MINI-REVIEW

RNA Interference: a Promising Therapy for Gastric Cancer

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Abstract

Gastric cancer (GC) remains a virtually incurable disease when metastatic and requires early screening tools for detection of early tumor stages. Therefore, finding effective strategies for prevention or recurrence of GC has become a major overall initiative. RNA-interference (RNAi) is an innovative technique that can significantly regulate the expression of oncogenes involved in gastric carcinogenesis, thus constituting a promising epigenetic approach to GC therapy. This review presents recent advances concerning the promising biomolecular mechanism of RNAi for GC treatment.

Keywords: RNA interference - therapy - gastric cancer

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Introduction

Despite the decline in the global incidence, gastric cancer (GC) remains the second leading cause of cancer death and fourth most common malignancy worldwide (Jemal et al., 2011). The GC prevalence is associated with environmental triggers, host genetic, and bacterial factors. In the last case, Helicobacter pylori infection is a major contributor to the development of this malignancy (Malakar et al., 2012; Zabaleta, 2012). Despite the common use of multimodal therapy [chemotherapy, radiation and surgery], there is a few long-term disease-free survival rate for GC patients, therefore, further significant studies are required in this field.

Before the 80s, RNA was only considered as an intermediate for the passive information transport between DNA and protein synthesis. With the discovery of catalytic properties of RNA in the early 80s, a Nobel Prize was shared between Tom Cech and Sidney Altman (Waldrop, 1989). Since then, several researchers are continuously striving for new strategies and future challenges.

Over the past decades, several tumor suppressor genes and proto-oncogenes have their role established in the development and progression of GC, thereby making these insights, a potentially attractive therapeutic target in this disease. Among these findings, an innovative and evolutionary mechanism called RNA interference (RNAi) is revolutionizing oncology (Wang et al., 2011). RNAi was discovered by Fire et al. (1998), and it was defined as a posttranscriptional gene-silencing mechanism produced by small double-stranded RNAs which include endogenous microRNA (miRNA) and exogenous small interfering RNA (siRNA) or short hairpin RNA (shRNA). These are all commonly cleavage by endogenous enzyme called Dicer that gives rise to the important transcription factors involved in the gene expression regulation. This methodology's greatest potential is to specifically repress the transcription of disease-causing genes thus avoiding undesirable effects. This is possible due to the fact that RNAi acts exclusively on the endogenous mechanism called RNA induced silecing complex (RISC) which acts as an endonuclease on target messenger RNA (mRNA). Thus, the gene expression is controlled at the post-transcriptional level through the recognition and cleavage of mRNA in association with RISC (Baulcombe, 2000; Matzke et al., 2001; Bader et al., 2010; Wilson and Plucinski, 2011). Figure 1 shows a simplified scheme of the functioning mechanism of RNAi.

It is estimated that there are about 900 human miRNA's not yet characterized with respect to their biological targets and cellular functionality. Among these, a number of miRNA are involved in carcinogenesis and other pathological conditions (Bader et al., 2010). shRNA is a double-stranded RNA by flipping back on the loop sequence, hence the term "short hairpin", and it can be used to silence target genes expression by RNAi technique. In this case viral vectors or plasmids are used (Rao et al., 2009).

RNAi has great genetic and epigenetic importance as it acts as a key component in cellular differentiation

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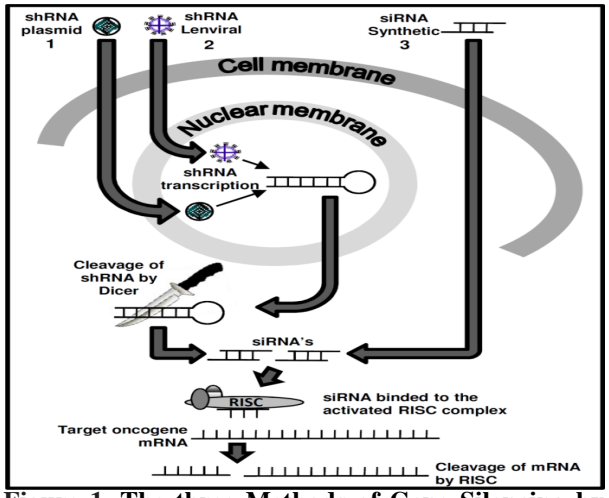

