An Overview: Genetic Tumor Markers for Early Detection and Current Gene Therapy Strategies

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ABSTRACT: Genomic instability is considered a fundamental factor involved in any neoplastic disease. Consequently, the genetically unstable cells contribute to intratumoral genetic heterogeneity and phenotypic diversity of cancer. These genetic alterations can be detected by several diagnostic techniques of molecular biology and the detection of alteration in genomic integrity may serve as reliable genetic molecular markers for the early detection of cancer or cancer-related abnormal changes in the body cells. These genetic molecular markers can detect cancer earlier than any other method of cancer diagnosis, once a tumor is diagnosed, then replacement or therapeutic manipulation of these cancer-related abnormal genetic changes can be possible, which leads toward effective and target-specific cancer treatment and in many cases, personalized treatment of cancer could be performed without the adverse effects of chemotherapy and radiotherapy. In this review, we describe how these genetic molecular markers can be detected and the possible ways for the application of this gene diagnosis for gene therapy that can attack cancerous cells, directly or indirectly, which lead to overall improved management and quality of life for a cancer patient.

KEYWORDS: Gene therapy, immune cell therapy, DNA vaccine therapy, cytokine therapy, tumor suppressor gene therapy

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Introduction

In our previous article, we described how liquid biopsy can be used for the detection of genetic tumor markers for the early diagnosis and prognosis of cancer.[1] In this current article, we will review various nucleic acid based biomarkers for gene diagnosis and clinical methods for gene therapy to cure cancer disease. Cancer is a multi-factorial genetic disorder and a complex group of diseases in which cells grow out of control and do not die after being damaged or useless, and show immortality.[2] There are many factors involved in the process of cancer generation such as; environmental factors, lifestyles, and infections, which can then lead to abnormalities in cancer-related genes at a molecular biology level. Abnormal changes in body cell molecules (genes) and their products can be detected and serve as genetic tumor biomarkers.[3,4] These direct tumor biomarkers of genetic origin offer an earlier and more precise diagnosis of cancer than the conventional protein-based tumor markers.[4] Another advantage of these gene-based tumor biomarkers is that they can also be used to see the prognosis of the disease if one or more of these biomarkers are monitored for changes during and after the therapy.[5] In addition, molecular abnormalities in the genes or DNA offer targets (cancerous cells) that can also be utilized to develop a specific gene-drug which recognizes and attack only such abnormal targets (cancerous cells) to cure cancer without producing side effects at all or minimum.[6,7] These genetic biomarkers can also be used to enhance the immune cell therapy against cancer, therefore, more effective and specific immune cells can be engineered in the laboratory by the application of gene transfer technology , consequently, these immune cells become effective cancer-attacking cells, then these engineered cells can be re-introduced into the body of a patient to attack and kill the cancer cells.[7-9] In this review, we explain briefly about gene tests such as concentration and fragment length of free DNA, DNA mutation detection test, DNA methylation test, and Gene expression test for early detection, diagnosis, and prognosis of cancer, we also describe different types of gene therapies performed to either directly attack cancer cells or to induce the immune system of a cancer patient to fight cancer. In the field of oncology, molecular genetics of cancer is becoming an important tool for screening, surveillance, treatment, and management.

Future research and improvements in cancer genetics will increase the ability to precisely and cost-effectively diagnose and treat cancer patients. The purpose of this report is to present the clinical work performed in our laboratory (HIC Clinic) and to review it with the current research in this area.

Gene Test for the Early Detection of Cancer

As mentioned above, there are cancer-producing alterations in genes and these abnormalities can be detected as genetic tumor markers to diagnose the presence of cancer in the body.[3,10] The progression of cancer involves multiple genetic and epigenetic events that disrupt the balance between cell division and apoptosis. Genes that affect cancer progression are known as cancer driver genes,[11] which can be classified as tumor suppressor genes (TSGs) and oncogenes (OGs) based on their roles in cancer progression.[12] Oncogenes are usually activated by gain-of-function mutations that stimulate cell growth and division. Whereas, tumor suppressor genes are recessive, anti-proliferative, and frequently found inactivated or mutated in cancer.[13] These genes are inactivated by loss-of-function (LoF) mutations (insertions/deletions and nonsense

Table 1. Gene diagnostic strategies.

GENETIC TEST	SIGNIFICANCE
Free DNA concentration	cfDNA concentration found higher in malignancies, other causes excluded.
Free DNA fragment length	This can help discriminate between the necrotic and apoptotic origin of cfDNA.
DNA methylation	DNA methylation in promoter region at CpG sites is an epigenetic tumor marker.
DNA mutation	DNA mutation is an important molecular marker for their role in malignant transformation.
Gene expression	Gene expression analysis is performed on cancer-related gene.

