synthetic peptides in the sera.

Western Blot results showed that the antiserum developed against Nanog and Fragilis detected a 35 kDa and 16 kDa bands respectively in cell extract of 3T3 cell line which used as a positive control to confirm the presence of antibody against the immunized peptides (Fig.2). For Fragilis, a 16 KDa band was detected in the Mouse embryonic cell 3T3. It belongs to the highly conserved fragilis protein family. It is an interferon-inducible transmembrane protein consisting of 2 putative transmembrane domains. It is associated with germ cell specification and development. It is located on the cell surface and might be important in mediating interactions amongst germ cells and their surrounding neighbors. It is expressed in early embryos and

nascent primordial germ cells. These proteins might be involved in anti-proliferation signaling and homotypic cell adhesion [14]. Antibody development against a few embryonic stem cell markers will meet the need for identification of ES cells. Therefore in this study antibody raised against stem cell marker and the expression of the antibodies were validated by immunocytochemistry using mouse embryonic cell line 3T3. Antibodies were developed against synthetic peptides made from carefully chosen epitopic region from sequences available in the National Center for Biotechnology Information [<https://www.ncbi.nlm.nih.gov>].

In immunohistochemistry analysis, the expression of human NANOG in the tails of spermatozoa of rat testes was detected, which are easily distinguished by their size, topological position and morphology (Fig. 3a). NANOG staining was primarily detected in the nucleus of the stained germ cells and was also weakly detected in the cytoplasm of a few cells. NANOG was strongly expressed by differentiated germ cells (spermatocytes) and weak expression was detected in spermatogonia. NANOG expression was observed in the nuclei of most spermatocytes, and following their differentiation to spermatids, it was translocated to the perinuclear region and cytoplasm (Fig. 3b).

3.2. Discussion

Western blot analysis showed that the anti-NANOG antibodies could identify proteins of definite sizes in the adult testis. The smaller fragment in NANOG immunoblots indicate towards truncated protein that may have arisen from the cytoplasmic translocated NANOG. Although amino acid sequences of NANOG (Accession Number(s): NP_079141.1; Human GeneID(s): 79923) show 88% homology to mouse NANOG (accession no. Q5TM83) our results show that NANOG is expressed in the primitive germ cells of adult rat testis. The pattern of expression was strikingly similar to that of the DBA binding in germ cells reported previously [9]. Strong NANOG expression, like DBA staining, was observed in undifferentiated germ cells of adult rat testis and it showed that it is not progressively lost with increasing age reported previously [8]. At adult age, many germ cells showed translocation of NANOG protein from the nucleus to the perinuclear region and cytoplasm.

NANOG expression was not only specific to germ cells but was also observed in Sertoli and interstitial cells of adult rat testes. NANOG expression was observed not only in PUC matrix cells but also in porcine fibroblast cell lines [5]. NANOG transcripts were detected not only in the ICM of porcine embryos but also in epiblast-derived somatic cell lines and adult organs [2]. Therefore, NANOG expression, unlike DBA binding, could not be used as a specific marker of pig undifferentiated germ cells in the testis or in culture. Nevertheless, the expression pattern of NANOG in the postnatal pig testis is a strong indication that it has a role in stem cell activity of undifferentiated germ cells. NANOG expression was localized mostly in germ cells in the adult rat testes, suggesting that rat gonocytes possess stem cell potential.

The cytoplasmic translocation of NANOG protein in the germ cells with increasing age is interesting because NANOG is a homeodomain-bearing transcription factor and nuclear localization of NANOG is essential for its function. Nuclear export of NANOG may result in the loss of function and quite possibly degradation by cytoplasmic machineries. Down-regulation of NANOG expression in goat blastocyst occurs by sequestration/degradation utilizing a nucleolar mechanism [12]. Although we failed to identify any such finding in this study, this discrepancy may be due to species difference or a cell-type-specific mechanism that exists in the testis. Interestingly NANOG expression was detected in somatic cells, such as Sertoli and interstitial cells in the adult testes. NANOG expression was detected not only in a few undifferentiated germ cells but also in differentiated germ cells such as spermatocytes in 10- and 20-week-old testis sections. In 20-week-old testis sections, the expression of NANOG protein was variable in the differentiated germ cells, suggesting that the expression pattern was much more dynamic. This is contrary to an earlier finding in mice, in which NANOG expression was restricted to PGCs and no germ cells stained positive in the adult testis or ovary [25]. This suggests that the expression pattern of NANOG varies among species. Intriguingly, NANOG protein localization changed from the nucleus to the cytoplasm in the differentiating germ cells in rat testes. Nevertheless, our finding that NANOG is expressed in the nuclei of spermatocytes and spermatids suggests that these proteins have some role in the meiotic stage of germ cells. Our finding suggests that NANOG expression was markedly upregulated in the gonocytes shows stem cell potential in adult testis. These results suggest that porcine gonocytes could proliferate *in vivo* and NANOG is essential for determining the stem cell potential of gonocytes. Nevertheless, these findings confirm that adult rat testis has a population of stem cells that can be act as biomarkers.

In conclusion, pluripotency-specific protein, such as NANOG, showed a unique expression pattern in developed adult rat testes. NANOG expression was abundant in primitive germ cells of adult testis such as gonocytes. NANOG protein strongly expressed in differentiated germ cells such as spermatocytes and weakly expressed in spermatogonia. The strong expression of NANOG protein in porcine gonocytes raises the possibility of using the gonocytes to establish a cell line that could have germline characteristics.

Furthermore, this study has provided a number of interesting findings regarding the development and differentiation of rat germ cells in the adult testis, including a very small population of PGCs with a molecular signature expressing NANOG. Being able to identify the molecular signature of PGC will provide valuable tools for isolating this distinct population of germ cells for cytological and molecular analysis. Of great interest is the period of development in germ cell fate studied here since it is the time at which human germ cells demonstrate the ability to form EGCs and ECCs. This information will be critical in future studies for identifying the progenitor cells of EGCs and ECCs, as well as the mechanisms involved in their

conversion.

4. Figures

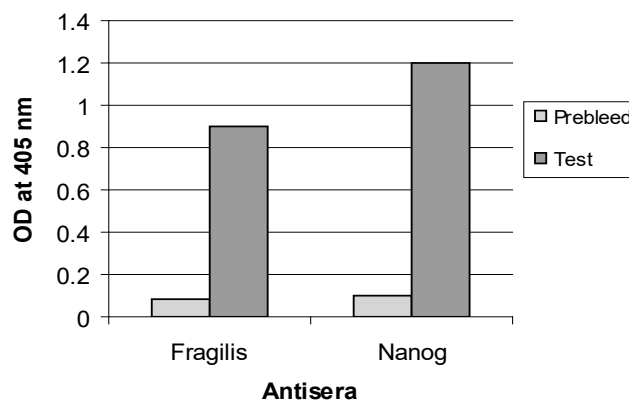


Figure 1: ELISA showing variation in absorbance at 405 nm to check the developed antibody titer in the sera. performed to check antibody titer in the sera. Data of ELISA showed the expression level of anti-Nanog serum was more than anti-Fragilis serum.

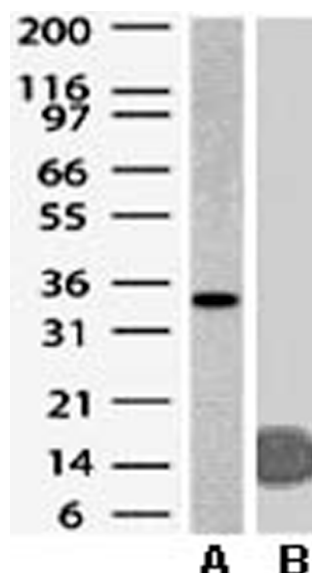


Figure 2: Western blot analysis of developed antibody against the immunized peptides. SDS-PAGE separated proteins were blotted onto a PVDF membrane and incubated with the immunized rabbit sera. Lane A, Western Blot results against Nanog; Lane B, Western Blot results against Fragilis in cell extract of 3T3 cell line. Molecular weight of standard protein is indicated on left margin (kDa).

