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shRNAs: A few keypoints about their applications

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Abstract
Epigenetics modifications regulate and process gene expression through small and non-coding RNAs mechanism. In fact this mechanism which is termed "RNA interference (RNAi)" is a protected phenomenon in post-transcriptional or pre-transcriptional level which regulates the expression of different genes through microRNA (miRNA) and interfering RNA (siRNA). mRNA degradation is done by siRNA mechanism including siRNAs and shRNAs. These synthesized dsRNAs have a wide range of applications in targeting variety of genes and are sufficient tools for therapeutics aims. In this paper, we reviewed RNAi molecular mechanism and its application in common human cancers including cervical prostate, breast, lung, gastric, colon, head, neck, skin and oral cancers.

Keywords: Gene silencing; RNAi; shRNAs; cancer

Introduction
Epigenetics is heritable and reversible alternations in gene activity and expression without any change in DNA sequencing, [1] transmitted through mitosis division into daughter cells [2] and has an impressive role in evolution and differentiation [3]. In fact, epigenetics regulates access to whole cell's genome. Epigenetics modifications which used as a marker for studying gene functions and expression, performed through four major mechanisms: Histone modifications, DNA methylation, chromatin architecture and small and non-coding RNAs [1].Inappropriate operations of these mechanisms lead to diseases such as cancer [2].
In non-protein-coding RNAs (ncRNAs) mechanism, involved in processing and regulation of other RNAs, RNA-based ncRNA (include miRNA, siRNA and PIWI-interacting RNAs (piRNA)) and some other long ncRNAs have significant impact on epigenetics and processing [4]. RNAi is a highly protected mechanism in post-transcriptional or pre-translational level that will be created in response to double stranded RNA and down-regulate the gene expression. Actually, it targets the endogenous mRNA and results in cleavage and gene knock down [2]. RNAi was discovered initially in nematode *Caenorhabditis elegans* (C. elegans). For the first time in 1998, Mello and Adrew Fire demonstrated the interference role of dsRNAs in sequence-specific degradation when they knocked out a gene for making a muscle filament protein by introducing double strand RNA encoding that protein. After that time, this silencing response has been found in other eukaryotes such as yeast [5-8]. RNAi can act through miRNA and siRNA [4]. siRNA was the first synthetic silencer which could successfully reduce the target gene expression in mammalian cells temporarily. But this method also faced with challenges such that this molecule could only be used in short-term extinction [9]. Until the year 2002, resistant short hairpin structure was made to be used for long-term studies [10]. Introduction of short hairpin RNA (shRNA) into cell through infection by viral vectors allows stable integration of genes, whereas the previous method including cytosolic delivery of siRNA, was limited to cell transfection and *in vitro* studies [11].
Silva and his colleagues presented a model of shRNA that has a structure similar to natural miRNAs of cell which are made by the cell. They termed the model "second generation of shRNAs" or microRNA-adapted shRNA (shRNAmir). shRNAmir has a similar structure to miRNAs and imitates the behavior of natural miRNAs of cells [12]. shRNAs can be divided into two main categories based on their design: simple stem-loop and microRNA-adapted shRNA.
In this review, article we have provided all molecular data about shRNA and siRNA and their functions through RNAi mechanism.

Molecular mechanism

RNAi or posttranscriptional gene silencing (PTGS) is a post-transcriptional and regulation event which inhibits the homologous mRNA translation [13] through two pathways. The first one is "antisense inhibition" through single-stranded RNAs (ssRNA) which lead to mRNA suppression by non-specific binding of 21nt miRNAs to target mRNA. The second one is mRNA degradation by dsRNAs which are more sufficient than ssRNAs [14]. These synthesized dsRNAs including siRNAs and shRNAs [9, 10]. siRNAs are double stranded sequences related to target gene, produced *in vitro* by transcription of both version of sense and antisense strands of the sequence [15]. shRNAs are specific double stranded RNAs in nucleus of transfected/transduced cells in vivo [14, 15, 16]. Therefore, a loop will be formed to make a hairpin structure known as small hairpin RNA. These shRNAs will be broken into siRNAs by Dicer enzyme [14]. Indeed, siRNAs are intermediates of RNAi mechanism and they will be processed within the same RNAi machinery [13].