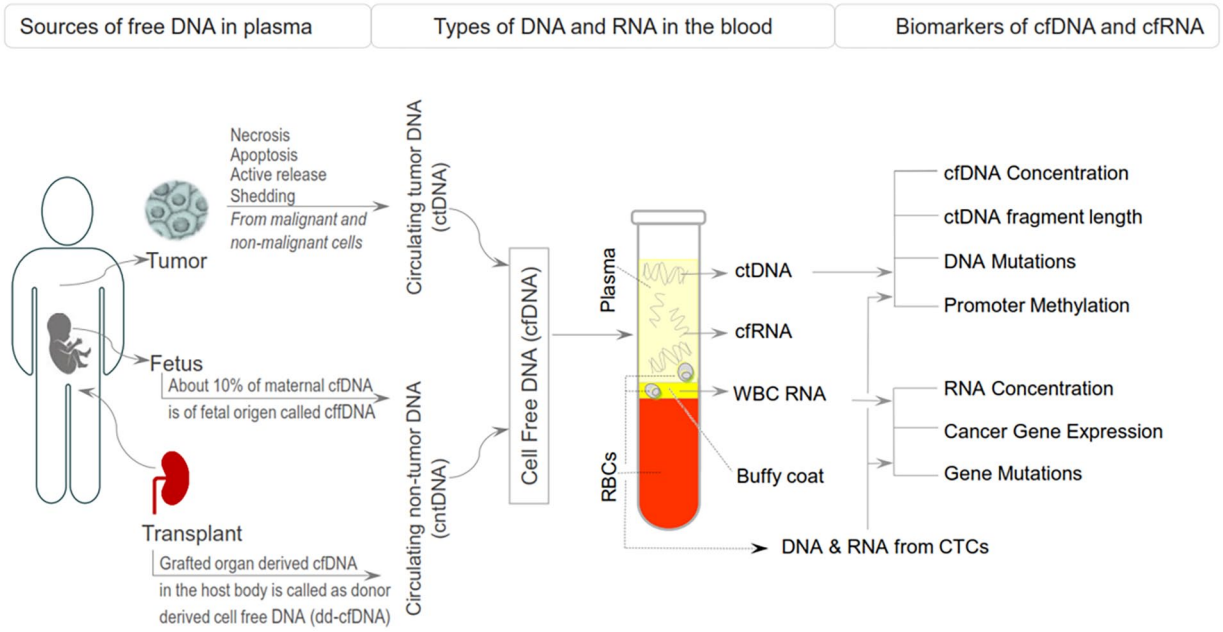

Figure 1. Demonstrate the release of CfDNA into the blood circulation by normal cells and cells involved in the pathogenic processes including cell death. The human bloodstream contains cell-free DNA, cell-free RNA, and proteins that can be used as biomarkers for various diseases.

mutations) that block tumor suppressor gene functions in inhibiting cell proliferation, promoting DNA repair, and activating cell cycle checkpoints.[14]

Depending upon the technology, sensitivity of the method, and analytical techniques used, these genetic cancer markers can be detected in various types of samples from a patient's body, such as blood, urine, tissue, saliva, or a biopsy sample. Minimally invasive detection of the tumor markers by the body fluids (blood or urine) will also prove a useful method for the follow-up monitoring of the therapeutic effects or prognosis of the disease.[15-17] Several gene test methods are used in Hirahata International Cancer Clinic (HIC Clinic) and we find these genetic markers are high in their sensitivity and specificity for cancer detection. Table 1 shows various gene diagnosis strategies in molecular biology to detect the nucleic acid tumor markers in a blood sample.

Free DNA concentration

Mandel and Metais discovered the presence of cfDNA in the blood of healthy individuals in 1948.[18,19] Decades later, many researchers extended Mandel and Metais' work and recognized tumor-derived cfDNA (also known as circulating tumor DNA-ctDNA) in the blood of cancer patients. It is mainly found in the bloodstream; now it can be derived from cerebrospinal fluid and saliva. It can be excreted through urine with extremely low content.[20,21] However, the amplifiable amount of circulating free DNA can be obtained from the blood plasma of healthy and diseased individuals.[22,23] Figure 1 represents the characteristic features of free DNA including its sources.

In general, cancer patients have an elevated level of circulating free DNA than healthy individuals.[24-26] In 1977, scientists made the significant observation of abnormally high levels of cfDNA in plasma and serum from cancer patients compared to healthy individuals.[26,27] Previous studies showed that the concentration of cfDNA in cancer patients increased significantly compared to a healthy individual that ranges from 0 to 1000 ng/mL of blood, with an average of 180 ng/mL. In contrast, cfDNA in healthy individuals ranges from 0 to 100 ng/mL of blood, with an average of 30 ng/mL.[26,28] The concentration of free circulating DNA in the plasma of cancer patients can be 3 to 10 times higher than the level of normal healthy samples of

plasma. The measurements and quantification of this free DNA can be performed by Real-time quantitative PCR, digital PCR, fluorescent dye method, and other molecular biology technologies.[29-31] Circulating tumor DNA analysis can be used as an early cancer screening test and can also be used for the monitoring of successful cancer treatment. For instance; a decrease in the quantity of circulating tumor DNA may suggest tumor size. Although the increased amounts of this circulating DNA (unless it is proved circulating tumor DNA abbreviated as ctDNA) are not specific to cancer, it can be utilized as a screening method to indicate the need for further complete biomarker assessment.[32-34]

Figure 2 represents the concept of free DNA concentration among cancer patients and normal healthy individuals.

Free DNA fragment length

Another important feature of ctDNA is the large variation in the fragment size. This characteristic can be referred to as the variety of mechanisms that underlie dead cancer cells. In healthy individuals, cfDNA is released predominantly through apoptosis with small and uniform fragments of around 185 to 200 bp.[35]

In cancer patients, longer DNA fragments are considered to result from necrotic cell death; necrosis creates a spectrum of DNA fragments of various sizes due to incomplete and random digestion of genomic DNA by the nuclease enzyme, interestingly; shorter fragments of ctDNA have been reported in some tumor types (eg, hepatocellular carcinomas) as well as large fragments of cfDNA of thousands of base pairs.[36-38]

Circulating cell-free DNA concentration and fragment length detection can be exploited for the prediction, prevention, early diagnosis, and prognosis of cancer in the field of oncology.[36-39]

DNA methylation

DNA methylation is an epigenetic mechanism involving the addition of a methyl group at the fifth position of the pyrimidine ring of cytosine: that is, 5-methylcytosine Cytosine of CpG islands at the promoter region (Figure 3). From previous

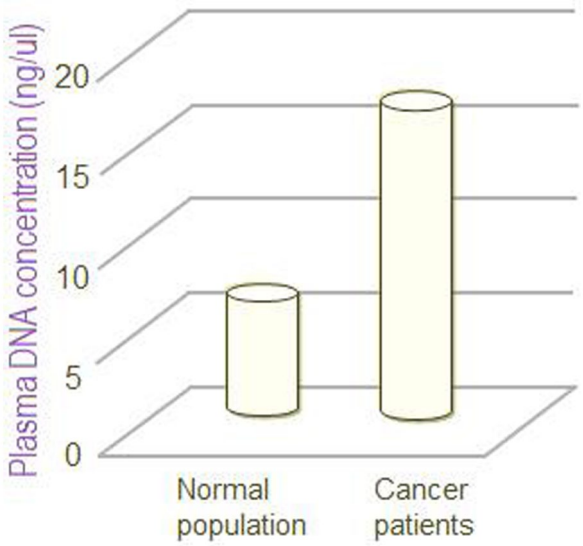

Figure 2. Diagrammatic representation of cfDNA concentration as a biomarker. The concentration is found higher in cancer patients than in healthy individuals due to the presence of necrosis or apoptosis in the body cells. Other causes of higher cfDNA concentration should be excluded, such as pregnancy, transplant, and intense physical activity.[35]

