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Review Article

Emerging Gene Knockdown Technology Using Small Interfering RNA in Ovarian Cancer

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Abstract

RNA interference (RNAi) is an emerging field of genetic intervention in difficult diseases where there is hardly any treatment. Micro RNA (miRNA) and small interfering RNA (siRNA) are two components of such RNAi. Exogenous double stranded small interfering RNA (siRNA) targets specific mRNA and prevents anomalous gene expression causing such disease. Ovarian cancer with its dismal prognosis due to recurrence and drug resistance is such a disease which can hardly be treated by conventional therapy. Hence siRNA is tried for recurrent and refractory ovarian cancer. With success already at hand in rare genetic disease it seems worthwhile that development of such therapy in ovarian cancer so far is critically analysed, hence this review.

Keywords: *RNAi; siRNA; Ovarian Cancer; Nanoparticle*

Introduction

Ovarian cancer is the second most common gynaecological cancer. It is the third most common killer of all such malignancies. It is also the sixth leading cause of cancer-related deaths amongst women [1]. Incidence increases with increasing age and reaches a peak in post-menopause up to 60 years after which it declines. Epithelial variety is most aggressive causing highest number of deaths [2]. This is intra-abdominal occult cancer with hardly any symptom to start with. When diagnosed it's already late, mostly in the third stage without any scope for definite surgery, hence cure. In spite of good chemotherapy and cytoreductive surgery micrometastases specially inside abdominal cavity are difficult to treat and cause recurrence. Ovarian cancer is very prone to develop resistance to common chemotherapy regimen used. In the second line and above response to therapy is very poor causing fatality. Thus, there is always an unmet need for newer modality of treatment for increasing survival. Genetic manipulation through epigenetics or other processes may precipitate regression of cancer. This may also make such cancer susceptible to therapy again. Manipulation of RNA i.e. RNA interference (RNAi) may prevent formation of proteins necessary for resistance to develop by blocking DNA. This could be a novel method to cure ovarian cancer.

RNAi

It is seen that mRNA may be suppressed by using short RNA strands in a sequence specific manner which in turn stops translation and transcription required for gene expression. Handling of RNA has improved a lot in the present time. As a result, RNAi has become feasible. This creates much hope and it seems using this technique it will be possible to fight against many intractable diseases including such

cancers where there is longstanding unmet need. RNAi technology includes microRNA (miRNA) and small interfering RNA (siRNA). Both are very small double stranded RNA causes silencing of gene after transcription by targeting messenger RNA (mRNA). However, miRNAs have more than one targets but siRNAs are specific as they target only a single mRNA. Hence, not only they have quite different mechanism of action, their clinical applications are also different. Subject of siRNAs with their many types of carriers is advancing at a rapid pace in recent times. Though there are many hindrances and setbacks there is a strong indication that it might be quite useful soon. These molecules need to be tailor-made to suit the goal of fighting against particular pathology. Also specific strategy to carry such RNAs to proper cells and tissue has to be chalked out so that it will be safe and useful.

Oligonucleotides of 20 - 25 base pairs are so prepared that it knocks down the target gene by targeted mRNA degradation. These oligonucleotides are called short interfering RNA or siRNA [3]. RNA-induced silencing complex (RISC) is that region of mRNA which after binding with siRNA causes required gene knockdown. RNAi will be more pronounced as siRNA will be efficiently bound to RISC. After formation of RISC with double stranded siRNA endonuclease separates it's sense strand due to unwinding and cleavage. This is followed by transcriptional silencing by the antisense strand left behind after separation by endonuclease. Antisense strand plus RISC complex works on mRNA to stop further transcription. The desired post-transcriptional gene silencing happens only after the formation of RISC caused by siRNA binding. RISC with antisense siRNA then works on mRNA by splitting or cleaving that portion which codes a target gene. This process is called silencing of that particular gene expression. As siRNA is biological product unknown to the cells to which this is introduced into, immunological reaction may occur when cells recognize them as foreign and even viral products. Then it may give rise to what is traditionally called innate immunity along with associated off-targeting, adaptation of immune response and saturation of RNAi machinery.