shRNAs enter the nuclei by bacterial or viral vectors then they were transcribed by DNA polymerase II or III (depends on promoter driving their expression) and form precursors, which were processed by Drosha (RNase III) and dsRNA-binding partner (DGCR 8) and results in production of pre-shRNAs. These pre-shRNAs were exported into cytoplasm by exportin-5 protein and cleaved by Dicer enzyme. Dicer enzyme converts the dsRNAs to 20-25 bp siRNAs, with a 2nt overhangs on each 3'-end, without any change in sugar-phosphate backbone. The products were loaded into the cytoplasmic RNA-induced silencing complex (RISC) contains dsRNA binding proteins, including protein kinase RNA activator (PACT), transactivation response RNA binding protein (TRBP), and Dicer [15, 17, 18]. The sense (passenger) strand is degraded. The antisense (guide) strand directs RISC to mRNA that has a complementary sequence. In the case of perfect complementarity, RISC cleaves the mRNA. In the case of imperfect complementarity, RISC represses translation of the mRNA. In both of these cases, the shRNA leads to target gene silencing (Figure 1) [18].

The advantage of shRNA over siRNA is the ability to utilize the viral vectors for delivery to overcome the transfecting certain cell type problems [14]. Indeed shRNA expression in cells is possible by plasmids delivery with viral, bacterial or nonbacterial vectors. An appropriate transporter must have the ability of penetration inside the cell and also inside nuclear membrane [11, 19, 20].

Since, cells have different sensitivities to introduction of nucleic acid, depending on the type of cells, scientists use various method for shRNAs delivery into the cells, including: transfection, electroporation and transduction using viral vectors.

In transfection method, shRNA are encoded on a plasmid and introduced into the cell by one of the following methods: lipid transfection, cationic polymer-based nanoparticles, and lipid or cell-penetrating peptide (CPP) conjugation. In electroporation, an electrical field was applied on the cell membrane that phospholipids with negative charge on the membrane creating holes and allow siRNAs entrance into the cell. In transduction using viral vectors, Retroviral and Lentiviral plasmids, Adenoviral vectors, and Adeno-associated virus (AAV) are utilized [11, 14, 19]. There are some concerns about biosafety immunogenicity and pro inflammatory effects of these viruses. But the Herpes simplex has a high capacity for gene transfer that is appropriate for gene therapy. Also, SV40 vectors are very sufficient for *in vitro* and *in vivo* packaging and are useful in delivery into a variety range of cells [21, 22]. Furthermore, shRNA technology is a cheap method with more resistant structure in comparison with siRNAs, which make them appropriate tools for long lasting studies.

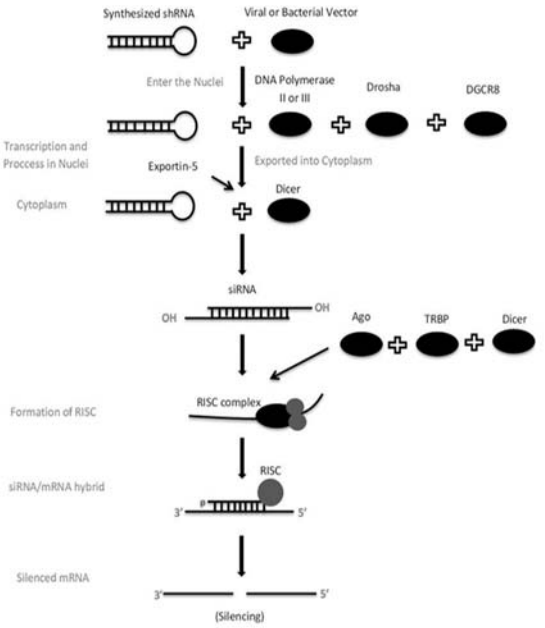

Fig 1: Molecular mechanism of shRNA.

Applications

Different applications of shRNAs are mentioned in table 1. Our major aim is to collect all data about their application in certain cancers. Cancer is a common disease all over the world and is the second cause of deaths in the United States [31, 32]. There are limiting numbers of drug available which can be used as a sufficient and safe treatment for cancer. To improve this situation, novel and specific methods of treatment are required [33].