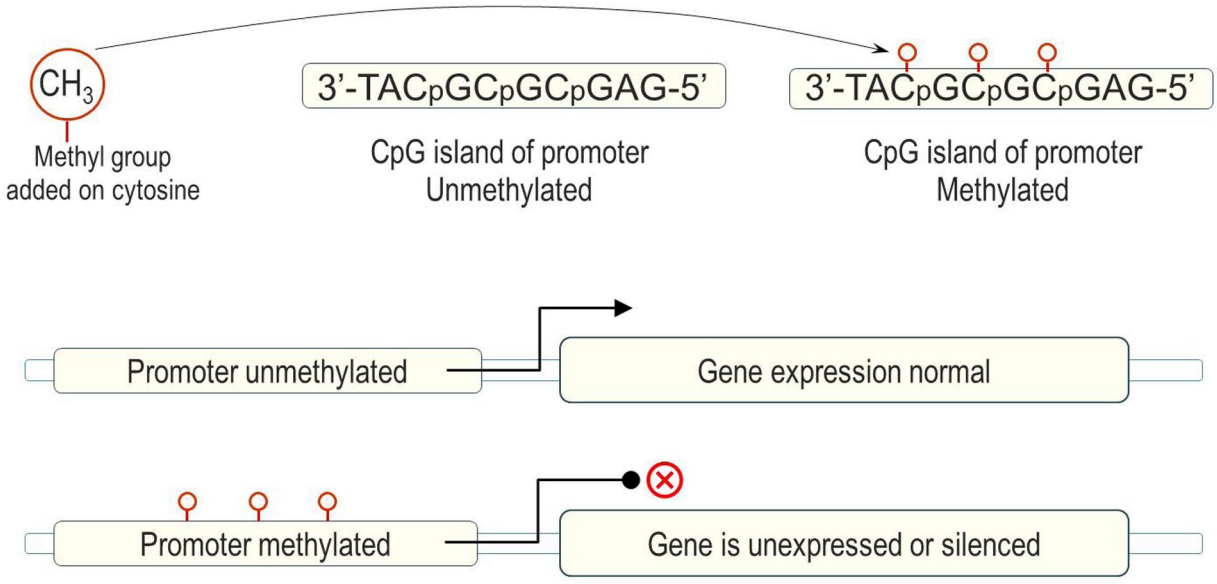

Figure 3. Promoter methylation and gene silence. Promoter methylation (CH_3 group addition on CpG islands) sometimes called hypermethylation, is an important factor in carcinogenesis when it can prevent the proper expression (functions) of a tumor suppressor gene.

research, it is obvious that DNA methylation plays many crucial roles in epigenetic regulation in cells, including gene expression, cell proliferation, differentiation, genomic imprinting, X-chromosome inactivation, embryonic development, and carcinogenesis.[40,41] The mechanisms underlying cancer development are very complicated and the malignant transformation of a normal cell is forced by both epigenetic and genetic alterations. It is also demonstrated that the initiation, maintenance, and progression of cancer involve epigenetic changes including key processes of DNA methylation, histone modifications, nucleosome positioning, and aberrant expression of non-coding RNAs, specifically microRNAs.[42,43] Disruption in epigenetic processes can lead to altered gene functions and cellular neoplastic transformation, therefore appropriate control of DNA methylation is not only essential for regulating gene transcription but also plays a pivotal role in maintaining the integrity of the genome and modulating immune response.[41] Loss of proper maintenance of epigenetic control may result in inappropriate inhibition of the involved gene and various signaling pathways, leading to a disease state, such as cancer. According to another report, aberrant DNA hypermethylation when occurs in the CpG island in the promoter regions of specific transcription factors contributes to the formation and progression of tumors. Consequently, DNA methylation may be a biomarker of early cancer detection.[44] Epigenetic modifications in the form of methylation within the promoter area may stop the function of a gene and if the gene is a tumor suppressor gene, then this can lead to cancer generation. Therefore, hypermethylation of the tumor suppressor gene can be a strong progression marker of cancer.[45,46] Several methods are in use for the detection of methylation as a biomarker with their advantages and disadvantages. Some of the methods are cost-effective but of medium or low sensitivity. Methylation-specific PCR (MSPCR) is a sensitive method for the analysis of DNA methylation patterns in CpG islands.[47-49] The methylation profile of DNA facilitates early diagnosis, prognosis, and screening for cancer as reported in previous studies.[44]

Table 2 shows some of the important genes found methylated in various cancers.

Table 2. Genes detected for methylation.

GENE	CANCER
P16	NSCLC, colon, bladder, esophageal, and stomach cancers
P15	AML, ALL, colon cancer, lung, and breast cancer
RAR-beta2	Colon, breast, and lung cancer
APC	Colon, gastric, and esophageal
DAPK	Lung cancer, lymph node metastasis
MLH1	Endometrial, uterine, colon, breast, and lung cancer
E-Cadherin	Thyroid carcinoma, breast, gastric, and liver cancers
H-Cadherin	Lung cancer and ovarian cancers
RASSF1A	Lung cancer
MGMT	Brain tumor, colon cancer, lung and breast cancer
BRCA1	Breast cancer and ovarian cancer
MAGE-A	Breast and esophageal cancer
GSTP	Prostate cancer
FHIT	Esophageal cancer
GATA	Lung cancer and esophageal cancer
DLEC1	Lung cancer
TSLC1	Lung cancer
NKEFB	Colorectal cancer

Involvement shows only the major data findings in our laboratory as well as in published articles.[50-55]