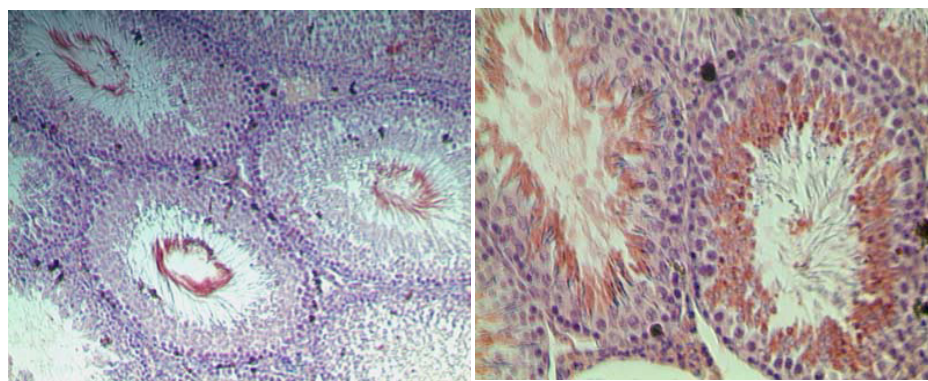


Figure 3: Immunohistochemistry analysis (a) Expression of NANOG in the tails of spermatozoa of rat testes, distinguished by their size, topological position and morphology; (b) NANOG expression in the nuclei of spermatocytes and their differentiation to spermatids.

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Advances in Biotechnology

Chapter 6

Production of Recombinant Proteins from Plants - An Overview

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1. Introduction

Since time immemorial, mankind has been developing crops to suit their needs by selective breeding. Cultivar development within a species has been done by selecting alleles present in a population. Sexual recombination allows allelic recombination during meiosis resulting in plants which can be selected with novel traits. The primary gene pool allows for crosses to be easily made, the hybrids will be fertile and chromosome pairing is normal, thus allowing Mendelian segregation of traits. The biological species is divided into two categories, (i) subspecies composed of lines used for agronomic use and (ii) subspecies, which contains weedy or wild relatives of subspecies A. The secondary gene pool refers to all biological species, which cross with a different species. Although mating and gene transfer are possible, there will be hybrid sterility or aberrations in chromosome pairing. Hybrids may be weak and may not reach maturity. The recovery of a desired phenotype will not be possible or might be difficult even when traits are crossed back. In the tertiary gene pool, although crosses are possible with the crop of interest, hybrids are lethal or completely sterile. Hybrids can be rescued by embryo rescue, grafting, the application of mutagens to break apart chromosomes or by doubling the chromosome number. The quaternary gene pool does not allow any transfer of DNA between the crop of interest or other organism by mating and sexual recombination. In this case, genetic transformation would be the only way to insert a DNA sequence from any biological or synthetic source into the crop plant of interest. The DNA can be chimeric where different segments e.g. the promoter, gene of interest and terminator can be taken from any source and put together in a functional manner so that the DNA cassette is expressed once transferred into the genome of the crop of interest.

Genetic engineering of plants have been done from the early 80s. This has become possible because of the emergence of new technologies which enables the incorporation of genes

into plant cells or organelles for transient as well as stable expression and exploiting the totipotency of the plant cell. The traits introduced into plants can be for increasing the agronomic traits like improved nutritional quality, altered metabolic pathways for nutraceutical production, reduce the maturation time of crops or altering the protein profile in food crops or develop plants as expression platforms for the production of recombinant therapeutic proteins. Plants produce large biomass hence plants can produce large quantities of recombinant proteins at low cost, this would be commercially viable. At the same time, care has to be taken about the contamination of food crops or products because of transgene integration and expression. Humans may develop immunotolerance due to oral dosage of vaccine as well, also illegal or unethical cultivation of GM plants have to be prevented. Hence regulatory issues have to be stringent.

Stable nuclear transformation involving the incorporation of exogenous gene into the nuclear genome of the plant can be done by either Agrobacterial infection or biolistic gene delivery. As a result of stable gene delivery, production costs are decreased and become more simple. The exogenous proteins thus produced can be targeted to various organelles for standard eukaryotic post translational modifications. For rapid production of large amount of recombinant proteins, transient expression is the best method. One method of achieving this would be by using viral coding sequences via *Agrobacterium tumefaciens*. The other is by agro infiltration, i.e., infiltration of a suspension of recombinant *Agrobacterium* into plant tissue [1,2]. This has been specially developed as a rapid and high yield strategy for the production of clinical grade biopharmaceuticals [3,4]. Plastid transformation is another efficient alternative. The major advantage here is that the public anathema against GM plants can be reduced; the transgene cannot be transferred as pollen does not contain chloroplast [5]. High yield of recombinant therapeutic proteins have been obtained (3-6% of TSP) using tobacco chloroplast transformation [6-15].

Using biotechnology, transgenic plants have been used to produce therapeutic proteins, edible vaccines, antibodies for immunotherapy and proteins for diagnostics [16-30]. In all these cases, the therapeutics or proteins expressed in the plant tissues are either purified and used or the plant tissue is processed to a form which can either be applied topically or taken internally. Fermentors and bioreactors can be replaced with green houses with appropriate biological containment or plant growth chambers which will reduce upstream facility. Plant tissues can be processed for oral delivery of edible proteins which will reduce downstream processing too.

2. Current Status

The first recombinant plant-derived pharmaceutical protein, human serum albumin, was produced in transgenic tobacco in 1990. The concept of plant-based expression has since ex-

panded to include industrial enzymes, blood components, cytokines, growth factors, hormones, therapeutics such as antibodies, and human and veterinary vaccines [31,32] leading to federal approval (US Department of Agriculture) of a vaccine against Newcastle disease developed by Dow AgroSciences and manufactured in genetically engineered plant cells.

Literature survey over the years for plant produced antigens or vaccines describe the expression of different vaccine antigens in different plant expression systems (**Table 1**). For the commercial production of pharmaceutical products the plants chosen should express the proteins with high efficiency in a large scale. Also such systems need to gain safety and regulatory approval.

The antigen can be expressed in the cytoplasm and remain there or localized into any plant compartments like vacuole, chloroplast or Endoplasmic Reticulum (ER) by means of specific signal peptides. However the stability of the expressed antigen in the appropriate compartment has to be checked. Also, the level of protein expression for economical extraction, apparently calculated to be 1% of TSP, is very rarely realized [33].

Table 1: List of various vaccine antigens expressed in plants

Protein	Transgenic species	References
Human growth hormone	<i>Nicotiana tabacum</i>	Barta et al. 1986 (34)
Human albumin	<i>Nicotiana tabacum</i>	Sijmons et al. 1990 (35)
Human albumin	<i>Solanum tuberosum</i>	Sijmons et al. 1990 (36)
Epidermal growth factor	<i>Nicotiana tabacum</i>	Higo et al. 1993 (37)
Human interferon α	<i>Oryza sativa</i>	Zhu et al. 1994 (38)
Eritropoietin	<i>Nicotiana. tabacum</i>	Matsumoto et al. 1995 (39)
β -casein	<i>Solanum tuberosum</i>	Chong et al. 1997 (40)
α and β hemoglobin	<i>Nicotiana tabacum</i>	Dieryck et al. 1997 (42)
Human mucarinic cholinergic receptors	<i>Nicotiana tabacum</i>	Mu et al. 1997 (43)
Interleukine 2 and 4	<i>Nicotiana tabacum</i>	Magnuson et al 1998 (44)
Human α 1-antitrypsin	<i>Oryza sativa</i>	Terashima et al. 1999 (45)(45)
Somatotropine	<i>Nicotiana tabacum</i>	Leite et al. 2000 (46)
Collagen	<i>Nicotiana tabacum</i>	Ruggiero et al. 2000 (47)
Lactoferrin	<i>Solanum tuberosum</i>	Chong et al. 2000 (48)
Somatotropin	<i>Nicotiana N. tabacum</i>	Staub et al. 2000 (49)
Human acetylcholinesterase	<i>Lycopersicon. esculentum</i>	Mor et al. 2001 (50)
Bovin aprotinin	<i>Zea mays</i>	Azzoni et al. 2002 (51)
Human collagen	<i>Nicotiana N. tabacum</i>	Merle et al. 2002 (52)
Lactoferrin	<i>Nicotiana N. tabacum</i>	Choi et al. 2003 (53)
Interleukine 18	<i>Nicotiana N. tabacum</i>	Zhang et al. 2003 (54)
Human granulocyte-macrophage colony stimulating factor	<i>Oryza sativa</i>	Shin et al. 2003. (55)

