Abstract: Adeno-associated virus EzH2 shRNA (AAV EzH2 shRNA) based treatment, incorporating targeted gene silencing of cancer metastasis promoting gene EzH2, through RNA interference (RNAi), mediated by recombinant adeno-associated virus vector, is provided. The present invention provides an efficient and safe therapeutic choice that can inhibit breast cancer progression in vitro and in vivo. The recombinant AAV vector covers the serotypes 1, 2, 3, 4, 5, 6, 7 or any homologous serotypes or hybrids thereof.
FIELD OF INVENTION

[001] The present invention generally relates to the field of interference RNA (RNAO) and more particularly to the treatment of cancer by RNA interference (RNAO-)

BACKGROUND OF INVENTION

[002] Diseases caused by an abnormal gene function are potentially manageable by silencing the specific gene mediated by small, non-coding polynucleotide molecules, particularly short interfering RNA (siRNA) through RNA interference. Silencing a specific gene, correlated with any metabolic or a malignant disease like cancer with a siRNA constitutes a potential therapeutic approach.

[003] Increasing evidences indicate that EzH2 (Enhancer of Zeste-2) is regarded as a metastatic determinant and a key component in tumor metastasis in several malignancies including breast cancer. EzH2 is a catalytic sub-unit of polycomb group of proteins, which catalyzes methylation of chromatin leading to transcriptional silencing of several tumor suppressor genes or anti-oncogenes. A compromised expression of anti-oncogenes contributes to tumor cell division, cancer aggressiveness and metastasis in several human cancers including breast cancer resulting in poor prognosis. Since there is an over expression of EzH2, a known metastasis promoting gene in many cancers in their metastatatic stages; its down-regulation mediated by a specific siRNA administered in a pharmaceutically acceptable carrier molecule or formulation is essential.
[004] RNA interference refers to the process of sequence-specific post-transcriptional gene silencing in animals mediated by short interfering RNAs (siRNAs). RNA interference with shRNA is an innate cellular process involving a set of enzymes which is activated by double stranded RNA (dsRNA) molecule causing the degradation of endogenous target mRNA.

[005] Many inventions are directed towards the use of viral vectors, which are generally more efficient vehicles in vivo than non-viral vectors. Several viral vectors such as adenovirus, retro- or lentivirus, and adeno-associated virus (AAV) have been successfully used for gene silencing therapy for RNAi. Although viral vectors permit the efficient delivery and stable expression of shRNA; establishment of safety, efficacy and potent gene silencing are crucial ingredients for selecting the viral delivery vehicle. Retro-lentiviral vectors randomly integrate into genome and generate insertional mutagenesis and are derived from pathogenic viruses. Adenoviral vectors have also been successfully used for the delivery of shRNA but they are well known to trigger unacceptable levels of immune response due to their large size.

[006] Adeno-associated virus (AAV) is one of most promising vectors for gene therapy. The recombinant AAV (rAAV) provides a non-pathogenic and latent infection by integration into the host genome. It also shows high transduction efficiency of both dividing and non-dividing cells and tissues with persistent transgene expression. Such recombinant AAVs have the advantage of exhibiting modified tropism, (i.e., being highly selective with respect to the tissues it infects), as well as having a higher rate of transduction efficiency when compared to native AAV transduction of selective tissues. The AAV vector harboring
the nucleotide sequence encoding a protein of interest, e.g., chimeric growth factor receptor, and a post-transcriptional regulatory sequence (PRE) flanked by AAV ITRs (Inverted Terminal Repeats), with a selective promoter such as CMV can be constructed by directly inserting the nucleotide sequence encoding the protein of interest and the PRE into an AAV genome which has had the major AAV open reading frames ("ORFs") excised there from. However, major challenges of delivery, specificity and efficacy need to be overcome before siRNAs can be used as therapeutic agents. RNAi technology could be further developed into therapeutics for cancer by selectively silencing aberrantly activated oncogenes. RNA interference is often seen as a promising way to treat cancer by silencing genes that are differentially upregulated in tumor cells or genes involved in cell division. A key area of research in the use of RNAi for clinical applications is the development of a safe delivery method, which to date has involved mainly viral vector systems similar to those suggested for gene therapy.