Figure 1. The three Methods of Gene Silencing by RNA Interference (RNAi) Used in the Experimental Treatment of Gastric Cancer (GC) by the Researchers. In method (1) cells can be directly transfected with short hairpin RNA (shRNA) carried by plasmids. In method (2) lentiviral particles are used. Both can be employed to transport and transcribe shRNA in the cell line of interest. After transcription, the expressed shRNA is released from the nucleus and cleaved by the Dicer enzyme in the cytoplasm, generating the siRNA. In (3) the synthetic siRNA can be directly transfected and enters the RNAi pathway. From this point, all siRNA in the cytoplasm is recognized by the RISC complex (RNA induced silecing complex), which, once activated, mediates cleavage and destruction of the target oncogene mRNA, thus silencing the protein responsible for GC

and progression processes in normal and tumor tissues (Fire et al., 1998), thus, several investigators have focused their attention to the possible antitumor activity of this new epigenetic mechanism in GC. The term "epigenetics" involves an inherited phenotype resulting from chromosomal alterations without changes in the DNA sequence. Briefly, epigenetic events in humans provide a more stable and precise control of gene expression over multiple generations (Berger et al 2009). These epigenetic events are important in all aspects of biology and research. In the last decade, they have been directly related to carcinogenesis and tumor progression. Currently, four different epigenetic systems are recognized. They are, in brief: Self-sustaining metabolic cycle (Ferrel, 2002; Thieffry and Sanchez, 2002), Chromatin-marking systems (Weissmann and Lyko, 2003), Structural inheritance (Collinge, 2001), and RNAi system (Hannon, 2002). All these epigenetic events are involved in the gene expression regulation, either by gene activation or silencing.

Thanks to recent technological advances, the RNAi is chemically synthesized and soon become an effective therapeutic tool against several diseases as it shows a huge therapeutic benefit in these experimental trials. From a practical perspective, the RNAi approaches are becoming promising as an attractive therapeutic alternative for GC patients, due to its ability to selectively inhibit the expression of oncogenes involved in human gastric carcinogenesis. Thus, it becomes necessary to better understand this revolutionary approach in several experimental models of treatments targeting future clinical applications. This review aims to bring together the latest advances concerning the promising biomolecular mechanism of RNAi for GC treatment, and besides, to report the future perspectives that might contribute to the further understanding and treating this disease. Additionally, to clarify a number of concepts and methods related to this innovative therapy.

Promising Therapeutic Targets for Treatment of Gastric Cancer by RNAi Silencing.

Epidemiologists and public health researchers from several institutions reported a global decline in GC mortality (Crew and Neugut, 2006; Jemal et al., 2011). However, the advances in new therapies and approaches, the GC treatment have been shown to have a limited impact on the overall survival of these patients.

The promising epigenetic therapy using RNAi is in development and several oncogenic targets involved in drug resistance, anti-apoptosis specific, angiogenesis and survival are being studied. Recently discovered promising therapeutic targets for GC treatment by RNAi silencing are listed below.

The homeobox protein Nanog is an important transcription factor in the pluripotency of embryonic stem cell and the preservation of self-renewal (Pan and Thomson, 2007). Expression of Nanog has also been correlated with the clinical classification of GC and invasion of GC cells, additionally, its overexpression correlates with poor prognosis patients with CG (Lin et al., 2012). In this way, Ji and Jiang (2013) used shRNA to inhibit the expression of the Nanog gene to study the effect on the tumor biological behavior of the SGC-7901 GC cell line. The results revealed that RNAi technology was able to inhibit the expression of Nanog, thereby inhibiting tumor cell proliferation, migration and invasion. This method may provide an experimental basis for a gene therapy approach for treating GC.

