Potential use of RNA interference in cancer therapy

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RNA interference (RNAi) is an evolutionary conserved mechanism for specific gene silencing. This mechanism has great potential for use in targeted cancer therapy. Understanding the RNAi mechanism has led to the development of several novel RNAi-based therapeutic approaches currently in the early phases of clinical trials. It remains difficult to effectively deliver the nucleic acids required in vivo to initiate RNAi, and intense effort is under way in developing effective and targeted systemic delivery systems for RNAi. Description of in vivo delivery systems is not the focus of this review. In this review, we cover the rationale for pursuing personalised cancer therapy with RNAi, briefly review the mechanism of each major RNAi therapeutic technique, summarise and sample recent results with animal models applying RNAi for cancer, and provide an update on current clinical trials with RNAi-based therapeutic agents for cancer therapy. RNAi-based cancer therapy is still in its infancy, and there are numerous obstacles and issues that need to be resolved before its application in personalised therapy focusing on patient-cancer-specific targets can become standard cancer treatment, either alone or in combination with other treatments.

Recent advances in understanding tumour survival signalling pathways have supported a migration of cancer treatment from untargeted cytotoxic therapy to selectively targeted therapeutics. Targeted therapeutics directed against specific molecules in malignant cells have been approved by the US Food and Drug Administration (FDA) and enable initial attempts at personalised cancer treatment. By analysing genetic abnormalities of a patient's tumour, unusual gene and protein expression can be determined and used to guide selection for specific targets.

RNA interference (RNAi) is a sequence-specific, naturally occurring mechanism for gene silencing induced by double-stranded (ds) RNA that was

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first discovered in the nematode Caenorhabditis elegans – work for which Andrew Fire and Craig Mello were awarded the 2006 Nobel prize for physiology or medicine (Ref. 1). The pathway of RNAi has been found in cells of nearly every multicellular organism and might have evolved as a defence against viral dsRNA (Ref. 2). Target-specific RNAi can knockdown a gene with high specificity and selectivity, thereby providing an important tool for personalised cancer therapy, as demonstrated in numerous animal xenograft studies (Table 1). However, many issues need to be resolved for RNAi treatments to translate from the laboratory to the clinic, including an effective targeted delivery system and a more comprehensive understanding of possible off-target effects (Ref. 3). RNAi-based approaches are reviewed here as part of a new division being established for cancer management.

Personalised cancer gene therapy

Human tumours show profiles of gene expression that are different not only from normal tissue, but also from each other. This can explain the differing responses to treatment (Ref. 4). Part of the differential gene expression involves altered levels of microRNA (miRNA) in malignant cells (Ref. 5). miRNAs are endogenous, noncoding RNA molecules that play a key role in regulating the gene expression pattern of a variety of developmental and physiological processes (Ref. 6). Classifying tumours by their miRNA levels provides further information on the aberrant gene expression pattern of individual tumours (Ref. 5). Recent studies have demonstrated that the pattern of gene expression can be classified into categories for clinical outcome prediction and for treatment response (Ref. 7).

In our evaluation, targets for therapy should be based on the degree of connectivity of dynamic genomic–proteomic nodes in a patient-data-based network model, rather than targeting the more highly active metabolic pathways in rapidly proliferating cancer cells in accord with traditional chemotherapy principles (Ref. 8). Highly connected targets are more vulnerable to attack because of the impact on the entire network (Ref. 9). The disordered cancer circuitry in malignant tissue can cause the cells to become highly dependent on a specific rewired pathway. The disruption of pathways that produce robustness to certain attacks often also causes increased fragility to other perturbations (Ref. 10). Therefore, in theory, knockdown of a rewired oncogenic tumour-specific pathway should lead to cell death in malignant tissues without significantly disturbing normal cell functionality (Ref. 3).

Each patient has a genetically different tumour, with a distinct rewiring of protein pathways. In order to find the optimal targets, malignant and nonmalignant tissue can be analysed in the context of global protein-interaction networks, providing information to create a prioritised list of potential gene and protein targets for each patient (Ref. 11). For example, we recently identified two scaffold proteins [RACK1 (GNB2L1) and stathmin 1 (STMN1)] as uniquely elevated within malignant tissue in comparison with matched same-organ nonmalignant tissue, and highly connected with several procancer gene signals (Ref. 11). We constructed and verified activity of a unique bifunctional short hairpin RNA (bi-shRNA) targeting stathmin 1 (Ref. 12) and are currently processing the Investigational New Drug (IND) application to treat patients demonstrating elevated stathmin 1 in their malignant tissue. RNAi therapy of patients demonstrating target signal upregulation is also under way in several clinical trials around the world (Table 2).

Mechanism of RNAi

Single-stranded antisense RNAi was initially shown to cause interference as a result of hybridisation of the antisense and the mRNA target site (Ref. 13). However, it was also observed that treatment with the sense strand caused a similar reduction of gene function (Ref. 14). It was then demonstrated that dsRNA caused much more interference than each strand individually. Only a few molecules of dsRNA were required to achieve this effect, which indicated hybridisation was not the only mechanism of gene knockdown (Ref. 1).