[007] Self-complimentary adeno-associated viral (scAAV) vectors are safe, clinically proven and efficient that ensures continuous production of RNAi molecules within the cell. One of the drawbacks of AAV vectors is that they often have low transduction efficiencies, which requires large doses of vectors to achieve desired effect. The reason is that the AAV capsids are phosphorylated at tyrosine residues in the cell, which leads to ubiquitin-proteasome degradation of majority of AAV particles leading to inefficient transduction.

20[008] Hence, there exists a need for improved treatment of breast cancer incorporating the expression of shRNA in a recombinant AAV vector with better transduction efficiencies.
OBJECT OF INVENTION

[009] The principal object of the invention is to provide an improved treatment of breast cancer by utilizing a recombinant AAV vector for down-regulation of EzH2.

5

STATEMENT OF INVENTION

[0010] Accordingly the invention provides a vector for down-regulation of EzH2 which is characterized by a vector of polynucleotide sequence of SEQ ID NO:1.

[0011] There is also provided a pharmaceutical composition for down-regulation of EzH2 which is characterized by a vector of polynucleotide sequence of SEQ ID NO:1 and a pharmaceutically acceptable carrier.

[0012] In another embodiment, the invention provides a method for down-regulation of EzH2 by administering a vector of polynucleotide sequence of SEQ ID NO:1.

[0013] In yet another embodiment, the invention provides a method for treating a patient having a disease associated with over expression of EzH2 by administering to the patient a therapeutically effective amount of a vector of polynucleotide sequence of SEQ ID NO:1.

BRIEF DESCRIPTION OF FIGURES

20[001] This invention is illustrated in the accompanying drawings, through out which like reference letters indicate corresponding parts in the various figures. The embodiments
herein will be better understood from the following description with reference to the drawings, in which:

[002] FIG.1A is a Schematic diagram depicting the construction of recombinant vector, AAV-EzH2-shRNA.

[003] FIG.1B-1, 1B-2 and 1B-3 are schematic diagrams depicting the consensus nucleotide sequence of chimeric virus vector, AAV-EzH2-shRNA.

[004] FIG.2 is a schematic diagram depicting a graphical illustration of the effect of AAV-EzH2-shRNA on the growth of MCF-7 breast cancer cell line.

[005] FIG.3 is a schematic diagram depicting immuno-histochemical detection of EzH2 in normal breast tissue and malignant breast cancer tissue under 400X magnification.

[006] FIG.4A is a schematic diagram depicting subcutaneous tumor growth in SCID mice by MCF-7 cells which are transduced by AAV-EzH2-shRNA.

[007] FIG.4B is a Schematic diagram depicting subcutaneous tumor growth in SCID mice transduced by control MCF-7 cells showing profuse tumors.
DETAILED DESCRIPTION OF INVENTION

[008] The embodiments herein and the various features and advantageous details thereof are explained more fully with reference to the non-limiting embodiments that are illustrated in the accompanying drawings and detailed in the following description. Descriptions of well-known components and processing techniques are omitted so as to not unnecessarily obscure the embodiments herein. The examples used herein are intended merely to facilitate an understanding of ways in which the embodiments herein may be practiced and to further enable those of skill in the art to practice the embodiments herein. Accordingly, the examples should not be construed as limiting the scope of the embodiments herein.

[009] It is to be understood that the present disclosure is not limited in its application to the details of construction and the arrangement of components set forth in the following description or illustrated in the drawings. The present disclosure is capable of other embodiments and of being practiced or of being carried out in various ways. Also, it is to be understood that the phraseology and terminology used herein is for the purpose of description and should not be regarded as limiting.