DNA mutation

A mutation is a change in the nucleotide sequence of a gene that can result from errors in DNA replication during cell division, exposure to mutagens, or viral infection.[56] With the development of new technologies for a more accurate understanding of the genome and potential gene therapies, the detection of mutations in the cancer samples of blood and/or tissue has an increasingly central role in terms of the genetic diagnosis of cancer. It is a very important genetic marker and the variation in sequence from normal can indicate the presence of a carcinogenic process.[57-59] As a powerful technique in molecular genetics, DNA sequencing provides analysis of genes at the nucleotide level. The main focus of DNA sequencing is to determine the sequence of small regions of interest (~1 kilobase) using a PCR product as a template. DNA sequencing could be used to check all small known and unknown DNA variations; Dideoxynucleotide sequencing or Sanger sequencing represents the most widely used technique for sequencing DNA.[60] Among chain-termination methods, capillary electrophoresis is used (SeqStudio Genetic Analyzer, ThermoFisher scientific) double-stranded DNA is denatured into single-stranded DNA with NaOH. A Sanger reaction consists of a single-strand DNA, primer, and a mixture of a particular ddNTP with normal dNTPs (eg, ddATP with dATP, dCTP, dGTP, and dTTP). A fluorescent dye molecule is covalently attached to the dideoxynucleotide. ddNTPs cannot form a phosphodiester bond with the next deoxynucleotide so they terminate DNA chain elongation. This step is done in 4 separate reactions using a different ddNTP for each reaction.[61] We use advanced technology for the detection of mutations utilizing free circulating DNA and at present, we analyze the full coding sequence of the following genes mentioned in Table 3.

Table 3. Genes sequenced for mutation test.

NAMES OF THE GENES SEQUENCED FOR MUTATION TEST		
EGFR	APC	BRCA2
HRAS	p53	NRAS
BRAF	BRCA1	ALK
ERBB2	KRAS	JAK1
EGFR1	BCL2	ERBB4
FGFR1	FLT3	MAPK2

Oncogenic mutations found in the cancerous cell, are an important nucleic acid tumor marker for cancer detection and progress assessment during and after the treatment (Table 3). Mutations can also be useful to determine the effectiveness and chemotherapy resistance.[62,63]

Gene expression

Altered gene expression (mostly hyper-expression of an oncogene) is a very common finding in nearly all types of cancers and can be used as a molecular marker for the detection of cancerous activity in a group of cells. We can detect the types of genes that are either up or down-regulated in specific cancer types with the use of microarray, real-time PCR, and digital PCR.

Microarray is useful for the mass analysis of oncogenes such as to make a profile; this data provides a snapshot of all the transcriptional activities in the biological sample. Expression measurement analysis leads to an understanding of genes that are being regulated under the disease conditions, including cancer, both in basic medical science and clinical medicine.[64] Cancer is one of the most dreaded genetic disorders, and it develops either through acquired mutations or epigenetic changes that lead to disruption in gene expressions of cancerous cells. Accordingly, microarray technology is allowing the construction of specific gene expression profiling that plays a role in the correct diagnosis of a specific cancer type. Gene expression analysis also provides insight to understand the oncogenic pathways and discover novel biomarkers for clinical diagnosis.[65] The power of these gene expression detection tools has been applied to a wide range of applications such as discovering disease subtypes and identifying underlying mechanisms of disease or drug response.[66,67] We analyze more than 100 oncogenes by the technology of our custom build microarray; real-time quantitative PCR, digital PCR, and northern blot are also used when required. RNA from cancer cells is utilized to detect the expression of oncogenes.

Table 4 shows the list of genes detected by microarray for their expression.[68-70]

Genes listed in Table 4 are often found with altered expression in tumor samples. The expression can be detected by DNA microarray, real-time quantitative PCR (qPCR), or digital PCR; which is more sensitive than qPCR.

Gene Therapy for Cancer

There are 3 types of standard therapies for the treatment of cancer; chemotherapy, radiotherapy, and surgical treatment. The prognosis of cancer with these treatments was never satisfactory and even with the remission achievement of 5 years; some of the cancer cells or cancer-related molecular changes remain undetected and can cause recurrence.

There are some other problems also associated with conventional therapies such as these conventional therapies are generally effective against early-stage localized tumors, whereas their effectiveness is not the same against a later stage of cancer and there are chances of frequent relapse after radical surgery and conventional therapies.[71,72] Further, drug resistance appears to be a serious problem in the treatment of cancer and a major reason for chemotherapy failure in cancer.[73] Moreover, various agents used in conventional therapies are harmful both to normal cells and cancer cells, which can lead to prominent side effects.[71,72] Another concern with the standard therapies is the quality of life of the patients during their remission or survival period. While improving and alleviating the severity of symptoms, these conventional treatment methods adversely affect the body and most of the time their side effects are systemic due to their unspecific nature.[74,75]

Therefore, novel approaches have raised hope to significantly improve the survival rate of patients with cancers such as immunotherapy, hormone therapy, gene therapy, and immune-gene therapy. Currently, gene therapy approaches predominately practice in research laboratories. It has been recognized as the capacity for genetic improvement through the correction of the altered (mutated) gene or site-specific modifications that have treatment as a target.[72,76,77]

Figure 4 demonstrates a summary of molecular gene therapy and immune-cellular gene therapy.

The main categories of antitumor immune-gene therapy that can be customized according to the patient's requirements are given below, followed by a brief description of each method.[72,76,77] Table 5 summarizes the categories of gene therapy.

Immune cell therapy

Cancer has been identified as a leading cause of death worldwide. In recent years, cancer treatment has been revolutionized with the development of immunotherapies that aim to boost the body's immune system to more efficiently target and destroy cancerous cells.[78] Traditional therapies such as chemotherapy and radiotherapy cannot distinguish between normal and cancerous cells, causing substantial toxicity to healthy tissue which can be a major limiting factor in their use, therefore, much attention is being paid to cancer immunotherapy which is a personalized therapy that activates the intrinsic ability of the immune system of the cancer patient through using the component of the immune system to prevent and inverse the evasion activity by tumor, thus boost the immune system to

Table 4. Genes detected in expression analysis.