Epitope of <i>C. diphtheriae</i> , <i>B. pertussis</i> , <i>C. tetani</i>	<i>Tomato</i>	Soria-Guerrra et al. 2007, 2011 (56,57)
E6 and E 7 of HPV	<i>Tomato</i>	Paz De la Rosa et al. 2009 (58)
PA of <i>Bacillus anthracis</i>	<i>Tobacco</i>	Lee et al. 2011 (59)
VP2 of CPV	<i>Tobacco</i>	Ortigosa et al. 2010 (60)
FMDV	<i>Nicotiana. benthamiana</i>	Andrianova et al. 2011(61)
NP of H1N1 Influenza A virus	<i>Vigna unguiculata</i>	Meshcheryakova et al. 2009 (62)
Hepatitis B surface antigen	<i>Maize</i>	Hayden et al. 2014, 2015 , Shah et al 2015(25,26,63)
Cholera toxin subunit B	<i>Maize</i>	Karaman et al. 2012(64)
Human epidermal growth factor	<i>Nicotiana. benthamiana</i>	Thomas and Walmsley 2014(65)
HPV type 16 L2 epitope,type 8, L1	<i>Nicotiana benthamiana</i>	Cerovska et al 2012(66) Waheed et al 2011(67,68) Matic et al 2012(69)

Stable integration, selection and maintenance of transgenic plants take time. Even then, the high level of expression is not maintained in subsequent generations. This might be due to post transcriptional gene silencing or si RNA mediated gene silencing. Expression can also be varied in nuclear transformation because of meiotic segregation. *Li et al* [70] reported the stability as well as immunogenicity of human rotavirus VP 7 protein expressed in transgenic potato for 50 generations. However this is the only report where expression study has been done for so many generations.

The steps involved in the production of recombinant proteins from plants include: (i) choice of the host species and optimization of coding sequence of the target gene in relation to the host, (ii) selection of expression cassette and creation of the expression vector, (iii) integration of the gene construct into the plant genome and regeneration of plants expressing the desired protein, (iv) identification and stabilization of the plant line for commercial production of the recombinant protein.

Selection of the host plant depends on the type of protein, i.e., the form of the recombinant protein which is to be finally used. The life cycle of the host, biomass yield, containment and scale-up costs are other deciding factors. Success largely depends upon the understanding of species- or tissue-specific factors that affect the recombinant product. Self-pollinating species are advantageous over cross pollinated plants as the spread of transgene through pollen can be reduced. This can also be addressed by using plants which can be grown in containment, e.g. Tomato which can be grown in green houses. Further, the use of plant cell cultures addresses the issue of containment where dedifferentiated cells such as in calli or cell suspensions are used and can be grown on industrial scale using fermenter.

Expression via seed specific promoters is preferred in many cases as seeds accumulate a large amount of protein, purification of proteins from seeds would also be easier. Recombinant

proteins can be stored for a longer time in storage and normally do not degrade under ambient temperature. The only disadvantage is that it takes a long time for seed set depending on the lifecycle of the plant, hence transgene expression can be assessed only then.

Several cereals including rice, wheat, barley and maize have been investigated [71,72,73]. The first plant derived commercialized product was produced in maize [74,75]. Cereal plants have been adopted as a production platform by the plant biotechnology enterprises like Ventria Bioscience ([http:// www.ventria.com](http://www.ventria.com)). A rice based cholera vaccine Muco-Rice CTB was shown to be stable at RT for 8 months, as well as resistant to pepsin digestion [75]. An ETEC subunit vaccine produced in soybean seeds was found to be stable for 4 years [76]. *Hayden et al* [24,25,26] biochemically and biophysically characterized maize derived HBsAg and showed that oral delivery of wafers made from HBsAg-expressing maize germ induces long-term immunological systemic and mucosal responses.

Various parameters have to be considered and optimized for commercial recombinant protein expression in plant cells

2.1. Internal factors

- a. Codon usage
- b. RNA structure
- c. Regulatory elements
- d. Fusion protein
- e. Specific tag
- f. Signal peptide

2.2. External factors

1. Glycosylation
2. Proteolysis
3. Response to unfolded protein
4. Activity of chaperons
5. Compartmentalization

2.3. Product nature

1. Isolation
2. Purification
3. Storage

2.4. Different stages of transformation

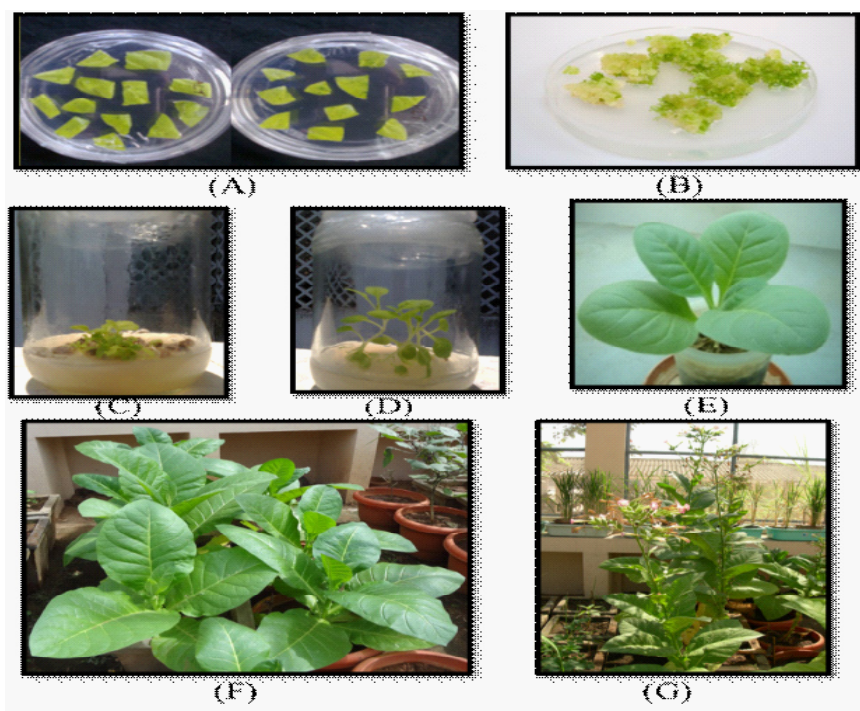


Figure 1: Different stages of transformed tobacco plant.

(A) Transformed Explants. (B) Callus induction from transformed leaflets. (C) Multiple shoots induction from callus. (D) Rooted transformed plants. (E) Transformed plant set for hardening. (F) Matured transformed plant. (G) Transformed plants-flowering.

3. A few examples where success has been achieved in recombinant protein production in Plants is mentioned below:

3.1. Plant made antibodies

There are reports of many plant produced antibodies in literature with applications ranging from diagnostics [30,77,78]; to cancer treatment [79,80,81,82,83,84]; prevention of tooth decay [86,87]; prevention of plant disease [87,88] and preventing sexually transmitted diseases [73,90,91]. Different subclasses of antibodies (IgG, IgM) have been expressed in different plant species but *Nicotiana* species predominate [89,93].

He et al. [89] demonstrated that WNV DIII antigen (West Nile Virus) and E 16 monoclonal antibody were produced at high levels, could be purified easily and were effective in identifying WNV and also in detecting human IgM response to WNV detection. *Ganapathy et al* [30] reported the efficacy of plant produced Wb SXP1 as comparable to *E.coli* produced WbSXP1 in the diagnosis of Lymphatic filariasis, a neglected tropical infectious disease. Immunological screening using clinical sera from patients indicates that the plant-produced protein is comparable to *E. coli*-produced diagnostic antigen. These reports further substantiated that plants could serve as cost effective platform for diagnostic protein production, especially towards infectious and parasitic diseases which are prevalent in tropical countries.