[0010] The use of "including", "comprising" or "having" and variations thereof herein is meant to encompass the items listed thereafter and equivalents thereof as well as additional items. The terms "a" and "an" herein do not denote a limitation of quantity, but rather denote the presence of at least one of the referenced item. Further, the use of terms "first", "second", and "third", and the like, herein do not denote any order, quantity, or importance, but rather are used to distinguish one element from another.
One embodiment is directed towards the construction and expression of a chimeric virus vector, AAV-EzH2-shRNA, wherein a short hairpin RNA specific for EzH2 is cloned in a recombinant adeno-associated virus vector serotype-2 and the subsequently expressed small interfering RNA selectively targets EzH2, a breast cancer metastasis promoting gene, a key factor in metastasis of breast and many other cancers. The vector based medicine with a pharmaceutically acceptable carrier, wherein the pharmaceutically acceptable carrier includes without limitation simple saline and buffer, is directly administered to the breast tissue of subjects resulting in an increased drug exposure that achieves tumor regression more significantly than systemic delivery.

The embodiments are better illustrated in the accompanying drawings, throughout which like reference letters indicate corresponding parts in the various figures: Referring now to the drawings, and more particularly to FIG. 1A, FIG.1B-1, 1B-2, 1B-3, FIG. 2, FIG 3, FIG 4A and FIG 4B, where there are shown preferred embodiments.

FIG. 1A is a diagram depicting the construction of AAV-EzH2-shRNA, a recombinant virus vector where an entire shRNA sequence of EzH2 with sense strand, stem loop structure and antisense is cloned into multiple cloning sites of AAV-shRNA. As such, the vector constructed is a modified AAV vector in which the expression of EzH2-shRNA is being driven by human U6 promoter. Adeno-associated viruses, from the parovirus family, are small viruses with a genome of single stranded DNA. The recombinant AAV does not contain any predominant viral genes but contains the overwhelming therapeutic gene and does not integrate into the genome. Instead, the recombinant viral genome fuses at its ends via the ITR recombination to form circular, episomal form, which are predicted to be the
primary cause of the long term gene expression. The vector also accommodates several marker genes to help the functional analysis of a gene of interest.

[0014] Recombinant AAV vectors containing the siRNA expression cassette can be packaged efficiently and can be used to successfully transduce the target cells at high frequency and with minimal toxicity. Adeno-associated virus (AAV) is currently being tested in several human gene therapy trials because of its several unique features that distinguish it from other gene therapy vectors. These include (i) ability to infect both dividing and non-dividing cells; (ii) a broad host range; (iii) wild-type AAV has never been associated with any disease and cannot replicate in infected cells; (iv) lack of cell-mediated immune response against the vector and (v) lack of insertional mutagenesis (vi) minimal influence on changing the pattern of cellular gene expression and the like.

[0015] FIG. 1B-1, FIG. 1B-2 and FIG. 1B-3 are diagrams depicting the consensus nucleotide sequence of a chimeric AAV-EzH2-shRNA vector with reference to the restriction endonuclease sites, EcoRI and PspOMI. According to an embodiment, a self-complementary adeno-associated virus (scAAV) vector also known as double-stranded AAV (dsAAV) is employed which significantly minimizes the vector load required to achieve sustained transgene expression. However, the efficiency of these vectors, in terms of the number of genome-containing particles required for transduction, is hindered by the need to convert the single-stranded DNA (ssDNA) genome into double-stranded DNA (dsDNA) prior to expression. This step can be entirely circumvented through the use of self-complementary vectors, which package an inverted repeat genome that can fold into dsDNA
without the requirement for DNA synthesis or base-pairing between multiple vector genomes.

[0016] Persistent siRNA expression is highly desirable to inhibit EzH2, which requires the intracellular expression of siRNA by continued production of siRNA. This is possible if a DNA template coding for a short, self-complementary RNA sequence in tandem, separated by a 'spacer or bridge (sometimes called stem-loop), is transfected into cells as part of a plasmid or introduced by recombinant viral vectors. This generates a single stem-loop of sense and antisense strands in the form of short hairpin RNA (shRNA) that will be cleaved by the Dicer to produce the active siRNA.