NAMES OF THE GENES DETECTED IN EXPRESSION ANALYSIS					
CYTO-7	TNFSF10	SESN1	CEA	PSMA	NOTCH1
MAGE-A3 6	MDM2	MAPK1	PSA	VEGFR	FAS
DDIT3	CDKN1A	BRCA2	Bcl2	AKT1	Mucin-6
Mucin-12	S100	TRC3	PMS2	NOD1	Abl/Bcr
MCL1	MAP7	KIT	HPRT1	ESR1	CXCL1
Ki-67	BRCA1	BAX	AKAP13	VEGF-A	TGFB1
RPL13A	RAB21	PGR	NLRP1	MMP2	MBD4
hk5	JUN	FOXA1	ERG	CTNNB1	mTOR
Bcr	ARAF	Topo-II	TP73	TERT	RORB
SCCA1	PGF	NFKB1	MMP13	MAS1	MAP2K5
ITGB3	C-FOS	ERCC1	CD38	CD40	BIRC5
AR	AFP	TP63	SRC	RB1	COX2
PALB2	MYC	MLH1	VEGFR3	Cyto-20	IGFBP3
FLT1	Her-2	CFLAR	CCND1	BIRC2	APC
Ab1	p53	SOCS3	CD43	CD10	NSE
Mucin-1	c-Met	MAPK3	Cyto-19	IGF1R	FGF1
EGFR	HSP70	CASP3	BCL2L1	ANGPT1	ABCG2

destroy cancer cell in the body.[79] Immune cell therapy is a most advanced and entirely new paradigm in the field of cancer treatment, different strategies such as immune cells, targeted antibodies, cancer vaccines, adoptive cell transfer, cytokines, tumor infecting viruses, and checkpoint inhibitors are used in immunotherapy.[80] It has been reported that various immune cell types contribute toward developing innovative anticancer strategies such as macrophages, neutrophils, NK cells, T-cells, and B-cells. The major form of cellular immunotherapies includes T-cell-based, NK cell-based, and Dendritic cell-based.[81,82] In this review, we describe briefly the major role played by NK, NKT, and dendritic cells (DCs) and the mechanism used by these cells to get rid of cancer from the body.

Immune system cells do not recognize the tumor cells to attack and as such, they cannot initiate an effective immune response against tumor cells as they do for bacteria and viruses, etc. In cancer, immune cells seem to be tolerant to the cancer cells like their normal body cells.[83,84] To remove this tolerance and to make the immune cells activated against the tumor cells, first, remove them from the patient's body and then train and cultivate these cells ex vivo in a GMP-grade facility to make them able to recognize the tumor cells. To produce clinical-grade immune cells, such as NK, DC, and NKT, strategies are developed to amplify these cells and modify them by transfection of these immune cells with cancer-recognizing elements such as tumor antigens, immune cell receptors, and co-stimulatory molecules.[84]

NK cell therapy. NK cells are an important subunit of the innate immune system that exhibits a potent tumor cytolytic function against physiologically stressed cells such as tumor cells and virally infected cells and induce an antigen-independent immune response.[85,86] The function of NK cells is similar to that of cytotoxic T-cells in the adaptive immune response of vertebrates.[87] Unlike the adaptive immune system, NK cells exhibit tumor cytotoxicity against tumor and virus-infected cells without prior sensitization via activation of NK cell-activating receptors against ligands present on tumor target cells.[88-90] As NK-mediated cytotoxicity develops in a TAA-independent (T cells target tumor-associated antigens) and MHC-unrestricted fashion, thus diverting the difficulty in identifying defined tumor-specific antigens, and maintaining the accurate distinction between normal and malignant cells.[91,92] Nk cells express several activating and inhibitory receptors that recognize the altered expression of proteins on a target cell and control the cytolytic function of NK cells. In patients with cancer, NK cell function is inhibited due to the reduced expression of NK cell-activating receptors, which results in impairing their cytolytic activity. In this regard, adoptive immunotherapy with NK cells has emerged as a promising solution against several malignancies.[93] Several approaches have been developed to generate NK cells for adoptive immunotherapy. One of these approaches involves using cytokines, such as IL-2, IL-12, IL-15, IL-18, and IL-21, to culture and expand NK cells.[94] These cytokines can upregulate the