Advanced plant and mammalian glycosylation differ in regard to types of sugar moi-

eties added and the type of linkages [95]. This difference in glycosylation might result in the identification of antibodies of non-human origin being seen as antigen by patients [96,129]. Plant specific glycosylation can also induce immune response. Plants now have been genetically modified to mimic typical animal glycosylation pattern by either inactivating the native enzymes or by expressing heterologous enzymes responsible for mammalian like glycosylation [97,98, 99,100].

Table 2: Examples of pharmaceutical antibodies expressed in plants

Antibody	Antigen specificities	Transgenic species	Application	References
IgG and SIgA	<i>Streptococcus mutans</i>	<i>N. tabacum</i>	Therapy (topical)	Ma <i>et al.</i> 1998(80)
IgG	Colon cancer antigen	<i>N. tabacum</i>	Therapy/Diagnostics	Verch <i>et al.</i> 1998(79)
IgG	Herpes Simplex virus HSV-2	<i>G. max</i> , <i>O. sativa</i>	Therapy (topical)	Zeitlin <i>et al.</i> 1998(117)
Humanized IgG	Respiratory Syncytial virus	<i>Z. mays</i>	Therapy (inhalation)	EPIcyte
IgG	<i>Clostridium difficile</i>	<i>Z. mays</i>	Therapy (oral)	EPIcyte
IgG (topical)	Sperm	<i>Z. mays</i>	Contraceptive	ReProtect LLC, MD
IgG	Hepatitis B antigen	<i>N. tabacum</i>	Therapy/Diagnostics	Valdes <i>et al.</i> 2003(118)
Diabody	Carcinoembryonic Antigen (CEA)	<i>N. tabacum</i>	Therapy/Diagnostics	Vaquero <i>et al.</i> 2002(119)
scFv	Non-Hodgkins(NHL) Lymphoma	<i>N. tabacum</i>	Vaccine	McCormick <i>et al.</i> 1999,2008 2011(120,121,122)
scFv	Carcinoembryonic Antigen (CEA)	<i>N. tabacum</i> <i>P. sativum</i> , <i>L. esculentum</i> , <i>O. sativa</i> , <i>Triticum sp</i>	Therapy/Diagnostics	Stoger <i>et al.</i> 2002(91)

Two successful plant made antibodies have made to human clinical trials. Planet Biotechnology Inc. produced the world's first clinically tested antibody CaroRx™ in tobacco which specifically binds to bacteria that cause tooth decay and prevent adhesion of the organism to tooth. This is currently approved for sale as a medical device in European Union. With support from the National Institute of Allergy and Infectious Diseases (NIAID), Planet Biotechnology Inc have also developed an immunoadhesin for treatment and prevention of anthrax (PBI-220) and have tested it successfully in anthrax-infected monkeys as a therapeutic with an 80% survival rate when treatment is started after disease symptoms appear.

Middle East Respiratory Syndrome (MERS) is a recently emerged disease caused by the MERS coronavirus (MERS-CoV) endemic to the Arabian Peninsula. It has already appeared in seven Middle Eastern countries and has traveled to European countries and South Korea as well. MERS-CoV causes a pneumonia-like disease that has a fatality rate approaching

40%. Planet Biotechnology Inc have created and produced in green plants an immunoadhesin (DPP4-Fc) that has improved binding to MERS-CoV and have shown that it prevents the virus from infecting human lung cells in culture. In June 2015, the company has been awarded a Phase II SBIR grant from NIAID to support development of this candidate immunoadhesin..

In July 2008, Large scale Biology Corp reported the success of first human clinical trials testing of a plant made vaccine against cancer. A transient expression system produced patient specific recombinant idiotype vaccine against follicular B cell Lymphoma in tobacco. 16 patients immunized with their own individual therapeutic antigen showed no serious adverse effects, 70% of the patients developed cellular or humoral responses, 47% developed antigen specific response. In 2009, Bayer started the clinical development of this plant made antibody vaccine submitting Phase I study protocol to US FDA .LSBC and Bayer Crop Science also had an agreement for research and development of plant based expression of Lysosomal acid Lipase (LAL) Plant based human enzyme which could breakdown lipids, supposedly a breakthrough for Orphan diseases but this fell through and LSBC has now filed for bankruptcy.

3.2. Plant made vaccines for viral and bacterial diseases

3.2.1. Respiratory infections

3.2.1.1. Influenza is a serious respiratory disease caused by influenza viruses. It is the root cause of pandemics worldwide and prevention of influenza is a challenge as rapid mutations in subsequent generations cause antigenic variation in haemagglutinin (HA) [103]. HA is a surface glycoprotein of the influenza virus which plays a key role in viral infectivity and pathogenesis. HA is also the main target for generating protective immunity against influenza virus [104, 105]. Recent outbreaks caused by the new H1N1 swine influenza virus infected a large number of humans and raised significant concerns as a global pandemic.

The virus, A(H1N1) pdm09, which is a triple reassortant with genes acquired from swine, avian and human influenza viruses , was first detected in humans in the United States in April 2009 [105]. The highly pathogenic avian influenza A virus (H5N1) caused pandemics in poultry. Rapid evolution of the virus and easy mode of spread leads to chances of global human infection of [106]. The antigenicity of the HA protein depends on its proper folding and trimerization. It also requires multiple post-translational modifications including disulphide bond formation and glycosylation [107]. The expression of HA without its transmembrane domain from the A/Hong Kong/213/03 (H5N1) influenza virus strain fused with an ER-targeting signal at the 5' end and the HDEL ER retention motif at the C-terminus resulted in its high-level accumulation in the ER (140 lg/g fresh weight, FW), N-glycosylation, protection from proteolytic degradation and long-term stability in Arabidopsis. Oral administration of freeze dried leaf powder expressing this HA antigen and the adjuvant saponin together elicited high levels of HA-specific mucosal IgA and systemic IgG responses in mice. It also led to the de-

velopment of neutralizing antibodies and cellular immune responses, conferring protection against a lethal viral challenge.

Normally trans-membrane domain is essential for the trimerization that is required for HA antigenicity, but plant-based HA without the transmembrane domain still could induce strong HA-specific immune responses in mice [106]. The influenza virus nucleoprotein (NP) is a highly conserved multifunctional RNA binding protein found in many different strains, making it a potential candidate for a universal vaccine. Oral immunization of maize-expressed H3N2 NP induced humoral responses in mice, showing the immunogenicity of this maize-based antigen and its potential as a universal flu vaccine candidate. The NP protein level in T1 transgenic maize seeds ranged from 8.0 to 35 lg/g of corn seed, and this level increased to up to 70 lg NP/g in T3 seeds. Cytokine analysis showed antigen-specific stimulation of IL-4 cytokines in splenocytes from mice orally administered with NP, further confirming a Th2 humoral immune response [108].

3.2.1.2. Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (MTB), a leading bacterial infectious disease has shown reemergence and is proving to be difficult to treat due to development of drug-resistance [109]. In 2013, there were 9.0 million cases of TB, with an estimated 480 000 multidrug-resistant TB cases and 1.1 million HIV-positive individuals with more than half (56%) of these cases in South East Asia and the Western Pacific. Of the patients who suffered from the disease, 25% were in Africa, which suffered the highest rates of cases and deaths relative to the population. China and India accounted for 11% and 24% of total cases, respectively (WHO, 2014a). The 6 kDa early secretory antigenic target (ESAT6) and culture filtrate protein 10 (CFP10) proteins are among the key cell virulence factors of MTB and have been expressed in transgenic carrot plants. ESAT6 makes up <0.056% and CFP10 composes 0.002% of the total storage protein in carrot storage roots. Oral immunization of mice induced both cell-mediated and humoral immune responses [110]. Fusion of the ESAT6 antigen with other tuberculosis antigens, such as Ag85B or Mtb72F (a fusion polyprotein of two TB antigens, Mtb32 and Mtb39), and use of a transmucosal carrier such as CTB, LTB and LipY (a cell wall protein) to facilitate bioencapsulation/oral delivery, and further expression in various plant species (*Arabidopsis thaliana*, tobacco and lettuce), have been attempted [109,110,111].