Discoveries between 2001 to 2002 proved that RNAi mechanism is active in mammalians too, and led to an exclusive phenomenon in which for the first time scientists could silence the expression of a target gene effectively by the use of its specified sequence as an approach for effective treatment purposes and other studies [34]. Recent studies illustrate that RNAi mechanism can be used in experimental studies widely for regulation or suppression of gene expression. Researchers have been able to reduce the expression of a vast variety of molecules in tumor cells by RNAi technique [35].

Table 1: Applications of shRNAs.

Applications	References
Utilized for Therapeutic aims (gene silencing and/or RNA-based drugs) in different diseases such as cancer	[23]
modulators of the mammalian circadian clock	[24]
host mediators of HIV-1 replication	[25]
regulators of cell migration	[26]
Producing artificial products(e.g. plants) for different aims such as increasing yields or protecting plant from insects	[27] [28] [29] [30]
Utilized for Engineering and biotechnological studies	[27] [28] [29] [30]
Studying genes function	[23] [31]

Cervical cancer

Gu and his colleagues confirmed the importance of shRNA in cervical cancer treatment. The *in vitro* and *in vivo* studies demonstrated that shRNA can lead to target gene expression silencing through RNAi mechanism in a specific manner [36]. Likewise, another study proved the prominent role of these synthesized materials in cervical cancer treatment. In this study, shRNA and siRNA were delivered by lentiviral vectors, shown as shRNA-LV and siRNA-LV. Then they have been tested on mouse model *in vitro*. The results indicated that shRNA can cause inhibition of the human papillomavirus (HPV) oncogenes E6 and E7 in cervical cancer cells [37].

Prostate cancer:

SATB1 is an essential protein in prostate cancer mechanism which lead to cancer growth and metastasis in this organ. During an investigation which was done to verify the role of shRNA in prostate cancer, ZD55 adenoviral vectors have been used for shRNA delivery (ZD55-shRNA) and then tested on LNCap and Du145 cells. Next experiment was carried out on mouse model. The investigation results demonstrated that shRNAs can decrease significantly the expression of SATM1 in both *in vitro* and *in vivo* experiments [38]. Besides, in another studies were done by Czauderha, expression of two catalic subunits of phosphatidylinositol 3 kinase (PI 3-kinase), p110α and p110β, were decreased significantly by shRNA [39].

Breast cancer

In 2013, Notch1 was targeted by shRNA during the studies which were done by Mao and colleagues on NCF-7 line cell. It demonstrated that by down regulation of Notch1, the expression of NF-κB, an anti- apoptosis protein, was decreased dramatically and therefore results in reduction in cell proliferation and reduction of apoptosis [40]. In addition, based on the experiments using Reverse transcription polymerase chain reaction (RT-PCR) and western blotting, proved that the expression of ALDH1, NICD, Hes1 and drug transporter ABCG2 were decreased significantly in both *in vivo* and *in vitro* by Notch1 knockdown. This evidence indicated the important role of Notch1 as a resistant agent to paclitaxel and also the importance of targeting it in breast cancer treatment.

ER81 is a transcription factor which is located in downstream region of HER 2. Several studies confirmed that the expression of this gene was increased in breast cancer lines, breast typical hyperplasia and primary breast cancer (triple negative). In one study carried out on primary breast cancer including triple negative, the expression of ER81 was decreased by shRNA. Then ER81-shRNA was transfected into MDA-MB-231 cell line and nude mice. The results proved that through successful silencing of ER81 by shRNA, cell proliferation was diminished gradually [41]. Another study by Han and colleagues asserts that shRNAs can be used in breast cancer treatment as a medicine resistance reducible agent. This research was performed by shRNA-lentiviral vectors, the expression of LAMP2A was reduced significantly and caused reduction in medicine resistance to paclitaxel [42].

Lung cancer

X-linked inhibitor of apoptosis protein (XIAP) is a protein whose expression was increased dramatically in lung cancer. During an investigation by Zhang and colleagues, a specific shRNA were delivered by plasmid vector into A549NSCLS cell line tissue made to knockdown XIAP. Likewise, celecoxiB (CXB) which is a sufficient medicine for lung cancer treatment particularly non-small cell lung cancer type injected into the target tissue. As a result, cell proliferation, migration and invasion were reduced significantly. This reduction results in induction of apoptosis. It proved that CXB and XIAP-shRNA can be proper medicines for nscLc treatment [43]. In another studies, two types of shRNA eukaryotic expression vectors namely Livin and Survivin were entered the cancer tissue simultaneously and led to induction of co-silencing. These studies demonstrated that co-transfer caused to inhibit cell proliferation in lung cancer cells and induction of apoptosis. Furthermore, it proposed that co-transfer of these two vectors led to significant reduction too [44]. In 2013, Lentiviruses were used for shRNA delivery into the target tissue in lung cancer type (SCLC) (small cell line cancer) to silence thePROX1 gene. It is concluded that PROX1 expression in SCLC cell line is high and can be reduced with shRNA lentivirus, thereby reducing the cell proliferation rate [45].