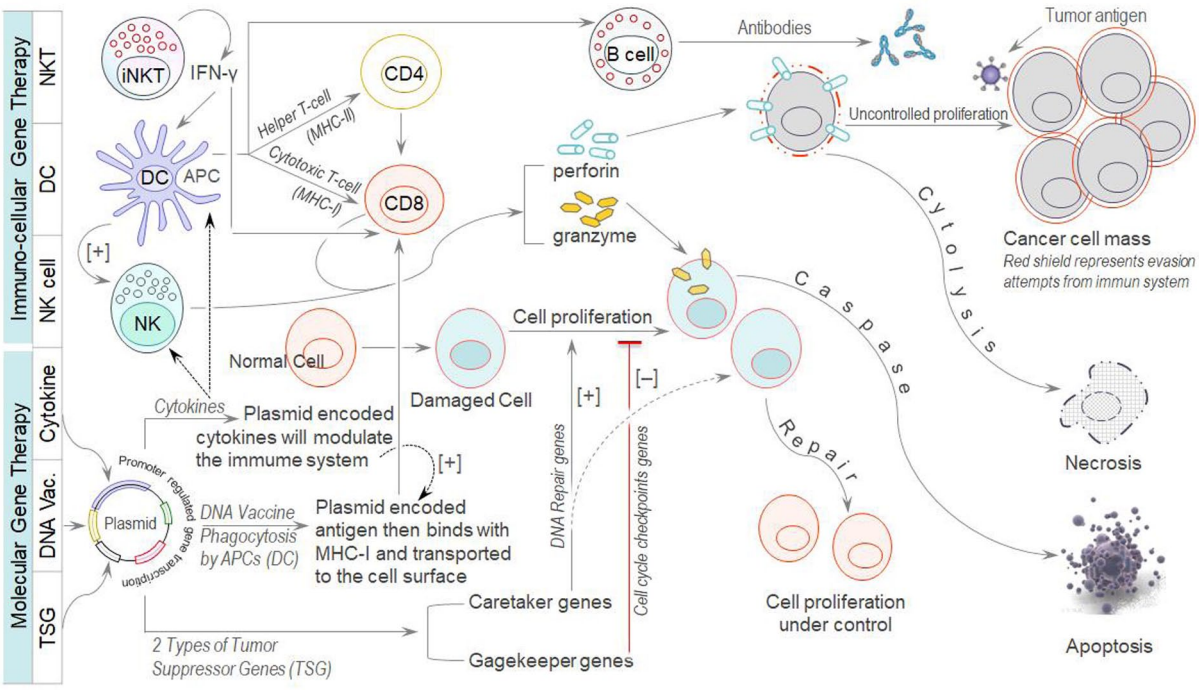

Figure 4. In a broad sense, gene therapy involves restoring or manipulating the altered genes found in the diseased cells, as detected by the gene test or gene diagnosis. In the molecular gene therapy approach, a gene can be constructed and inserted in a mammalian expression plasmid and then infused into the body to replace the function of a damaged or mutated gene, such as wild type TP53 gene or any other tumor suppressor gene (TSG). A plasmid can also be used to deliver the antigenic sequence of a tumor antigen (DNA Vaccine) which produces the peptide to present on the cell surface with a major histocompatibility complex (MHC) molecule to activate the cellular immunity against the tumor. Cytokines are another option that can be constructed in the plasmid to encode and modulate the immune system cells. Immuno-cellular gene therapy is an approach that modulates the immune system to work against cancerous cells; both humoral and cellular arms can be manipulated. Anti-tumor immune cells such as NK, NKT, and DC can be activated and educated against the tumor in vivo by plasmid-based cytokines or DNA vaccines, these anti-tumor cells can also be taken out of the body, expanded in vitro, educated by tumor peptide culture method or by transfection method and then re-infused into the body.

Table 5. Major categories of gene therapy.

CATEGORY	SUBCATEGORIES
Immune cell therapy	NK cell therapy
	Dendritic cell therapy
	NKT cell therapy
DNA vaccine therapy	Dendritic cell vaccines
	Plasmid DNA vaccines
Cytokine therapy	IL-24 therapy
	IL-12 therapy
	IL-21 therapy
Tumor suppressor gene therapy	P53 gene therapy
	TRAIL gene therapy
	FUS1 gene therapy

expression of NK cell-activating receptors present in NK cells, thereby enhancing the anti-tumor activity of NK cells against the cells that express the respective receptor ligands. Culturing NK cells with growth-inactivated feeder cells may also be used to enhance NK cell proliferation and activation. Various NK cell sources are used for the adaptive immune system such as autologous NK cells, allogeneic NK cells, NK cell lines from peripheral blood and stem cells, and genetically engineered NK cells.[95,96]

Dendritic cell therapy. Dendritic cells (DCs) represent a heterogeneous family of immune cells that act as a messenger between the innate and adaptive immune systems and are critical for the induction of protective immune responses against pathogens.[97] These cells are considered the optimal antigen-presenting cells that capture, process, and present antigenic material on major histocompatibility complex (MHC) class I and II molecules to lymphocytes thus activating the adaptive immune response.[97,98] Under resting conditions, DCs are inactive and in an immature state in blood circulation, once activated and mature they migrate to secondary lymphoid tissues such as lymph nodes where they interact with T-cells and B-cell to initiate adaptive immune response.[99,100] Maturation of DCs is crucial to transport antigens to tumor-associated draining lymph nodes for initiation of T-cell activation which is characterized by upregulation of MHC class I and II, cytokines, and chemokines production that are necessary for activation of T-cell responses.[101]

In DC-based immunotherapy, DC is obtained from the patient and modulated ex vivo to induce the immune system toward tumor elimination.[97] However, host immune systems involving DCs are restricted in tumors due to several mechanisms, including the low number of DCs in the tumor, reduced ability for antigen presentation, and limited access to tumor antigens. Recent advancements in cell culture techniques demonstrate that GM-CSF and IL-4 trigger monocytes in the peripheral blood to induce significant activation of DCs. Therefore, artificially induced DCs can be administered intratumorally or subcutaneously to effectively stimulate DC-mediated host immune responses.[102,103]

NKT cell therapy. NKT cells are another specialized subset of lipid and glycolipid reactive T-lymphocyte that play an important role in immunosurveillance and antitumor immunity, therefore they play an important role in the development of effective immunotherapy against cancer. These cells are found in very small numbers in the peripheral blood and they share the molecular characteristics of both natural killer cells and T cells.[104,105] Unlike traditional T-cells that recognize peptide antigens in the context of MHC I or II, NKT cells recognize glycolipids presented within the context of the MHC I-like molecule CD1d. These cell cells can be classified into 2 major subsets: type I and type II NKT cells based on TCR rearrangements and glycolipid reactivity. Type I, invariant NKT (iNKT) cells, are the well-characterized subset of CD1d-restricted T-cells and express an invariant Vαβ TCR. Following antigen recognition, iNKT cells rapidly secret cytokines and chemokines which can influence both innate and adaptive immune systems. Furthermore, activated NKT cells can directly induce cell death in tumor cells and infected cells, therefore, play a critical role in autoimmune disease, infection, transplant immunology, and cancer.[105-107] NKT cells can be engineered and cultivated to those subpopulations of NKT cells ex vivo that are proven to be most effective against cancer cells. They can also be made to activate the adaptive T cell immunity in the body.[108,109]

DNA vaccine therapy

Cancer vaccines represent a promising strategy to induce a specific and long-term immune response against tumor antigens (TAs). TAs are proteins overexpressed in tumor tissues and play a critical role in tumor initiation, progression, and metastasis.[110,111] Cancer vaccines can be given to prevent the development of cancer in an otherwise healthy person and in such a case these are called preventive vaccines, other vaccines that are given to treat cancer are called therapeutic vaccines.[112] Vaccination can be cell-based such as dendritic cell vaccines presenting tumor-associated antigen epitope or plasmid-based which can be inoculated with or without liposome.

DNA Cancer Vaccine has been considered a very promising immunotherapeutic approach to activate the immune response against cancer due to its simplicity, stability, and safety. These vaccines are based on the bacterial plasmid and contain genes encoding tumor antigens coupled to CMV promotor.