The STMN-1 gene, which encodes the Stathmin-1/oncoprotein, its function as an essential regulatory protein of microtubule dynamics has been typified (Sobel, 1991) and was found overexpressed in a large number of human cancers, such as leukemia (Melhem et al., 1991), prostate (Friedrich et al., 1995), breast (Brattsand, 2000), and lung cancer (Chen et al., 2003). To establish the silencing effects of Stathmin-1, some researchers used the shRNA to knockdown, STMN-1 mRNA in MKN-45 GC cell line. The results showed that the silencing of stathmin1 has led to the significant decrease in proliferation and inhibition of cell migration *in vitro* and in nude mice (Akhtar et al., 2013). Stathmin-1 oncoprotein has been shown to be a potential oncogene target that which is a promising strategy to treat GC.

Coiled coil domain containing 134 (CCDC134) is a promising gene therapeutic method and studies have shown that it regulates the Erk1/2 and JNK/SAPK pathways in HeLa cells (Huang et al., 2008). From this, other investigators wondered if this gene could be play a role in GC development or tumor progression, and thus used siRNA technology and CG cell lines in this investigation. Notably, these reports have demonstrated that CCDC134 siRNA knockdown caused a significant

increase in invasion and migration of AGS CG cell lines (Zhong et al., 2013). The CCDC134 may be a candidate for a therapeutic target of GC.

The MUC1 mucin core protein is aberrantly expressed in malignancies, such as in stomach cancer (Ng et al., 2008) and recently its overexpression was reported to be involved in trastuzumab resistance. In that study, siRNA of MUC1 into drug-resistant MKN45 GC cell line was used to observe the silencing effects. The authors observed that the sensitivity to trastuzumab under MUC1 siRNA conditions was significantly increased (Deng et al., 2013). Therefore, this resistance can be cancelled by using the RNAi approach.

Some researchers showed that the overexpression of Bcl-2 gene can inhibit tumor cell apoptosis induced CCDC134 (Bilim et al., 2008). Therefore, Liu and Lu, (2013) synthesized siRNA to transfect BGC823 GC cell line and observed the influence of inhibiting Bcl-2 gene expression on the apoptosis and radiosensitivity. These investigators demonstrated that Bcl-2 knockdown can effectively inhibit the expression of Bcl-2 gene and thus enhance the radiosensitivity and apoptosis of GC BGC823 cells. These findings indicate that Bcl-2 gene silencing may be a promising novel approach for treatment of GC.

Ras-related protein (Rab-25) is a newly identified protein encoded by the RAB25 gene belonging to the Ras oncogene family and currently recognized as CATX8, in additional, Rab25 was closely related to tumorigenesis and metastasis (Chia and Tang, 2009). In a study with GC cell lines using siRNA silencing, Cao et al., (2013) showed that Rab25 protein expression positively associated with the development of GC and that Rab25 is involved in the invasion and metastasis of GC cells. Those authors hypothesized that knockdown of Rab25 may be a potential future biological treatment strategy for inhibition of invasion and metastasis of human GC.

Vascular endothelial growth factor A and C (VEGF-A/C) were considered as most important factor in the angiogenesis, and further, the GC patients that overexpression of VEGF were correlated with peritoneal dissemination and poor prognosis (Aoyagi et al., 2005). Recently, Wang et al. (2013) correlated the VEGF-A/C expression with clinicopathologic parameters and prognosis in patients with GC using the plasmid shRNA therapy targeting VEGF-A. These researchers established that the expression of VEGF-A/C predict worse prognosis of GC patients and the silencing of VEGF-A/C markedly suppresses cancer growth than silencing of VEGF-A or VEGF-C (Wang et al., 2013). Their findings provide a novel strategy for the treatment of GC using the VEGF-A/C gene as target.

AKT1 (akt murine thymoma viral oncogene homolog 1) enzyme is a serine/threonine kinase encoded by AKT1 gene. It plays a key role in cell metabolism such as growth and survival. Some studies confirm that AKT1 is overexpressed in a variety of human cancers including GC and may be related to chemoresistance to drugs (Oki et al., 2005; Lindsley, 2010). AKT1 expression was then silenced in GC cell lines using the shRNA method. This silencing increased chemosensitivity to cisplatin in those cell lines by *in vitro* and *in vivo* apoptosis induction (Zhou et al., 2012). The results suggested that the AKT1 gene could be considered as a therapeutic target in combination with cisplatin in GC patients.