Being precise in targeting siRNA can pinpoint gene code, so it's highly effective to prevent the pathology with great specificity and least harm. Problem with siRNA is that RNAse can easily lyse and degrade it if it is introduced and exposed without a carrier. As gene knockdown requires presence of sufficient amounts of siRNA in the vicinity inside the cell they need to cross the plasma membrane. But unfortunately they are unable to do so making this process difficult to be used if siRNA is used alone. So, they need to be altered in such a way that makes them stable, active and less immunogenic. siRNAs were this put inside nanolipid particles so that they may remain stable in blood.

Other nano vectors evolved which might facilitate delivery and cellular uptake of siRNA. As a result their concentration would be high in the cell where they were required. Also there would be less destruction of the compound [4]. Strangely enough siRNAs are classified according to the nanovector by which they are carried. Though nanovector was lipid based to start with, gradually non lipid organic and also inorganic vectors emerged. These three are the main types of carriers. Plan of siRNA in ovarian cancer use started in first decade of new millennium [5]. There are quite a few good general review on siRNA in clinical use and also in ovarian cancer. But they specialises mostly on carriers of siRNA and chemistry involved in manufacturing such vehicles [6-8].

Chronology of targeting

This review will provide an overview of targets for siRNA in ovarian cancer. As already discussed delivery systems being of foremost importance in such remedy, they will also be described. But we will prefer using chronology of targeted genes as the subject evolved. That approach will clarify the targets attempted so far along with delivery system as used. Lipid based nanoparticle though, was used to start with, gradually other organic vehicle like polyethyleneimine (PEI) based nanoparticle, polysaccharide based nanoparticles, dendrimer based nanoparticle, inorganic silicon based nanoparticle and special kind of microbubble based nanoparticle were also used.

When reports were analysed we could see that siRNA targeting effort as preclinical experiment for ovarian cancer started in the beginning of last decade and till date many targets with different carriers are used.

In 2011 researchers used lipid based nanoparticle. Goldberg., *et al.* targeted *PARP1* for mice xenograft tumour of *BRCA1* deficient ovarian cancer cells [9]. Among solid nanoparticles, reconstituted high-density lipoprotein (rHDL) was developed in the same year by Shahzad., *et al* [10]. Focal adhesion kinase (*FAK*) and signal transducer and activator of transcription 3 (*STAT3*) are another two gene targets. They help in tumour development and progression. Targeting them should help in killing cancer cells [11]. siRNAs targeting three MDR genes including *survivin*, *Bcl-2,* and *P-glycoprotein* were used in 2015 when their pooled mixture were adsorbed electrostatically on a cationic lipid layer. This layer covered lipid based nanoscale coordination polymers (NCP-1) particles with trigger release properties. *NCP-1* is linked with cisplatin prodrug-based bisphosphonate bridging ligands with Zn^{2+} metal connecting points. It reduced tumour sizes of cisplatin-resistant SKOV-3 subcutaneous xenografts [12]. Anti *Survivin* gene siRNA added to Paclitaxel uses a polymeric micelle (PM) as nanovector. PM is lipid based self assembly formulation with ability to load siRNA and chemotherapy combination. When siRNA of *survivin* silenced it's gene the cell would again be sensitive to added paclitaxel causing cell death [13]. PEM specific action of Lipid based YSK05-MENDs is worth mentioning as strangely they had no effect if injected inside the abdomen but had intense activity for PEM. Knockdown of genes also occurred when this was introduced inside the peritoneum [14]. In a cell culture experiment with ovarian cancer cell line SKOV3 *Notch1* gene was targeted by its siRNA using cationic cholesterol. Cholesterol based nanovector increased both cellular uptake and local *Notch1* siRNA concentration enormously [15]. Programmed cell death in the ovarian cell line caused inhibition of growth.