There are optimally designed antitumor vaccination strategies are clinically tested and developed in the laboratory. These vaccines can be delivered by a variety of different routes such as intramuscular (IM), intradermal (ID), subcutaneous (SC), and mucosal.[113,114] Once, a vaccine is delivered in the nucleus, the antigen encoded by the DNA vaccine is expressed and presented on major histocompatibility molecules (MHC) for T cell activation.[115]

Dendritic cell vaccines. Dendritic cell (DC) vaccination is an alternative form of immunotherapy and is a prime candidate to enrich the treatment possibilities for cancer. A dendritic cell vaccine (sipuleucel-T) was first approved by FDA in 2010 and consists of isolated PBMCS which is cultured with a GM-CSF/Prostatic acid phosphate fusion. The use of sipuleucel-T prolonged overall survival among men with metastatic castration-resistant prostate cancer.[116] DC-based vaccination is an approach to produce an immune response to eliminate tumor cells and is safe, highly immunogenic, and able to activate an anti-tumor immune response. To generate the whole cell DC vaccines, monocytes (immature cDCs) are isolated from the patient's peripheral blood. In the case of monocyte isolation, immature moDCs are generated by culturing the isolated cells in GM-CSF and IL-4. Once immature DCs are obtained, they are activated ex vivo using a variety of cytokines and loaded with tumor-specific antigens. The matured DCs are then injected back into the patient, usually via subcutaneous or intradermal injections, although intravenous or direct injection into lymph nodes, thus present antigens to activate cytotoxic T cells and can also stimulate the B cells for antibody production, so DC vaccines can induce both innate and acquired immune system components. These vaccines are associated with limited toxicities, are therefore recognized as a relatively safe therapeutic approach, and are being extensively evaluated in the clinic.[117-119]

Plasmid DNA vaccines. Plasmid DNA-based vaccines are Third-Generation Vaccines. Fundamentally a non-viral vector (plasmid) is designed with a gene of interest (DNA or RNA), a promoter, and antibiotic resistance gene and cloning sites. These plasmid DNA vaccines can be administered into the body through different routes, such as intramuscular, intravenous, or intradermal injection. A delivery vehicle is required to transport the plasmid to the target cells. Non-viral Liposomal delivery system is used to carry DNA vaccine plasmid to the cancer cells. These vaccines are proven to be relatively specific and produce high levels of transgene expression, as well as inducing no vector-related immune responses (Figure 5).[120,121]

Cytokine therapy

Cytokines such as interleukins and interferons are the key regulator of the innate and adaptive immune system that has

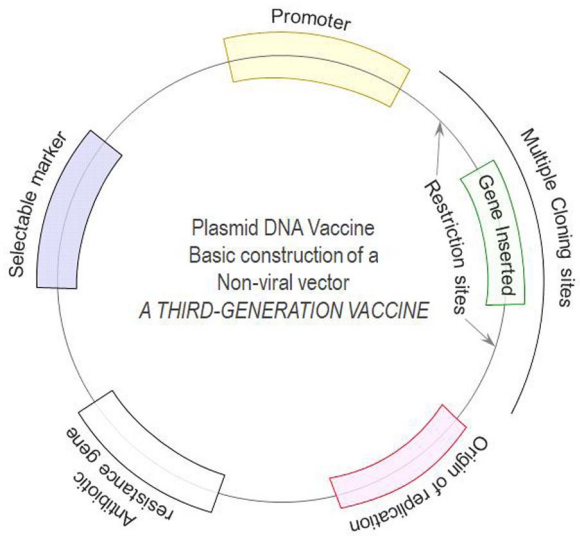

Figure 5. Basic components of a plasmid. A plasmid is constructed to have a gene of interest (expression of which is required), origin of replication (at which replication is initiated), multiple cloning sites (restriction sites for DNA insertion), antibiotic resistance gene (to select the transformed bacteria), and a promoter (transcription of an inserted gene).

therapeutic effects in various types of tumors they can be given with or without other therapeutic agents.[122,123] The function of cytokines to activate the immune system in cancer was first studied in a patient with metastatic melanoma and renal cancer. The interleukin-2 (IL-2) family of cytokines comprised of IL-2, IL-7, IL-5, and IL-21 is the most targeted family of cytokines in cancer immunotherapy,[124] IFN-α was the first cytokine approved for the treatment of human cancer. In recent years, many cytokines, including interleukin (IL)-2, IL-12, IL-15, IL-21, granulocyte-macrophage colony-stimulating factor (GM-CSF), and interferon (IFN)-α have been evaluated in clinical trials for the immunotherapy of cancer.[125] Cytokines are given with a non-viral delivery system such as liposome and without liposome depending upon the requirement. These cytokines improve the overall reaction of the immune system against the cancer cells and they can also stimulate NK cells, B-cells, and T-cells.[122,123] The IL-2 family of cytokines activates cytotoxic T-cells and natural killer cells through the activation of STAT5 which is a transcription factor responsible for cellular differentiation.[124]

Tumor suppressor gene therapy

Tumor suppressor genes play a pivotal role in maintaining genome integrity and regulating cell proliferation, cell-cycle progression, differentiation, and crucial signaling functions including apoptosis, autophagy, and necrosis. Tumor suppressor gene therapy has been observed to suppress tumor growth via induction of apoptosis and cell cycle arrest.[126] These genes are inactivated by loss-of-function (LoF) mutations that change or block tumor suppressor genes' normal functions. Their loss-of-function mutations are related directly to the development of cancer. Therefore, the use of tumor suppressor genes as anticancer therapeutics has been investigated in both experimental and clinical research, it has been reported that therapeutic strategies targeting tumor suppressor genes may help in preventing the progression of pancreatic cancer.[127] Further, novel approaches including synthetic lethality and collateral vulnerability screens have been developed to target gene defects in p53, PTEN, and BRCA1/2. In addition, various studies have shown that a combination of tumor suppressor gene therapy with conventional cancer therapy can produce synergistic therapeutic benefits.[128,129] Tumor suppressor genes have been given intravenously with a target-specific delivery system and can reduce cancer. Synthesis and construction of the complete coding sequence of wild-type plasmids of various tumor suppressor genes are possible; these tumor suppressor gene plasmids have various functions such as apoptosis induction, cell cycle arrest, and anti-angiogenesis. All of these functions lead to the inhibition of tumor cell growth.[130]

Challenges and future perspective

Cancer gene therapy involves the modification and manipulation of gene expression to cure cancer. Over the past few decades, it has developed as a potential and effective modality for the treatment of cancer disease. Nevertheless, there are some challenges including non-specific expression, low-efficiency delivery, biosafety, and difficulty in utilizing newly discovered potential genetic markers for clinical data and trials. Even though, various novel genetic strategies have been developed to make transgene expression and gene delivery safe and more effective. However, there are still several issues that limited the success of cancer gene therapy applications. Among them, the crucial factors are targeting efficiency and safety. Recent studies suggested that these issues could be resolved with the technique of targeted gene expression, including specific promotor–operating expression and distinct delivery systems. These specific promotors can be classified into 3 different categories: tissue-specific, cancer-specific, and tumor-specific.[131,132] According to previous studies, cancer- and/or tumor-specific promoters rather than tissue-specific could be a better choice to avoid non-specific expression in a normal cell, as they can restrict the expression of therapeutic genes in tumor cells, meanwhile avoiding nonspecific expression and adverse effects in normal cells.[133] Moreover, the selection of an appropriate tumor-specific promoter for a specific tumor is a critical factor for the specificity, efficiency, and safety problems of target gene therapy in cancer.