Compared with nuclear transgenic plants, the expression levels of CTB-ESAT6 and CTB-Mtb72F in transplastomic plants reached up to 7.5% and 1.2% of TSP, respectively, increasing antigen accumulation >100 fold [109]. CTB-ESAT6 was expressed up to 0.75% of the total leaf protein in transplastomic plants. Western blot analysis of lyophilized lettuce leaves stored for up to 6 months at room temperature revealed the stability of the CTB-ESAT6 fusion protein, which retained proper folding. ESAT6 is one of the secreted proteins in the ESX-1 system, which is involved in membrane pore formation during infection. A haemolytic assay indicated the ability of chloroplast-derived ESAT6 to lyse red blood cell membranes in

a dose-dependent manner [109].

3.2.1.3. Dengue, another potential public health problem is increasing in temperate regions due to dramatic climate change. The rice codon-optimized consensus domain III of dengue virus envelope glycoprotein (E) has been fused to the M cell-binding peptide via agroinfiltration with a plant virus-based expression system. Carrying these results a step further, Kim et al [113], generated an Ebola RIC-based DENV vaccine in tobacco plants using a Gemini viral vector expression system and reported its immunogenic properties as a self-adjuvanting dengue vaccine candidate. Previously, Phoolcharoen *et al* [114] reported that plant-expressed Ebola RIC protected mice against a lethal Ebola virus challenge.

The current epidemic caused by the Ebola virus in West Africa has brought attention to a plant-produced antibody cocktail. This cocktail, called ZMAPP, was recently shown to reverse advanced Ebola disease in 100% of tested Rhesus macaques.

4. PubMed listings for therapeutic antibodies produced in plants

Antibody (generic name)	Antibody (commercial name)	Plant species	Reference
Cetuximab	Erbitux	Zea mays	Lentz et al 2012(115)
Nimotuzumab	BIOMAb EGFR, TheraCIM, Theraloc, CIMAher	Nicotiana tabacum	Rodriguez et al 2005 (116)
Palivizumab	Synagis	Nicotiana benthamiana	Zeitlin et al 2013(117)
Rituximab	Rituxan, Mabthera	Lemna minor	Gasdeska et al 2012 (123)
Trastuzumab	Herceptin	Nicotiana benthamiana	Grohs et al 2010(124)
ZMAPP		- Nicotiana benthamiana	Qui et al 2014(125)

5. Gastroenteritis and Hepatitis

Diarrhoeal infectious diseases (DID) are a major problem in developing countries, where poor sanitation prevails and food and water may become contaminated by faecal shedding [126]. Traveller's diarrhoea and cholera, caused by enterotoxigenic strains of *Escherichia coli* (ETEC) and *Vibrio cholerae*, respectively, are two enteric diseases resulting in high mortality, especially in young children [127]. CTB was expressed in maize seeds driven by a c-zein promoter and accumulated in the endosperm of transgenic maize kernels with an expression level of 0.0014% of the total aqueous soluble protein (TASP) in the T1 generation and significantly increased to 0.0197% of TASP in the T2 generation.

Anti-CTB IgG and IgA were detected in the sera and in faecal samples from orally

administered mice, and the mice were protected against CT holotoxin challenge [127]. Inclusion of a heat-stable (ST) toxin into vaccine formulations is required, as most ETEC strains can produce both LT and ST enterotoxins. Transgenic tobacco plants carrying the LTB: ST gene accumulated up to 0.05% of TSP, and oral dosing with transgenic tobacco leaves elicited specific mucosal and systemic humoral responses in mice [128]. In comparison, lettuce chloroplast-derived CTB-AMA1 and CTB-MSP1 expressed up to 7.3% and 6.1% of TSP, which is >100-fold higher expression than from the nuclear genome. CTB-proinsulin expressed up to 70% of TSP, suggesting that the fusion protein, not CTB, determines the expression level. CTB-specific antibody titres were incredibly high (up to 10 000IgA, >800 000 IgG1) and also conferred protection against CT challenge in mice, providing long-term immunity [129].

5.1. Hepatitis B virus

Hepatitis B virus attacks the liver resulting in both acute and chronic disease. Despite the availability of an effective vaccine, it still remains as a major global health problem. Hepatitis B infection causes approximately 780000 deaths every year, split up between acute hepatitis B and from liver cancer and cirrhosis due to chronic hepatitis B infection [130]. In the case of plant-derived HBV vaccines, the first report was on the expression of the small hepatitis B surface antigen (S-HBsAg) in transgenic tobacco plants. The HBsAg produced in transgenic tobacco was antigenically and physically similar to the HBsAg particles derived from human serum and recombinant yeast [131]. The yeast-derived HBsAg is clinically used for HBV vaccination. Afterward, many research groups attempted HBsAg expression in different tissues and plant species, such as tobacco, potato, lettuce, soybean, lupine, maize, tomato, peanut and *Laminaria japonica*. In the transgenic tobacco plant transformed with the S-HBsAg gene controlled by the 35S promoter, expression levels were very low: less than 0.01% total soluble protein and less than 10 ng/g fresh weight in leaf tissues. The expression levels of S-HBsAg in other plant species were not significantly higher; in some species, expression levels were even lower than in tobacco.

To improve vaccine production in plants, the most widely used strategies involve: (1) suitable promoters, such as strong constitutive promoters, tissue-specific promoters and promoters that are inducible by environmental factors; (2) targeting systems to specific organelles; (3) optimized codon usage; (4) alternative polyadenylation signals; (5) increased translation efficiency using leader sequences; and (6) different vector systems. Many HBsAg-overexpressing transgenic plants have been developed using strong constitutive promoters, such as the 35S promoter with enhancer [138,140]. In addition to tissue-specific promoters, the patatin promoter for potato tuber [137,139], globulin promoter for maize seed [146] and fruit-specific promoters [142,143] were used. Specific organelle-, endoplasmic reticulum (ER)-, vacuole- and chloroplast-targeted strategies have also been tried [138,144].

The HBsAg has been expressed in non-edible plants, such as tobacco, using four different expression cassettes: the HBsAg gene without ER retention signal (HBS), the HBsAg gene with ER retention signal (HER), and each gene controlled by the ubiquitin promoter (UBQ) or ethylene forming enzyme promoter (EFE) [145]. The maximum expression level (19.4 ng/g FW of leaves) was observed in EFE-HBS transformed plant growth in vitro, but a higher proportion of the particulate form of the antigen was observed when it was expressed with an ER retention signal. HBsAg has also been expressed in vegetative crops, such as potato, tomato, soybean and lettuce. The expression level of transgenic potato tubers was 1–11 µg/g FW. The highest expression in a tuber was developed using a construct driven by the CaMV 35S promoter with dual enhancers, the tobacco etch virus 50-UTR, and the 30 region from the soybean vegetative storage protein gene [140]. Expression level of HBV-protein in potato was little increased when controlled by the tuber specific promoter [138].

The expression level of the major surface antigen of hepatitis B virus (P-HBsAg) reached 0.003–0.09% of TSP in transgenic potato. Mice produced specific faecal IgA and serum IgG antibodies against P-HBsAg after oral administration [140]. Herbicide-resistant lettuce was engineered to stably express the small surface antigen of hepatitis B virus (S-HBsAg) [141]. The progeny of these plants accumulated up to micrograms of antigen per gram of FW, and the S-HBsAg antigen was able to form VLPs [141]. Oral delivery of lyophilized lettuce containing low levels (100 ng) of VLP-assembled antigen with a long, 2-month interval between priming and boost administrations without adjuvant elicited both mucosal and systemic humoral anti-HB responses at the nominally protective level in mice. Lyophilized material, both as a powdered, semi-finished product or after conversion into tablets, preserved the S-HBsAg content for at least 1 year of room-temperature storage [141]. Bioencapsulated HBsAg expressed in maize reached between 0.08 and 0.46% of TSP and induced serum IgG and IgA in mice after oral administration [146]. High levels of HBsAg were obtained in maize grains, and supercritical fluid extraction (SFE)-treated maize material was used to form edible wafers. After feeding wafers containing approximately 300 lg/g HBsAg, mice showed robust serum IgG (20 000 mIU/mL) and IgA responses. Additionally, all mice administered the SFE wafers showed high sIgA and salivary IgA titres (142 mIU/mL) in faecal material, whereas Recombivax_ Merck & Co., Inc., Whitehouse Station, NJ, USA (an injected commercial vaccine)-treated mice showed no detectable titre [26]. Furthermore, mice boosted with orally administered HBsAg wafers displayed long-term memory mucosally and systemically, as evidenced by sustained faecal IgA and serum IgA, IgG and mIU/mL over 1 year [25]. Freeze-drying of S-HBsAg expressed in lettuce leaf tissue without any purification step was shown to be an important factor affecting S-HBsAg preservation. This reproducible process provided a product with VLP content up to 200 lg/g dry weight. Long-term stability tests showed that the stored freeze-dried product was stable at 4 °C for 1 year but degraded at room temperature. Animal oral immunization trials induced systemic IgG in mice (293 mIU/mL), confirming the preservation of antigenicity

and immunogenicity [161].