In 2012 organic polymer based nanocarrier was introduced. First came polyethyleneimine (PEI). Polyethylene glycol (PEG), Polyethyleneimine (PEI), and poly ε-caprolactone (PCL) along with folate (FA-PEG-PEI-PCL) conjugation formed a ternary copolymer capable of automatically holding *Bcl-2* gene siRNA and a chemotherapy doxorubicin [16]. Nanovector while combining siRNA with chemotherapy served both the purpose of gene targeting and specific cell killing. Tagging with folate caused recognition of those SKOV3 cells which had it's receptors in place. There this *Bcl-2* nanovector caused gene silencing whereas Doxorubicin caused programmed cell death thus potentiating the anticancer effect. They improved their experiment further in 2014 when, X-linked inhibitor of apoptosis protein gene (*XIAP* gene) could be silenced by its siRNA combined with PEI based *Her2*-targeted delivery. This effectively caused cell death both *in vitro* and *in vivo* [17]. siRNA-doxorubicin in PEG-pAsp(AED)-PDPA combination reducible micelles (triblock) was tried in the same year by this group [18]. In pH sensitive poly(2- (diisopropyl amino)ethyl methacrylate) (PDPA) core doxorubicin was put. The middle layer of poly(N-(2,2′-dithiobis(ethylamine)) aspartamide (PAsp(AED)) is sensitive to reduction. It contains *Bcl-2* siRNA and is kept secured by the outer layer of PEG. In 2015 three reports were a) based on PEI carrier targeted *PKM-2* and *MDR-1* sensitizes multidrug-resistant ovarian Cancer cells to paclitaxel [19], b) CD44 based PEI mediated *MDR1* siRNA delivery [20], c) *PD-L1* siRNA targeted delivery from folic acid-functionalized polyethylenimine [21]. In 2016, PEI based folate receptor tagged delivery of siRNA of *TLR4* along with paclitaxel was used to correct *TLR4* driven chemotherapy resistance in ovarian cancer cells showing encouraging results [22].

In 2013 use of polysaccharide was another polymer based approach that started following PEI. Stable Doxorubicin/nanoparticle and siMRA of *MDR1*/nanoparticle were created using two different lipid-modified dextran derivatives. This polysaccharide based siRNA was tested on drug-sensitive ovarian cancer (SKOV-3) cell cultures with success [23]. Organic polymer is made of polysaccharide of chitosan. It contained D-glucosamine and was cationic. It was capable of holding siRNA electrostatically forming a nanovector. Hypoxia-inducible factor-1α (*HIF-1α*) siRNA and chitosan oligosaccharide lactate (COL) combined to form nanovector. It is known that *HIF-1α* has a role in cancer progression and it is found in higher quantities in ovarian cancer cells [24].

Enhancer of Zeste Homolog 2 (*EZH2*) is a gene which has cancer growth promoting potential. In high expression growth and aggressiveness both are enhanced. Its siRNA in nanoparticles of TPP (Sodium tripolyphosphate) chitosan was used to show cancer cell apoptosis by Gharpure., *et al* [25]. In this study, TPP chitosan siRNA nanoparticles were combined with docetaxel-loaded nanoparticles.

P62/SQSTM1 (P62) is a protein of proteasome and autophagy regulation. It has an immense role in the development of drug resistance. Proteasome-ubiquitin system related β5 lowering along with increase in P62 protein may be responsible for making ovarian cancer cells refractory to cisplatin. *P62* siRNA, *β5* plasmid DNA together with cisplatin was made available from inside a biodegradable multifunctional nanoparticle (MNP) system. They were treated in CDDP resistant 2008/C13 ovarian cancer cells. Here in MNP, *P62* siRNA (si *P62*) and/or β5 expressing plasmid DNA (*pβ5*) attached to cationic chitosan formed the outer layer (26) whereas polylactic acid nanoparticle bearing cisplatin (CDDP) formed inner core [26]. This is also a polysaccharide based attempt in 2014.