[0017] FIG.2 is a graph depicting the effect of AAV-EzH2-shRNA on the growth of MOF-7 breast cancer cell line. These cancer cells are cultured for indicated time intervals after AAV-EzH2-shRNA transduction. Cell growth is assessed by CCK-8 cell proliferation assay method. As such, MCF-7 cells are transduced with two different clones of AAV-EzH2-shRNA and cultured over a period of three days. Both clones result in time dependent inhibition of cell proliferation and induced cell death. The extent of cell death is around 80% with clone AAV-EzH2-shRNA-2 showing efficient cell killing than clone AAV-EzH2-shRNA-1. EzH2 is one of a set of 70 genes whose expression predicts a poor outcome in breast cancer and most patients with high EzH2 exhibit this poor prognosis signature. EzH2 over expression correlates with late stage disease and can even be an independent predictor of aggressive breast cancer.

[0018] FIG.3 is a diagram depicting immuno-histochemical detection of EzH2. The immuno-histochemical detection of EzH2 depicts a normal breast tissue and a
malignant breast cancer tissue 304 under 400X magnification, according to an embodiment.

The over-expression of EzH2 is associated with vascular invasion and aggressive MCF-7 cell line through immuno-histochemical staining of EzH2 antibodies in breast cancer biopsies along with adjacent normal tissue. As shown in FIG. 3, the extent of EzH2 expression (percentage of positive cells) is higher in the cases of patients who have advanced disease with lymphatic and vascular invasion with minimal staining in control breast cancer sections. IHC figures clearly indicate that the extent of EzH2 expression is directly proportional to the aggressiveness of the disease.

[0019] FIG. 4A is a diagram 400a depicting the tumor-forming ability of MCF-7 cells when they are transduced with EzH2-shRNA. Tumor-forming ability of MCF-7 cells is substantially reduced clearly indicating that EzH2 is needed for the growth of breast tumors in vivo.

[0020] FIG. 4B is a diagram 400b depicting MCF-7 cells which produce palpable tumors. It is observed that tumors formed by transfection with control MCF-7 cell after subcutaneous injection of 3 million cells in CB17/ICr-SCID mice is without any notable rejection.

[0021] According to an embodiment, targeted gene silencing of breast cancer metastasis associated gene EzH2, by RNA interference (RNAi), mediated by recombinant vector rAAV-2-EzH2-shRNA, can inhibit breast cancer progression in vitro and in vivo. The siRN20 expressed from viral vectors in vitro and in vivo specifically reduce expression of stably expressed plasmids in cells, endogenous genes and transgenes in animal models. The ability of viral vectors based on AAV to transduce cells efficiently in specific tissues,
coupled with effectiveness of virally expressed siRNA will extend the application of siRNA to viral-based therapies and to basic research.

[0022] As will be appreciated by a person skilled in the art, the present invention provides a variety of advantages. As per an embodiment, shRNA of EzH2 is cloned in a self-complementary AAV-2 vector to express siRNA that selectively targets and silence EzH2, a metastasis promoting gene in breast cancer. It offers an efficient and safe therapeutic option to arrest the progression of metastasizing breast cancer and other metastasizing cancers in vitro and in vivo. This recombinant vector is administered in a pharmaceutically acceptable carrier molecule or formulation which can be used as an adjuvant therapy after surgery or radiation therapy. It can be used for direct infusion to breast cancer tissue for targeted and faster elimination of breast cancer cells. The present inventory compound can evade various cytotoxic side effects associated with many chemotherapeutic agents and is specific to cancer cells that do not cause severe side effects. The present invention as a drug can be administered directly into the breast tissue, which exposes the tumor to very high doses of the drug than systemic infusion. An increased drug exposure achieved by the vector based medicine results in tumor regression more significantly than systemic drug delivery and also eliminates any possible side effects on other organs. Using a shRNA cloned into the AAV provides a possibility of enforceable and stable expression of shRNA in vector systems that ensure an efficient EzH2 silencing activity.

20[0023] According to an embodiment, application of new generation of rAAV vectors for gene silencing that are not only self-complimentary AAV-2 vectors (scAAV-2) provide for efficient transgene expression. Moreover, capsid mutations in these vectors help in
circumventing cytosomal degradation and enhance the transduction by approximately 20 fold further leading to high-efficiency transduction at low doses.