The biggest advantage of edible plant-derived vaccines is their easy application to oral delivery. The benefits of plant-derived edible vaccines are as follows: (1) during oral delivery, plant-derived vaccines are protected in the stomach by plant cell wall and slow release in the gut; (2) the plant tissue expressing antigen may be used as raw or dried food; (3) capsules can also be made from partially or fully purified vaccine proteins; (4) no need for cold chain systems for storage and delivery of the plant tissues or extracts; and (5) the plant-derived vaccines are cost efficient compared with traditional vaccines.

Edible plant-derived HBV antigens have been administered by oral injection or feeding in mice with/without adjuvants [138,139,140,146,148]. An oral vaccine candidate has also been administered to human volunteers in small-scale clinical trials without adjuvants. The first trial was administered to three human volunteers in raw lettuce leaves in two doses (0.5–1 µg of S-HBsAg/dose) without the use of an adjuvant. All volunteers responded, with two of them having serum responses in excess of the protective minimum level (10 mIU/mL of serum). However, the antibody levels declined rapidly [151,152,153]. In the second trial, previously vaccinated human volunteers were fed two or three doses of 100 g of raw potato tubers (approximately 1 mg of the S-HBsAg/dose). More than half of the subjects showed increased antibody titers [153]. The animal experiments and trials showed the potential for plant-derived HBV antigens to be used as an oral vaccine for the prevention of HBV, but there remain many problems to be solved for practical application, such as the administration of bulky plant material, declining long-term responses, individual differences in the immune response and the difficulty of defining the antigen dose [154].

The expression level of plant-derived HBV antigen is only 1/20–1/25 of the expression of yeast-derived HBV antigen; however, the expression yield and plant production scale are still increasing [154,155,156]. Tomato can be eaten without processing or cooking, hence it is an attractive candidate to develop as an oral vaccine. According to the study to date, the expression level of HBV antigen was very low as 10 ng/g FW. The maximum titers of anti-HBsAg antibody in serum is 300 mIU using oral application. This antibody yield was high compared to the expression level of HBV antigen in tomato fruits [157]. HBV antigen expression in maize produced much higher levels of antigen, and the palatability and digestibility were better than for potato. In addition, the maize system induced a strong immune response with 4632 mIU of maximum titer by both injection and oral administration [146,25]. This result suggests the possibility of providing a raw material for thermostable formulation at \$0.01 per dose [146]. Plant components such as saponin, flavonoids, and plant oils also function as adjuvants [158,159,160] and help maintain the immune response in the long term [26].

The lyophilization method is an excellent way to increase the stability and shelf life of

the plant-derived vaccines. In the previous study, the storage stability of lyophilized powder form was limited at 4°C [161]. In a recent study, successful long-term storage at 3°C was achieved through improvements in the process [162]. It is easier to control the concentration and standardize antigen doses and process the antigen into a tablet or capsule form using a powdered tissue instead of freeze-drying [141].

5.2. Human papillomaviruses

Cervical cancer caused by HPV infection is the fourth most common cancer among women worldwide and has become a global concern, particularly in developing countries, which bear approximately 80% of the burden [163]. Furthermore, HPV type 16 is by far the most prevalent type and is correlated with 54% of cervical cancer cases [164]. Higher levels of specific IgG and IgA levels (<1 : 1000 for the L1/LT-B group and <1 : 500 for the L1 group) of HPV-16L1 (major capsid protein) were induced when mice were immunized with transgenic tobacco-derived HPV-16L1 combined with LT-B by the oral route [165]. A novel HPV 16L1-based chimericvirus-like particle (cVLP) expressed in tomato plants contains a string of T-cell epitopes from HPV-16 E6 and E7 fusion at the C terminus.

Long-lasting specific IgG antibodies with neutralizing activity were detectable for 12 months after induction by immunization with cVLPs. Efficient long-term protection and tumour growth inhibition were elicited by TC-1 tumour cells expressing HPV-16 E6/E7 oncoproteins, whereas significant tumour reduction (57%) was observed in mice administered with these cVLPs [166].

5.3. Rabies

Rabies virus is an enveloped, negative-sense, single-stranded RNA virus of the genus *Lyssavirus* in the family *Rhabdoviridae*. This zoonotic disease causes acute, progressive, incurable viral encephalomyelitis and is usually transmitted through the bite of an infected animal, resulting in 40000–100000 human deaths annually worldwide [167]. The expression level of the rabies virus glycoprotein protein (G protein) in transgenic maize kernels reached 25 lg/g FW. Neutralizing antibodies in sheep were induced after oral immunization with maize-derived G protein. Further, the degree of protection achieved with 2 mg of maize-based G protein was comparable to that of a commercial vaccine [168]. Transgenic hairy roots of *Solanum lycopersicum* were engineered to express the rabies glycoprotein fused with ricin toxin B chain (rgp-rtxB) antigen driven by a constitutive CaMV35S promoter. The expression level of the RGP-RTB fusion protein in different tomato hairy root lines ranged from 1.4 to 8 lg/g of tissue. A partially purified RGP-RTB fusion protein was able to induce an immune response in BALB/c mice after intramucosal immunization, but the IgG titres were low [169].

5.4. Malaria

Malaria is a mosquito-borne infectious disease caused by *Plasmodium* parasites [170]. Despite decades of intensive research efforts, at present there is no vaccine that provides sustained sterile immunity against malaria. In this context, a large number of targets from the different stages of the *Plasmodium falciparum* lifecycle have been evaluated as vaccine candidates. None of these candidates has fulfilled expectations, and as long as we lack a single target that induces strain-transcending protective immune responses, combining key antigens from different lifecycle stages seems to be the most promising route toward the development of efficacious malaria vaccines. *Plasmodium falciparum* is responsible for the majority of the over half a million malaria deaths per year, which are predominantly children under the age of five that live in indigent African nations [171]. A chloroplast-derived dual cholera and malaria vaccine expressing CTB fused with the malarial vaccine antigens apical membrane antigen 1 (AMA1) and merozoite surface protein 1 (MSP1) accumulated up to 13.17% and 10.11% of TSP in tobacco and up to 7.3% and 6.1% of TSP in lettuce, respectively. The AMA and MSP titres were lower than those of CTB, suggesting that the CTB antigen could saturate the immune system. Significant levels of antigen-specific antibody titres in orally immunized mice not only cross-reacted with the native parasite proteins in immunofluorescence studies and immunoblots, but also completely inhibited the proliferation of the malarial parasite [172]. Oral immunization of mice with the MSP1 and circumsporozoite protein (CSP) fusion protein (MLC) chimeric recombinant protein expressed in *B. napus* successfully elicited antigen-specific IgG1 production. Th1-related cytokines interleukin 12 (IL-12, a cytokine involved in the differentiation of naive T cells into Th1 cells), TNF (tumour necrosis factor, a cytokine involved in the inflammatory process and apoptosis) and IFN- γ were significantly increased in the spleens of immunized mice [173].

Spiegel *et al* [174] demonstrated the use of a plant transient expression platform based on transfection with *A. tumefaciens* as essential component of a malaria vaccine development workflow involving screens for expression, solubility and stability using fluorescent fusion proteins.