The APRIL (A Proliferation-Inducing Ligand) gene belongs to the TNF (tumor necrosis factor) family and was first identified in cell lines and primary samples from various tumor lesions. It is also recognized for its ability to stimulate tumor cell proliferation (Hahne et al., 1998). As a result, an siRNA specific for APRIL mRNA was designed and transfected different GC cell line cultures. The researchers successfully demonstrated the inhibition of cell viability and colony formation due to cell cycle arrest in the G2/M phase (Cui et al., 2012). These findings provide new clues to the investigation of GC malignant proliferation using the APRIL gene as target. In the same way, other authors (Ni et al., 2012) constructed a specific shRNA vector aimed at human APRIL gene and transfected into GC sgr-7901 cells. The results showed that APRIL gene silencing can increase the apoptotic rate of GC cells. Their study suggests that APRIL gene silencing may be useful in GC treatment.

CDX2 (Caudal type homeobox 2) is a gene encoded in the villi of intestinal cells. It plays a crucial role in the regulation of cell proliferation and differentiation in the intestine, besides being involved in resistance to chemotherapy (Suh and Traber, 1996; Lorentz et al., 1997; Silberg et al., 2000) and being overexpressed in GC (Mizoshita et al., 2003). In a study with GC cell lines using siRNA silencing, it was demonstrated that the CDX2 gene plays a critical role in the proliferation, invasion and apoptosis of GC cells. The researchers thus succeeded in reducing both *in vitro* and *in vivo* progression (Wang et al., 2012). Their study suggests new basis for the treatment of GC with gene manipulation by using siRNA.

Recently, genes that express ion transport proteins have been studied in several cancers, including the CLIC1 (chloride intracellular channel 1) gene, which encodes the intracellular chloride channel (Kunzelmann, 2005; Schönherr 2005). RNAi technology was used to target the CLIC1 gene in GC cell culture. The results showed that siRNA can efficiently inhibit CLIC1 expression and suppress *in vitro* GC migration and invasion (Peng-Fei et al., 2012). However, this silencing maintained GC cell proliferation and reduced apoptosis.

The CTGF (Connective Tissue Growth Factor) gene expresses connective tissue growth factor and plays a major role in many biological processes, besides being associated with various cancers development and progression (Xie et al., 2001; Yang et al., 2005; Sala-Torra et al., 2007). Chinese researchers have investigated the role of CTGF gene in GC cell lines and cells obtained from tissues of patients undergoing gastrectomy. After transfection with an siRNA targeted to the CTGF gene mRNA, they demonstrated that gene silencing promoted the adhesion of GC cells to the peritoneum, thereby hindering spread and metastasis in these *in vitro* assays (Jiang et al., 2012). Thus, the CTGF gene may be a new and promising therapeutic target.

The EPHA2 (ephrin type-A receptor 2) receptor is a protein encoded by the EPHA2 gene. It belongs to the protein tyrosine kinase family and has been initially

related to events in the nervous system (Sulman et al., 1997). Emerging evidences demonstrate EPHA2 gene overexpression in tumor progression, angiogenesis and metastasis in many cancers (Duxbury et al., 2004; Ireton and Chen, 2005). To further define the EPHA2 gene function in malignant invasion, researchers used the siRNA technique to inhibit this gene expression in GC cell lines. The results showed that silencing EPHA2 receptor expression inhibited proliferation by stopping the cell cycle and decreased *in vitro* invasion (Yuan et al., 2012). Therefore, this silencing may be a valuable approach to GC therapy.

hMex-3A (human Mex-3A) is an RNA binding protein that plays essential roles in its metabolism, such as transportation, surveillance and translation. This protein is overexpressed in several diseases, including cancer (Buchet-Poyau et al., 2007). A recent study has demonstrated for the first time that the silencing of hMex-3A by siRNA effectively inhibits cell proliferation and the migratory ability of GC cell lines. The results suggested that this gene functions as a candidate oncogene in the development and metastasis of GC (Jiang et al., 2012). Therefore, the hMex-3A gene may be suitable as a potential target for the epigenetic treatment of GC.