In addition, the success of gene therapy in cancer treatment relies not only on a good genetic strategy but also depends on a safe, efficient, and specific gene delivery system. Accordingly, a wide variety of genetic vectors have been developed for therapeutic gene delivery, among them, viral

vectors have shown the highest potential for efficient gene therapy but have some limitations like immunogenicity, less specificity to the target cell, toxicity, insertion mutagenesis, and limited genetic load, these limitations have encouraged researchers to focus on the development of non-viral vectors, supported by nanomedicines.[72,134] Physicochemical characteristics of nanoparticles have developed a delivery system that utilizes passive, active, and stimuli-responsive targeting strategies to improve drug delivery efficacy.[135] Because biosafety is the main concern in the design of gene delivery systems. Ideally, a vector needs to have excellent biosafety, biocompatibility, and low toxicity, as well as, should undergo complete metabolization. Non-viral vectors have an important biosafety advantage over viral vectors. Among non-viral vectors, nanostructure complexes called liposomes have been considered to be promising vectorization strategies for gene delivery, which exhibit low toxicity, and non-immunogenicity and are feasible to produce on a large scale.[136,137] Although many viral vectors are effective delivery vehicles for clinical gene therapy, some are considered risky for oncogenesis.[138,139] Therefore, further research is required with a focus on biosafety risk management; risk assessment, and mitigation plans.[140-143] Biosafety specialists play an important role in defining and meeting the need for an effective containment framework, mitigation plan, well-managed gene therapy protocol, biosafety strategies, and appropriate procedures in handling gene therapy products for health care personnel.[144] Another important obstacle in the way of successful cancer gene therapy implementation is the hurdle in the utilization of genetic markers in clinical practice. The large and expanding literature on cancer biomarkers asserts that these biomarkers can serve many clinical needs from risk stratification to prognosis, diagnosis, screening, monitoring, drug toxicity assessment, and prediction of therapeutic response, even many markers display promising results in the laboratory but they are not translated from the academic research laboratory in routine clinical care. Previous studies showed that insufficiency in sensitivity and specificity value makes them less desirable in clinical practice. Nevertheless, the new biomarkers are often clinically unfavorable and information provided by the these biomarkers is either weak or not essential for clinical acceptance. Moreover, some biomarkers show reasonable sensitivity and specificity and can address a clear clinical unmet need, but the strength of their predictive ability is below a clinically defined threshold, therefore, not persuade clinicians to adopt them. Another hindrance to biomarkers reaching the clinic is the limited evidence about their performance in a clinical setting and inadequate systemic evaluation of the available evidence, or false discovery.[145,146]

To introduce genetic biomarkers to the clinic, it is indispensable to show a useful clinical application that is supported by the validation data. The current cost of genetic tumor marker testing is another factor related to their clinical application as it is very costly to conduct a necessary trial to obtain approval from the Food and Drug Administration (FDA).[145]

The Early Detection Research Network (EDRN), is an organization with a mandate to discover, and validate cancer biomarkers and introduce them to the clinic, so that they can contribute to better patient care through the personalized management of cancer.[147] EDRN has implemented measures to improve biomarker discovery and validation such as data sharing, the use of common data elements, collaborative studies, and a strong emphasis on quality control. A well-designed strategy to accomplish the mandate of the EDRN will lead to decreased incidences of false discovery as well as better usage of funding and time, by focusing improvement on well-validated and high-performance biomarkers that will increase their implementation in the routine clinical care of cancer patients. The use of careful study design, large datasets, continuous progression in genetic technologies and bioinformatics methodologies, better reporting, careful clinical validation, and large collaborative studies facilitates the collection of valuable information about unmet clinical needs and improves the clinical utility of biomarkers.[145,146,148] Moreover, larger clinical trials are needed to fully explore the potential of genetic tumor markers for early diagnosis, prognosis, and drug selection. Professionals, as well as researchers and scientists, play a crucial role and make concerted efforts to earn and safeguard public trust in clinical trials and take a step to engage the public through current discussions/forums, and provide them understandable information about gene drive technology.

Moreover, gene therapy will play a pivotal role in future cancer care as part of multimodality treatment in combination with/or following other forms of cancer therapies such as chemotherapy, radiation therapy, and surgery. It has been observed that tumor suppressor gene therapy asserts synergistic effects in combination with conventional therapy.[129,149-151] Further research should be focused on well-designed experiments in combinational therapy studies including gene therapy with chemotherapy, molecular inhibitors therapy, radiation therapy, and immunotherapy will help in the development of cost-effective therapeutic strategies to improve treatment outcomes for cancer patients and decrease the death rate from cancer.

Conclusion

Gene therapy is one of the intriguing approaches in medical genetics for the treatment of cancer which enables genetic improvement through the correction of the defective (mutated) gene or site-specific alteration that targets treatment. Cancer gene therapy offers several potential treatment methods to fight this disease and has advantages over conventional therapies because high therapeutic doses can be administered via gene therapy technique without systemic adverse effects. Many of the past obstacles to treating cancer have been overcome with the use of gene therapy techniques and its potential for precision medicine is worthwhile and its expectation for

curing and preventing cancer disease is promising. It has been recognized as the most emerging approach in the arena of biotechnology to the current status of progress as well as future possibilities. Nevertheless, there is still a need for more rigorous research in cancer genetics to improve the clinical utility of emerging gene therapy technologies to ensure cancer elimination and normal cell maintenance. All scientists, pharmaceutical companies, and medical professionals must work together to ensure that safe, life-long, and cost-effective gene therapies become available to help treat patients with cancer.

Author Contributions

R.Q. and A.Q conceptualized, researched, and wrote this manuscript. All authors have read and agreed to the published version of the final manuscript.

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