6. Plant Made Pharmaceuticals for Veterinary Use

Vaccination based on the programming of the specific mechanisms of warm-blooded animal protection against pathogens is the most efficient method for the struggle against infectious diseases, which often result in a mass mortality. In agriculture, there is no alternative to livestock vaccination, because there are no anti viral drugs that are suitable for a wide use in animal husbandry. The importance of animal vaccination indirectly affects human health, because the use of vaccines significantly reduces the amount of pharmaceuticals in the food chain.

As a rule, animal immune mechanisms are activated by the direct introduction of infectious agents or their components. At present, most of used vaccines are preparations on the basis of inactivated agents. Although these vaccines manifest the high immunogenicity, they are not without serious shortcomings. Among such disadvantages is the increased sensitivity of the organism to them, the large load on the immune system, the reactogenicity of vaccines (side effects), their toxicity etc. Manufacturing of medicinal preparations for veterinary use is a very important and dynamically developing field of world industry.

Pathogen	Antigen	Plant	Reference
Group A rotovirus of cattle	Protein VP6 Protein VP4	Solanum tuberosum cv mayqueen Nicotiana benthamiana S.tuberosum cv Bintje Medicago sativa	Matsumara et al 2002(175) O'Brien et al 2000(176) Yu et al 2003(177) Wig dorovitz et al 2004(178)
Bovine Herpes Virus BHV 1	Glycoprotein	N.benthamiana	Perez et al 2003(179)
Foot and mouth disease	Protein VP1 VP1 and VP7	M.sativa, Chenopodium quinoa,N.benthamiana, Stylosanthes guianinses cv Reyan II	Wigdorovitz et al 1999(178) Wang et al 2007(180) Yang et al 2006(181)
Rinderpest virus of cattle	Haemagglutinin H	N.tabacum, Arachis hypogaea	Khandelval et al 2003, 2004(182,183)
Canine parvovirus	Peptide from protein VP2	Arabidopsis thaliana	Gil et al 2002(184)
Newcastle disease virus of birds	Proteins F and NH Glycoprotein F	<i>S. tuberosum</i> cv. Kennebec <i>Z. mays</i> <i>Oryza sativa</i>	Beristine 2006(185) Yang 2007 (187) Guerrera 2007(186)
Avian influenza H5N1	Haemagglutinin M2 Haemagglutinin rHA0	N.benthamiana	Nemchinov et al 2007(188) Kalthoff et al 2011(189)
Japanese encephalitis virus(horses, cattle, pigs)	Glycoprotein E	O.sativa cv Nipponbare	Wang et al 2009 (190)
Rabies	G protein G and N proteins G protein N protein G protein	<i>L.esculentum</i> var.UC 82b <i>N.benthamiana</i> , <i>Spinacea oleracea</i> , <i>N.tabacum</i> <i>cvSamsun</i> , <i>Zea mays</i> <i>D carota</i> var. <i>Rendidora</i>	McGarvey et al1999(191) Modelska et al1999(192) Yusibov et al2002(197) Loza-Rubio et al 2008, 2012 (132,168) Perea-Aranga et al 2008(195) Rojas-Anaya et al 2009(196)

Dow Agrosiences in 2006 received the first regulatory approval for plant made vaccine against Newcastle Disease Virus from USDA. As a part of the approval process, USDA verified that the plant produced protein is equivalent to other Newcastle vaccines. This vaccine

was composed of recombinant hemagglutinin neuraminidase expressed in transgenic tobacco suspension cells. Although this never came forward as a commercially available product, the formulation advanced through USDA Center for Veterinary biologics regulatory approval. Hernandez et al [198] had also showed the efficacy of orally delivered papaya produced anti-cysticercosis vaccine and its potential as a low cost alternative of immunization. Major et al [199] showed that intranasal vaccination with a plant-derived H5 NA vaccine protected mice and ferrets against highly pathogenic avian influenza virus challenge. An edible potato based vaccine has also been developed against chicken infectious bronchitis virus [200]. Chickens were immunized by oral delivery of sliced tubers expressing S1 glycoprotein in doses over two weeks. These immunized birds developed virus specific antibody response and were protected against IBV. Another research group succeeded in vaccinating chickens against infectious bursal disease virus (IBDV) with plant made VP2 protein. Chickens orally immunized with *Arabidopsis* crude leaf extracts or transgenic rice seeds were protected to a similar level achieved with a commercial injectable vaccine [201]. Recently, the VP2 antigen was produced transiently in *Nicotiana benthamiana* and induced neutralizing antibodies in immunized chickens [202].

Transgenic maize seeds expressing envelope spike protein of Transmissible gastroenteritis corona virus (TGEV) were seen to raise neutralizing antibodies in piglets. This antigen was also stable during various stage conditions [203]. Enterotoxigenic *E. coli* (ETEC) causing post-weaning diarrhea in piglets has been a target for a plant-made vaccine. The major subunit protein of ETEC F4 fimbriae has been expressed in the leaves of tobacco [204], alfalfa [205] and in seeds of barley [205]. This subunit vaccine was shown to be immunogenic and partially protective after oral delivery to weaned piglets [205].

7. Veterinary Vaccines in Various Stages of Clinical Trial

Product	Disease	Plant material	stage	Developer
Fused proteins containing rabies virus epitopes	Rabies	Spinach	Phase 1 over	Thomas Jefferson University
HN Protein of NDV	NDV of birds	Tobacco cell suspension	Approved by USDA	Dow AgroSciences
Mixture of antiviral vaccines	Diseases of horses, dogs and birds	Tobacco cell suspension	Phase 1	Dow Agrosciences
Vaccine for Birds	Coccidiosis	Modified rape plants	Phase 2	Guardian Biosciences, Canada

8. Plant Made Therapeutics for Human Use

There have been many reports of therapeutics expression in plants including anticoagu-

lants [207]; thrombin inhibitors [207] ; HIV [208]; Diabetes [209]; Liver cirrhosis and burns [210]; Hepatitis [207,211]; anemia [210]; hemophilia [212]; organophosphate poisoning [207]; Hypertension [213] etc. Shenoy et al [214] reported that the oral delivery of Angiotensin-Converting Enzyme 2 and Angiotensin-(1-7) bioencapsulated in plant cells attenuates pulmonary hypertension. Further this also provided proof-of-concept for a novel low-cost oral ACE2 or Ang-(1-7) delivery system using transplastomic technology for pulmonary disease therapeutics.

Taking advantage of the high number of chloroplast genomes per cell, Daniell's group optimized technology for chloroplast transformation and gene expression. Oral administration of factor VIII or FIX antigens expressed in transplastomic tobacco plants suppressed inhibitor formation and anaphylaxis in hemophilic mice. A combination of protection from digestion offered by bio encapsulation in plant cells and fusion to the transmucosal carrier cholera toxin B (CTB subunit, thereby targeting gut epithelial cells) resulted in efficient tolerogenic delivery.

The first plant made therapeutic to reach phase II human trials, Locterin, by Biolex therapeutics, was a plant made controlled release interferon alpha treatment for chronic hepatitis [211]. First plant made therapeutic to reach phase III trials was a therapeutic developed by Protalix BioTherapeutics against Gauchers disease expressed in carrot suspension cells [213]. Human cerebroside expressed by carrot cells (human pr GCD) had high batch to batch enzymatic activity. In December 2009, Pfizer and Protalix entered an agreement to develop and commercialize pr GCD. However in early 2011, FDA declined the approval of the drug asking for additional data from existing studies, but not asking for additional trials.

U.S. Food and Drug Administration granted approval for ELELYSO, a product of Protalix Biotherapeutics and Pfizer for injection in May 2012 as a hydrolytic lysosomal glucocerebrosidase-specific enzyme ELELYSO™, which is branded as UPLYSO (Taliglucerase alpha) in Latin America, which is a plant cell-expressed form of the glucocerebrosidase (GCD) enzyme. This enzyme is indicated for long-term enzyme replacement therapy (ERT) for adults with a confirmed diagnosis of Type 1 Gaucher's disease. Approvals have also been granted by the applicable regulatory authorities in Uruguay, Mexico, Australia, Canada, Chile and other countries. (www.protalix.com) Sembiosys has also completed Phase I and II trial of safflower produced insulin grown in seed bioreactor Using Seed crops, ORF Genetics also produces various growth factors and cytokines in transgenic barley for use in cosmetics.