Interleukin-8 (IL-8) is a well-known cytokine in gastric carcinogenesis as it attracts neutrophils to sites infected with the Helicobacter pylori causing chronic gastritis, gastric atrophy, intestinal metaplasia, dysplasia and finally GC (Lindley et al., 1988; Matsushima et al., 1988; Dixon, 1991; Crabtree et al. 1994). Therefore, siRNA was specifically synthesized to hybridize and destroy IL-8 mRNA in GC cell lines. It was demonstrated that in absence of IL-8 there was no tumor cell migration or invasion. On the other hand, GC cells have developed resistance to oxaliplatin in *in vitro* chemosensitivity assays (Kuai et al., 2012). These findings may contribute to the development of new GC treatments with the IL-8 gene as a therapeutic target. However, this technique which may lead to oxaliplatin chemoresistance.

KLF8 (Kruppel-like factor 8) is a protein that plays an important role in the regulation of epithelial-mesenchymal transition, which comprehends homeostatic mechanisms that regulate tissue repair, inflammation and wound healing. On the other hand, this protein is also expressed during metastasis and can contribute to treatment resistance due to its antiapoptotic effects (Thiery et al., 2009). Recently, KLF8 protein was found overexpressed in GC tissues and cell lines. In that study, shRNA was used to observe the silencing effect of KLF8 gene on *in vitro* and *in vivo* CG cell growth. The authors observed the inhibition of cell proliferation both in culture and in rats that were injected with GC cells (Liu et al., 2012). These results indicate that KLF8 gene silencing may be useful in GC treatment.

Versican protein, a component of the extracellular matrix found on tumor stroma and cancer cells, is encoded by the VCAN gene. Its activity is regulated by several cytokines, including IL-11, which performs an important role in the progression to GC. Versican overexpression promotes cell proliferation, differentiation and migration, thus contributing to the invasion and metastasis related to this neoplasia (Wight, 2002; Ricciardelli et al., 2009). Cultured GC cell lines and fourteen fresh frozen tissue samples from GC patients were treated with siRNA targeting the versican mRNA. The results revealed that low versican expression may create a hostile microenvironment for tumor progression by preventing subsequent stages of IL-11 mediated inflammation (Zhang et al., 2012). The study suggests a novel treatment possibility for GC by epigenetic avoidance of versican overexpression.

Researchers proudly obtained and demonstrated outstanding antitumor results, such as induction of sensitivity to drugs; inhibition of cell viability; reduction of tumor cell proliferation, invasion and progression; inflammation and migratory capacity blockage and finally, impairement of GC cell angiogenesis and metastasis. The studies that indicate the importance of RNAi in regulation and its antitumor effects in gastric carcinogenesis are briefly described in Table 1.

Table 1. Gene Knockdown Studies using RNA Interference Technology, and its Antitumor Effects in Gastric Carcinogenesis

Gene	RNAi mechanism	Antitumor effect	Study type	Reference
NANOG	plasmid shRNA	Inhibition of cell proliferation, migration and invasion	In vitro	Ji and Jiang, 2013
STMN-1	shRNA	Inhibition of proliferation and cell migration	In vivo/In vitro	Akhtar et al., 2013
CCDC134	synthetic siRNA	Increase in invasion and migration	In vitro	Zhong et al., 2013
MUC1	synthetic siRNA	To increase sensitivity to trastuzumab	In vitro	Deng et al., 2013
BCL-2	synthetic siRNA	Enhance the radiosensitivity and apoptosis	In vitro	Liu et al., 2013
RAB25	synthetic siRNA	Inhibition of invasion and metastasis	In vitro	Cao et al., 2013
VEGF-A/C	plasmid shRNA	Suppression of cancer growth	In vitro	Wang et al., 2013
AKT1	lentiviral shRNA	To increase chemosensitivity to cisplatin	In vitro	Zhou et al., 2012
APRIL	lentiviral shRNA	Cell viability and colony formation inhibition	In vitro	Cui et al., 2012
APRIL	plasmid shRNA	Increase the apoptotic rate	In vitro	Ni et al., 2012
CDX2	plasmid shRNA	Decreased proliferation, invasion and progression	In vitro	Wang et al., 2012
CLIC1	synthetic siRNA	To suppress GC migration and invasion	In vitro	Peng-Fei et al., 2012
CTGF	synthetic siRNA	Hindered metastatic spreading and dissemination	In vitro	Jiang et al., 2012
EPHA2	synthetic siRNA	Inhibited proliferation and decreased tumor cell invasion	In vitro	Yuan et al., 2012
hMex-3A	synthetic siRNA	Inhibition of proliferation and migration capacity	In vitro	Jiang et al., 2012
IL-8	synthetic siRNA	Interruption of tumor cell migration and invasion	In vitro	Kuai et al., 2012
KLF8	lentiviral shRNA	Inhibition of proliferation	In vivo/In vitro	Liu et al., 2012