[0024] In another embodiment, the AAV vector contains mutation(s) resulting in an amino acid substitution of the capsid protein at critical amino acid positions. According to an embodiment, recombinant AAV vectors which show increased transduction efficiencies can be achieved by replacing amino acid residues located, at critical positions (Y: Tyrosine; F: Phenylalanine; Y444F, Y500F and Y730F).

[0025] siPvNAs have favorable pharmacokinetic properties and can be delivered to a wide range of organs. siRNA based therapeutics offer a highly selective gene therapy to severM) metastatic cancers including breast cancer in subjects who failed to respond to conventional therapies through a specific post transcriptional gene silencing mechanism.

[0026] For purposes of this invention, by "AAV vector", "recombinant adeno-associated virus vector", "adeno-associated viral vectors" or "rAAV vector" is meant a vector derived from an adeno-associated virus serotype, including without limitation, AAV-1, AAV-2, AAV-3, AAV-4, AAV-5, AAV-6, AAV-7, AAV-8 etc. or any other virus or serotype which is substantially homologous in its capsid protein sequence to the AAV2 or capsid protein sequence.

[0027] While specific embodiments of the invention have been shown and described in detail to illustrate the inventive principles, it will be understood that the invention may be embodied otherwise without departing from such principles.

[0028] The foregoing description of the specific embodiments will so fully reveal the general nature of the embodiments herein that others can, by applying current knowledge,
readily modify and/or adapt for various applications such specific embodiments without departing from the generic concept, and, therefore, such adaptations and modifications should and are intended to be comprehended within the meaning and range of equivalents of the disclosed embodiments. It is to be understood that the phraseology or terminology employed herein is for the purpose of description and not of limitation. Therefore, while the embodiments herein have been described in terms of preferred embodiments, those skilled in the art will recognize that the embodiments herein can be practiced with modification within the spirit and scope of the embodiments as described herein.
WE CLAIM:

1. A vector for down-regulation of EzH2 comprising a polynucleotide sequence of SEQ ID NO:1.

2. The vector for down-regulation of EzH2 as claimed in claim 1, wherein the vector is a recombinant AAV vector of serotype selected from the group consisting of serotype 1, 2, 3, 4, 5, 6, 7 or 8 or any homologous serotypes or hybrids thereof.

3. The vector for down-regulation of EzH2 as claimed in claim 2, wherein the vector is AAV2 vector.

4. The vector for down-regulation of EzH2 as claimed in claim 2, wherein the recombinant AAV vector comprises of a modified capsid.

5. The vector for down-regulation of EzH2 as claimed in claim 4, wherein the modified capsid comprises of at least one amino acid substitution.

6. The vector for down-regulation of EzH2 as claimed in claim 5, wherein the amino acid substitution comprises of substituting tyrosine with phenylalanine.

7. The vector for down-regulation of EzH2 as claimed in claim 6, wherein the amino acid substitution is at least one of Y444F, Y500F and Y730F.

8. A cell line comprising a vector for down-regulation of EzH2, wherein said vector comprises of a polynucleotide sequence of SEQ ID NO: 1.

9. A pharmaceutical composition for down-regulation of EzH2 comprising:

   a vector of polynucleotide sequence of SEQ ID NO: 1; and

   a pharmaceutically acceptable carrier.
10. A method for down-regulation of EzH2 comprising: administering to a subject the vector of polynucleotide sequence of SEQ ID NO: 1.

11. A method for treating a patient having a disease associated with over-expression of EzH2, comprising:

administering to the patient a therapeutically effective amount of the vector of polynucleotide sequence of SEQ ID NO: 1.

12. The method as claimed in claim 2, wherein the disease associated with over-expression of EzH2 is breast cancer.
FIG. 1A

AAV-EzH2-shRNA
6839 bp

Left ITR
CMV IE enhancer
Hybrid Intron
EGFP
SV 40 poly-A
U6 promoter
EzH2 shRNA
f1 origin
Right ITR
lacZ cassette
AmpR
pUC
Sequence Listing