9. Against Helminths and Protozoans

Toxoplasma gondii, an intracellular parasitic protozoan can cause complications in pregnant women and in immunodeficient individuals like HIV positive patients and organ transplant recipients [215]. Recent studies have shown that chronic toxoplasmosis infection can play major role in the aetiology of certain mental disorders, such as schizophrenia [216].

Expression of the *T. gondii* dense granular protein 4 (GRA4) antigen via chloroplast transformation (chlGRA4) led to its accumulation to approximately 6 lg/g FW (0.2% of total protein) in tobacco plants. Oral immunization with chlGRA4 elicited both mucosal and systemic immunity (<1000 IgG titre) and also showed a 59% decrease in the brain cyst load of mice. Chloroplast-derived GRA4 elicited a protective immune response against *Toxoplasma* infection by reducing parasite loads in mice, correlating with a mucosal and systemic balanced Th1/Th2 response [217]. *Toxoplasma gondii* main surface antigen (SAG1) fused with the 90-kDa heat-shock protein from *Leishmania infantum* (LiHsp83) as a carrier expressed in transplastomic tobacco plants reacted with human seropositive samples in a functional analysis. Oral immunization with chLiHsp83-SAG1 also induced a significant reduction in the cyst burden in mice, which correlated with an increase in specific anti-SAG1 antibodies [218].

Cysticercosis, an endemic parasitic disease caused by *Taenia solium*, affects human health and the economy in developing countries. Cysticercosis cysts in the central nervous system produce neurocysticercosis (NCC), a common cause of acquired epilepsy [219]. The S3Pvac vaccine components (KETc1, KETc12, KETc7 and GK1 [KETc7]) and the protective HP6/TSOL18 antigen were expressed using a Helios2A polyprotein system through the 'ribosomal skip' mechanism. The 2A sequence (LLNFDLLKLAGDVESNPG-P) derived from the foot-and-mouth disease virus induces self-cleavage events at the translational level, releasing the distinct antigens in a single transformation and expression event. Plant-derived Helios2A accumulated up to 1.3 lg/g FW in transgenic tobacco leaf tissue and was recognized by antibodies in the cerebrospinal fluid from patients with NCC in a functional assay. Further, orally immunized mice elicited an immune response, but antibody titres were not reported [220].

Lymphatic filariasis caused by *Brugia malayi*, *Brugia timori* and *Wucheraria bancrofti* though not fatal; still is the second leading cause of permanent and long term disability worldwide. Filariasis has a spectrum of disease manifestation and infectivity found among the infected individuals; it also goes unnoticed for years. In areas where it is endemic, humans become infected in early life and are persistently infected by the presence of adult worms in their lymphatic system. The ability to survive long term in the hostile mammalian system shows that the parasite has evolved evasion mechanisms to avoid being attacked by the mammalian immune system. Ganapathy et al [29] reported the transformation of *N. tabacum* with *Brugia malayi* Abundant Larval Transcript-2 (Bm ALT-2), a major antigen produced from recombinant *E.coli* found to be experimentally successful as potential vaccine candidate against lymphatic filariasis. The level of expression varied from 50 to 90 ng/lg of total soluble protein for ALT-2. Immunization of mice with plant-extracted protein indicated that the plant-produced protein had immunological characteristics similar to the *E.coli*-produced protein. Antibody titers produced by plant produced recombinant ALT 2-immunized mice were on par with those immunized with recombinant protein produced by *E.coli*. Antibody isotype assay showed that

plant-produced recombinant ALT-2 induced significant IgG1, whereas E.coli-produced recombinant ALT-2 induced IgG3.

The same group also reported the expression of WbSXP-1, a diagnostic antigen for the easy detection of lymphatic filariasis, isolated from the cDNA library of L3 stage larvae of *Wucheraria bancrofti*, in tobacco plants [30]. The immunoreactivity of the plant-produced WbSXP-1 was assessed based on its reaction with the monoclonal antibodies developed against the E. coli-produced protein. Immunological screening using clinical sera from patients indicates that the plant-produced protein is comparable to E. coli-produced diagnostic antigen

10. Challenges

The selection of the host plant depends upon the nature of the recombinant protein. Hence depending on the protein, a suitable plant would have to be chosen and strategies would have to be optimized in that plant for maximum yield. Thus development of a common plant platform where any protein can be expressed is not possible. The selection of the suitable host plant also has to be based on economic issues like storage property, scalability of protein production after optimization of the conditions, transportation, cost effectiveness of downstream processing, preferably short timescale in production and also edibility. Efficient transformation and regeneration protocols would also be a criteria to choose a suitable plant host. Some recombinant proteins might also affect the natural metabolic pathway in plants or cause toxicity to natural plant proteins. It can also retard plant growth or reduce the production of some key proteins which are required for normal plant function. These toxic side effects can be decreased by the identification of intermediate metabolites involved in toxicity and altering them or targeting them to organelles.

11. Safety Issues

Production and wide distribution of biopharmaceuticals is hindered by a number of circumstances. The first of them is related to the problem of biosafety – the cultivation of genetically modified plants in the field can lead to the accidental introduction of foreign genes into crops grown for human consumption. Therefore, companies producing biopharmaceuticals focused on plant species, which are absent from the food chain of humans and animals and also on growing of genetically modified plants preventing their crosspollination with other crops. The second difficulty is related to the necessity of plant material treatment for the removal of various undesired compounds, such as lignin, proteases, phenolic compounds, and pigments, especially in the case of plant species, which are not consumed. All these result in the requirement of additional studies. The third circumstance is due to the fact that until now all aspects of maintaining and growing of plants producing biopharmaceuticals are not settled at the legislative level.

Biosafety issues can be covered by the application of chloroplast transformation and/or growing the plants in contained facilities [221,222]. Furthermore, an inducible system can be used to control the transgene expression when required. Transgenic plants can be grown at the site where the vaccine is needed. This advantage can save the costs related to transportation and cold storage. Plant-derived vaccines have the potential to be used as oral vaccine, thus evading the costs related to sterile needles and trained medical staff.

11.1. Stability

Plants-derived vaccines are likely to be more stable. A recent report shows that a chloroplast-derived vaccine candidate was stable at room temperature for 20 months [223]. Moreover, mice immunized with the vaccine stored at room temperature showed similar IgA/IgG levels as those of mice immunized with the vaccine stored at 4°C. This characteristic is very important for the development of a vaccine for developing countries where cold chains are difficult to maintain in remote areas.

12. The Future

Though the benefit of plant made pharmaceuticals have been pointed out reportedly it is being implemented only now due to investment by big pharmaceutical companies. Plant based systems have been able to reproduce a wide variety of human proteins, including those that have multiple subunits expressed and assembled in plants as well as proteins and vaccines requiring Co expression of additional modifying enzymes. While raw edible vaccines are unfeasible for human therapy, it may not be necessary to fully isolate the target protein from plant material. A middle ground of dried and ground plant material may be more suitable for oral delivery of some vaccines and therapeutics. This would be an excellent option for the production of veterinary medicines where recombinant feed could contain vaccine antigens and would be useful and cheap for developing nations [224,225]. If yields can be standardized, there is potential for delivery of therapeutics in unprocessed plant material, especially in veterinary field where the dosage has a wide active range. The use of vaccines and prophylactics for the control of infectious diseases in the livestock industry will grow as antibiotics applications diminish. Plants as bioreactors comprise a valuable option for production of recombinant protein therapeutics for animal health. In recent years numerous studies demonstrated the feasibility and advantages of plant-based production platforms for various proteins with therapeutic use, including complex antibodies, subunit vaccines and immunogenic virus-like particles. Plant made therapeutic products are currently on the cusp of widely entering biotechnology markets. Interaction and concerted actions of the plant biotechnology sector with veterinarians and regulatory authorities will facilitate development of novel approaches to sustainable, antibiotic-free livestock agriculture.

13. Acknowledgement

Research reported from the authors laboratory was funded by WOS –A Scheme Of DST, Govt of India for MG and ICMR Emeritus Medical Scientist grant to PK. The authors wish to thank Director, Centre for Biotechnology, Anna University for additional support.

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