2013 also saw introduction of dendrimer as nano-vehicle. Dendrimers (Greek word δένδρον, dendron meaning "tree") are symmetrical branched polymer molecules arranged around a central point or core. They have spherical shape in most cases. Polypropyleneimine based dendrimer tagged with GnRH carrying *CD44* siRNA was used by Shah in 2013 [27]. *Via* electrostatic interactions with such dendrimer, siRNA targeting the serine/threonine kinase, *Akt* [28] and later siRNA targeted to superoxide dismutase 2 (*Sod2*) ovarian cancer cell kill was attempted [29]. They used nanocomplex of a generation 6 PAMAM (dendrimers based on amine structures like polyamidoamine) with a triethanolamine (TEA)-core and it's modification respectively. Huang., *et al*. [30] then did almost a paradigm shift [30] when they targeted an mRNA binding protein called HuR. They used DNA based dendrimer and created a hybrid of siRNA of *HuR* with folate on it.

Silicon based nanoparticle was used in another two siRNA against the ephrin receptor A2 (*EPHA2*) and against *VEGF* in 2013 [31,32]. Ephrin receptor A2 (ephrin type-A receptor 2) is protein encoded by *EPHA2* gene belonging to the ephrin receptor subfamily of the protein-tyrosine kinase. It increases cell-extracellular matrix adhesion and anchorage-independent growth, thus promoting angiogenesis and metastasis. Lyophilized mixture of siRNA/*EPHA2* and lipids with water were retained in the 40 - 65 nm pores of discoidal mesoporous silicon nanoparticles (MSNs) of roughly 1000 × 400 nm in size, creating a multistage vector system. This experiment came up in 2013. siRNA induced *EPHA2* knockdown for up to 9 days. In 2015 Fe_3O_4 nanoparticles in a mesoporous silica matrix was prepared which showed properties of controllable magnetic heating and drug delivery ability. This is called magnetic mesoporous silica nanoparticles (M-MSN). It could be easily loaded with *VEGF* siRNA. The resultant nanovector would have theragnostic property and even the ability to decide on dose required.

In 2013 microbubble technology was introduced by Florinas., *et al*. [33,34]. siRNA directed against *VEGF* is loaded through electrostatic interactions of anionic microbubbles and cationic arginine grafted polymer (ABPs). Perfluoro crown ether (PCE) gas core would be created as microbubbles of 3 μm in diameter within an album in shell. Then ABP will be attached so that a disulfide linked polyamidoamine backbone with arginine functionalities is made. Resultant < 200 nm nanoparticles are stable polyplexes of siRNA and ABPs. The microbubble shell ruptures by the flickering of the inner core of the gas caused by application of ultrasonic rays. Uptake is promoted by this way which is reciprocally enhanced. Firstly, cells are penetrated by transient perforation caused by ultrasound; thereby allowing siRNA inside cells. Secondly, siRNA gets injected in micro quantities into the cell by the power that perforated the cells. However, this complex technology has not yet been clinically feasible since ovarian cancer resides in the abdominal cavity. Efforts are now made by taking advantage of ultrasound as it can be used for targeting even very small metastasis, or bigger metastasis and can be applied for the whole abdomen.

siRNA success story

Familial ATTR amyloidosis with polyneuropathy is a very rare (50000 cases worldwide) and fatal disease caused by mutation of transthyretin (TTR). Patisiran, sold under the brand name Onpattro, is a siRNA developed by Alnylam pharmaceutical. It is the first FDA approved siRNA and first treatment for such a condition. This drug was considered as an orphan drug, got fast track approval with priority review and break through therapy by FDA. It is first of its kind in the history of siRNA drug category [35]. It is a siRNA against transthyretin working by gene knockdown thus preventing production of wrong transthyretin.

Conclusion

Use of siRNA technology in ovarian cancer is a new and a contemporary subject. It has a combination of varied nanovector manipulation and a complex wide range of mRNA targets. All methods are not equally successful but some are definitely showing some promises. Drug delivery systems is itself full of complexity. Completely separate branch of chemistry and biochemistry are working towards fulfilling the cherished goal of getting optimum delivery system which will be useful in concerned clinical perspective. They need to be compared in terms of efficacy versus harm thus choosing efficient system while discarding less efficient one. Specific targeting with less and less untoward effect is what is required to advance this field.

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