RNAi can be modelled by both cleavage-dependent and cleavage-independent mechanisms (Fig. 1). It can be induced by small interfering RNA (siRNA; dsRNA of 21/22 nucleotides with a two-nucleotide overhang on the 3’ end), microRNA (miRNA; endogenously produced noncoding RNA of approximately 22 nucleotides in length), or short hairpin RNA (shRNA; a DNA plasmid encoding short RNA with a stem–loop structure).
The mechanism of RNAi begins with the cleavage of long dsRNA molecules into siRNAs by the RNAse III enzyme Dicer (DICER1) in the cytoplasm. Dicer protein contains an N-terminal helicase domain, an RNA-binding domain called Piwi/Argonaute/Zwille (PAZ), two RNAse III domains, and a dsRNA-binding domain (Ref. 15). The PAZ domain of Dicer specifically

RNA interference (RNAi) pathways
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Figure 1. RNA interference (RNAi) pathways. (See next page for legend.)
Figure 1. RNA interference (RNAi) pathways. (See previous page for figure.) Cleavage-dependent and -independent RNAi pathways are illustrated here with reference to bifunctional shRNA. The shRNA expression vector is shown entering the cell cytoplasm via a liposome delivery system, and enters the nucleus for expression. After nuclear entry, shRNA expression vectors are transcribed into primary transcripts by host RNA polymerases, and the transcripts are then processed by a microprocessor complex in the nucleus into a stem–loop structure and transported into the cytoplasm. In the cytoplasm, the stem–loop structure is further processed into mature shRNAs by Dicer in the RISC-loading complex, resulting ultimately in a single guide strand loaded onto the RISC for activity. In the bifunctional shRNA approach, two stem–loop RNA molecules are encoded, with the aim of increasing the efficiency of gene knockdown through efficient loading onto multiple types of RISC loading complexes: one has perfect complementarity for the target sequence, which results in cleavage of the target mRNA; the other has imperfect complementarity, which results in knockdown via translational repression. By contrast, siRNA and conventional shRNA rely on cleavage-dependent RISC for activity, whereas cleavage-independent RNAi is the predominant mechanism undertaken by miRNA. Note that RNAi via shRNA and vector based miRNA-mimics requires expression from engineered constructs in the nucleus, whereas siRNAs or oligonucleotide-based miRNA mimics or antagonists remain in the cytoplasm seeking entry into the RISC.

miRNA mechanism
The miRNA family of RNA is transcribed by RNA polymerase II (pol II) into a capped and polyadenylated primary transcript (pri-miRNA). The pri-miRNA transcript is processed by the nuclear RNase III enzyme Drosha (RNASEN) and its cofactor DGCR8 (a ds-RNA-binding domain protein) (Ref. 28). Drosha also generates a two-nucleotide 3′ overhang that can be recognised by Dicer for further processing in the cytoplasm (Ref. 25). This pre-miRNA is exported to the cytoplasm by exportin 5 (XPO5) (Ref. 28). Dicer cleaves the pre-miRNA to form mature miRNA and integrates the miRNA duplex with argonaute-containing RISC, much as it does for siRNA (Ref. 29). In addition to Ago2, the argonaute proteins Ago1, Ago3 and Ago4, which do not have endonuclease activity, can also be assembled into RISC for cleavage-independent interference (Ref. 30).

mRNA target recognition by miRNA usually requires a perfect nucleotide match in the seed sequence of the miRNA (Ref. 31). If the miRNA is perfectly complementary to the mRNA, Ago2 cleavage of the mRNA will occur just as it does

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for siRNA (Ref. 32). However, mRNA that binds to miRNA with imperfect complementarity experiences translational blockage due to the formation of a bulge sequence in the middle of the A-form helix, making it unsuitable for cleavage. This cleavage-independent mechanism of RNAi is the predominant mechanism undertaken by miRNA (Ref. 33). As a result of the ability to affect translation of mRNAs with imperfect complementarity, a single miRNA can act on several mRNA targets and mRNAs can be the target of multiple miRNAs (Ref. 31). Up to a third of protein-coding mRNAs are susceptible to regulation by miRNAs (Ref. 34).

**shRNA mechanism**

shRNAs are synthesised in the nucleus of cells, further processed and transported to the cytoplasm, and incorporated with the RISC for silencing activity (Ref. 35) The processing of shRNA is presumed to be very similar to the maturation pathway of miRNA, so studies on miRNA have provided the foundation of our understanding of shRNA synthesis (Ref. 36). shRNA is transcribed by either RNA pol II or III through promoters on the expression cassette. RNA pol II creates a transcript with a hairpin-loop structure that is processed in the nucleus by the microprocessor complex (Ref. 37). This creates pre-shRNAs containing a two-nucleotide 3′ overhang, which are transported to the cytoplasm by exportin 5, much like pre-miRNA. The loop of the hairpin is removed by the complex containing Dicer and TRBP/PACT to form ds siRNA with a two-nucleotide 3′ overhang, which is loaded onto RISC as described earlier (Ref. 38) Pre-shRNA has been found to be a part of the RLC, so it is possible that pre-shRNA directly associates with the RLC by a different Dicer–TRBP/PACT complex (Ref. 39). After loading onto RLC, and passenger-strand departure, shRNA should behave the same as siRNA or miRNA, causing cleavage of mRNA or translational blockage.