Gastric cancer

Taolin conducted a study on human gastric cancer in 2013 and indicated that RNAi technique can reduce the expression rate of Bcl-2 protein which is a carcinogen protein and results in cancer growth and radiotherapy resistance in gastric cancer cells. Likewise, these studies demonstrated that sensitivity rate of these cells to x-ray was increased, so their resistance to radiotherapy was decreased. This investigation is an evidence for prominent role of Bcl-2 gene specific siRNAs in gastric cancer treatment [46]. In 2014, Jin-Hee and his colleagues proposed that lentivirus-mediated shRNAs can target the cyclinD1 and as a result it can be used as a new approach for sufficient treatment of gastric cancer [35].

Colon cancer

According to studies done on catalytic subunit of human telomerase reverse transcriptase (hTERT) in 2014, the carcinogenic role of this enzyme in colon cancer was proved. hTERT has a prominent role in construction and preservation of telomerase on the end of the chromosomes and also cell proliferation. They utilized a designed siRNA targeting catalytic subunit of hTERT to study its effects on growth of telomerase-positive human colon carcinoma SW480 cells *in vitro*. hTERT knockdown and reduction in telomerase activity led to inhibition of cell growth and proliferation and could be a novel approach for colon cancer treatment [46]. Likewise, during a study in 2007, recombinant adenoviruses that express a U6 promoter-driven shRNA targeting Bcl-XL (Ad/Bcl-XL shRNA) led to Bcl-XL protein knockdown *in vivo*. Bcl-Xl is an anti-apoptotic of Bcl-2 family and overexpresses in colon cancer [47].

Head and Neck cancer (HNC)

A study showed that demmoglein 3 (DSG3) was overexpressed in patient who suffering from HNC. These results are based on the different expression of RNA and protein levels of this gene. In conclusion, suppression of DSG3 by RNAi results in significant reduction in cell growth and colonies formation in three HNC cell lines [48]. Also, Jung and his colleagues applied a shRNA targeting C-Met gene (sh-C-Met) for treatment of solid tumors in a nude mouse model with human Head and Neck cancer [49].

Skin cancer

P53 and Fas-ligand (FasL) (a tumor necrosis) repair the mutations and DNA damage of keratinocytes and melanocytes. These damages result in some common skin

tumors including Basal cell carcinoma (BCC), Squamous cell carcinoma (SCC) and Melanoma. The transformation of keratinocytes and melanocytes led to FasL re-expression and to tumor development. FasL has a key role in immune evasion and those genes which control apoptosis resistance such as Gli2. Therefore, silencing of these genes using siRNA or shRNA is an appropriate approach in skin cancer treatment in early stage [50].

Oral cancer

In 2011, anticancer effects of poly midoamine (PAMAM) dendrimer-mediated shRNA against hTERT in oral cancer was studied. During this investigation, hTERT silencing by Dendrimer-mediated shRNA *in vitro* led to cell growth inhibition and induction of apoptosis [51]. In other study, KLF8 was knocked down by Lentivirus mediated siRNA infection in human adenosquamos carcinoma CAL27 cells. Consequently, the gene expression and the number of cell proliferation and colonies were reduced. This experiment indicated that KLF8 which is an important factor for oncogene. It is essential for growth CAL 27 cancer cells in oral cancer [52]. Likewise, Yang and his colleagues targeted the bcl2 gene by *Salmonella typhimurium* carrying shRNA-expressing vectors and led to suppression of the gene in murine melanoma cells. These evidences provide novel approaches for cancer treatment [53].

Conclusion

The synthesized dsRNAs (shRNAs and siRNAs) could be sufficiently designed for targeting a specific gene. Also, based on their mentioned applications in common cancers they could be used as novel therapeutic methods for other diseases and have an important role in treatment of some diseases.

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