Despite the amazing advances of RNAi biomolecular technology in causing significant antitumor effects, some of the investigators frustrations should be pointed out. Peng-Fei et al., (2012) prevented the *in vitro* migration and invasion of CG but unfortunately did not prevent the proliferation of these cancer cells. Similarly, Kuai et al., (2012) were able to contain CG migration and invasion, however, they induced a significant increase in chemoresistance related to oxaliplatin use.

Several countries are currently initiating their studies, since most of the RNAi technological advances have been led by Asian researchers, especially in which concerns unraveling the relationship between genes and their functions in gastric carcinogenesis. This scientific revolution also occurs in various human cancers as the increased expression of certain genes is not unique to CG. Thus, controlling this expression by silencing appears as a hope for the development of new therapies for other cancers. Therefore, various medical and biological disciplines could investigate in developing countries this innovative RNAi technology.

It is estimated that some of the innovative RNAi therapy properties could benefit clinical and industrial applications and consequently public health. These benefits would include: improved toxicity profiles due to its specificity for the target, exclusively eliminating the expected protein; individualized treatment as RNAi would be rationally designed to block any target gene expression; low production cost, due to its relative ease of synthesis compared to antibodies or recombinant growth factors.

For these reasons, the RNAi mechanism is a promising class of medication effective against GC. On the other hand, some challenges may limit the overall effectiveness of this technology, such as the delivery system and the ideal RNAi release. This system should be formulated in order to be able to escape the endothelial reticulum in the tumor environment trajectory, thus preventing RNAi degradation and, consequently, treatment failure (Li and Huang, 2010).

Important steps were taken to develop an efficient siRNA molecule delivery system in cells or target tissues for the successful application of RNAi. The lentiviruses belong to the family of retroviruses and can be used to deliver RNAi (now called shRNA). This shRNA is transfected and integrates into the cell genome, and after gaining stability in prolonged shRNA expression, it keeps the gene silenced (Naldini et al., 1996; Fish and Kruithof, 2004), this being one of the exogenous genes delivery systems preferred by some of the researchers cited in this review (Cui et al., 2012; Liu et al., 2012; Zhou et al., 2012). Several strategies are theoretically proposed for targeting these molecules in order to ensure a better specificity in the target tissue, but tests are necessary which allow demonstrating efficacy in practice.

Final Considerations

Our review reveals how fast researchers are currently investigating new therapeutic possibilities for GC treatment with RNAi technology. In the last two years, substantial progress has been made in RNAi technology such as the interruption of important stages in gastric carcinogenesis related to the discovery of target oncogenes for future treatments in this area.

Nowadays the main use of this technology is related to the investigation of new drugs and therapeutic targets in experimental trials. In the short term, we may probably be able to follow the steps to the development and validation of methodologies to determine the ability of these drugs in humans. It is estimated that in the future these clinical applications will be able to solve the reversal of multidrug resistance in the gastrointestinal oncology field, and the adverse effects of current chemotherapy such as toxicity and low survival rates, besides the high cost currently related to GC treatments.

To contribute to the understanding of the RNAi technique, we attempted to synthesize the most recent available data and evaluate the research advances in GC treatment, which could be a solution to public health problems caused by this type of cancer.

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