1  CCTGAGGCA  GCTGCGGGCT  CGCTGCTCTA  CTGAGGGCCG  CGGCGAAAG
51  CGGCGGGTGC  GGGCGGACTTT  TGCTGCCCCG  GCTCTAGTAG  GGGCGGGAGC
101  GGGCGAGAGG  GGAGTGAGACT  GGGAGAACAT  GAGACATTTT  GGATTAAAAA  CAAGACTTTT
151  CCTAAAGAGA  ATTTGCTTTT  CAGATTTATT  GGATTAABA  AAAGACTTTT
201  CTATAACGCGG  CGCGTGCGTGC  CGACCTGACG  CAATTCTCAT  GTTGAGACGC
251  TTATCATCAGC  AGATCAGCGG  AACGTTTGGG  CCATTGCGTG  AGGCGGAGAA
301  GTGCTAGGTA  GGGAAAGACT  ATACATGGAAT  TAATAGTTT  CAATAGTTAG
351  TATAGTACAT  TGTTATATATA  GCATATAATCA  ATATGTAGCT  TTGGCCATTG
401  CATAGTGTTG  ATCTATATAC  TAATAGTTAG  ATTTATATTT  CTAATGTGCC
451  AATATGACCC  CGATGTGGAG  ATTTTGATTAG  GACTAGTTAT  TAATAGTTAT
501  CAATTTACGGG  GTCAATTAGTT  CATTAGCCCT  TATAGGAGGT  CGCGGTACACA
551  TAATCCTACGA  TAAATGCCCC  GCTTGCGTGA  CGCCCCAAAG  ACCCGCGCGC
601  ATTTAGCCTCA  ATATACTAACT  ATGTCCCCT  AGTAACCGCA  ATAGGGCCTT
651  TCCATTGACG  TCAATGGGCTG  GAAGTTTATC  GCTAAATGAC  CCACTTGCGA
701  GTACATCAAG  TGATATCATAT  GCCAAAGTCCG  CCCCTATGAT  AGTCAATGAG
751  CGGTTAAATGG  CGCGCTGCGG  ATATTGCCCC  GACATGAGCC  TACGAGGCCT
801  TTCTCTACTTG  GCAGTACTAC  TACGTTATTG  TACGCTGCTAT  TACGATGTTG
851  ATGCAGTTTTG  GGCAGTAGAC  GAATGCGCGT  GGAATGGGGG  TTGACTCGAG
901  GGGAGTTTCA  GGTCTCCTAC  CAGTTGGAGT  CAATGCGGAG  TTGTTTGGGC
951  ACCCAAATCG  AGCCGACCTTT  CCAAAATGTC  GTAAATACCC  CGCCCCGTTG
1001  AGCCAAATGGG  GCCTGATGCCG  TGTACCGGTTG  GAGGTCTATA  TAAGCAGAGC
1051  AGCCAAATGGG  GCCTGATGCCG  TGTACCGGTTG  GAGGTCTATA  TAAGCAGAGC
1051  TCGTGTGATG  AACGCTGAGA  TCCTAGAGA  CTGGAACCAG  CAGAGGCGCC
1101  TTAATATCTTAC  CATGGTGGAG  AAGCAGATCC  TGAAGAACAC  CGGCCGTCAG
1151  GAGACATGTA  GCTTCAAAGT  GAACCTGGAG  GGGCTGGTGTA  ACAACCAAGC
1201  GTCTCACAGT  GAGCGTGGCG  CAAAGGCGCA  CATCGTTGTC  GGGCCACGCG
1251  TGCGGAGGAG  AAGGGGCACC  CCCCTGGCCTT  CGCTCGTCGAC
1301  ATCCGGGAGC  CGGCTGGTCC  TACGCGGCCAC  CGCCACCTCA  CCAGATACCC
1351  CGGAGACATC  AGGCACTTCT  TCATCCGAGG  CTCCCGCGCCG  GCTTGGTGT
1401  AGGAGGCGAC  CCTGCGCTTAC  GAGGACGGGG  GCTGCTGGAG  GATCCCCAGC

FIG. 1B-1
FIG. 1B-2
FIG. 1B-3
FIG. 2

Cell Survival (% Control) over Days

Control, AAV-EzH2-shRNA-1, AAV-EzH2-shRNA-2

FIG. 3

Images 300, 302, 304