**Bifunctional shRNA mechanism**

A bifunctional shRNA is designed to induce both the cleavage-dependent and cleavage-independent mechanisms of RNAi (Ref. 12). It consists of two stem–loop shRNA structures: one of fully matched passenger and guide strand to incorporate with the cleavage-dependent RISC, and one composed of mismatched strands for incorporation with the cleavage-independent RISC. This design should increase efficacy as a result of the unique loading into the RISC complex. A higher level of efficacy (greater knockdown at equivalent dosing) and altered kinetics in a head to head comparison with siRNA to the same target has in fact been observed (Refs 3, 12).

**Delivery methods**

One of the biggest obstacles for RNAi use in vivo is achieving efficient delivery of the dsRNA required to induce RNAi. Delivering shRNA has more in common with traditional gene therapy than with the delivery of siRNA or antisense oligonucleotides because the nucleic acid must be delivered to the nucleus (Ref. 40) Therefore, previous research on delivering plasmid DNA can be adapted for the delivery of shRNA, and antisense delivery technology can be modified for the delivery of siRNA (Ref. 41). Numerous methods of delivery are being used; natural and synthetic polymers, lipid complexes, and viral vectors are the most common (Ref. 42). In addition, effort is being made to create targeting systems so the nucleic acid is delivered only to tumour cells. Delivery vehicles can be complexed with monoclonal antibodies, peptides, small-molecule ligands, or aptamers to recognise markers on the cell surface (Ref. 43).

**In vivo use of RNAi**

RNAi has become a standard procedure for gene knockdown in cells, which can be used to determine gene function by inhibiting a specific gene and analysing the phenotypic change. This knowledge of gene function is being used to characterise and identify signalling pathways for tumourigenesis and to evaluate possible targets for drugs or future RNAi therapy (Ref. 44). There have been numerous in vivo studies for cancer treatment using RNAi targeting a plethora of genes, and some of them are summarised here (Table 1). Moreover, systemic administration of cancer-relevant miRNAs or antagomirs (antagonists of miRNA) may provide opportunity for further novel cancer therapy investigation.

**p21-activated kinase 6**

Malignant prostate tissue overexpresses p21-activated kinase 6 (encoded by PAK6). The effect
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<th>Cancer</th>
<th>Model</th>
<th>RNAi molecule</th>
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Table 1. Examples of in vivo use of RNAi (continued)

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<th>Cancer</th>
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<th>RNAi molecule</th>
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Abbreviations: AAV, adeno-associated virus; miRNA, microRNA; RNAi, RNA interference; shRNA, short hairpin RNA; siRNA, small interfering RNA; STAT, signal transducer and activator of transcription; VEGF, vascular endothelial growth factor.
of PAK6 inhibition with siRNA was investigated in vivo in combination with the chemotherapy drug docetaxel. The siRNA was delivered intratumourally to nude mice with prostate tumour xenografts. After six weeks, the control group’s average tumour size was 475 mm\(^3\); mice treated with docetaxel alone had an average tumour size of 210 mm\(^3\); the group treated with siRNA alone averaged 68 mm\(^3\); and the combination treatment led to an average tumour size of 47 mm\(^3\). The PAK6-siRNA inhibited cell growth of prostate cancer cells by causing cell cycle arrest at the G2–M phase. Tumour growth was inhibited by both PAK6-siRNA and docetaxel alone, yet groups treated with both PAK6-siRNA and docetaxel showed a larger decrease in tumour volume than either treatment alone (Ref. 45).

Cytokine-induced antia apoptotic molecule
Hepatocellular carcinoma (HCC) overexpresses cytokine-induced antia apoptotic molecule (CIAPIN1). CIAPIN1 knockdown in HCC cells in vivo by adenovirus-mediated siRNA (AdsiCIAPIN1) delivery inhibited cell growth by blocking entry into the S phase of the cell cycle. Nude mice with HCC xenografts treated with AdsiCIAPIN1 injected intratumourally had tumours that were more than sixfold smaller than the control group after eight weeks. The treatment was also shown to induce apoptotic cell death in the tumours (Ref. 46).

\(\beta\)-Catenin
\(\beta\)-Catenin (CTNNB1) is overexpressed in many forms of cancer, especially oesophageal cancer. A plasmid with U6-promoter-driven expression of shRNA was used to induce RNAi to silence \(\beta\)-catenin expression in oesophageal squamous cell carcinoma (ESCC) cells. Nude mice xenografted with ESCC cells showed an average tumour size of 909.3 mm\(^3\) when treated with the \(\beta\)-catenin-silencing plasmid, compared with 2684.4 mm\(^3\) in mice treated with a different plasmid, and 2722.6 mm\(^3\) in untreated mice. \(\beta\)-Catenin silencing showed growth inhibition by G0–G1 cell cycle arrest but did not induce apoptosis (Ref. 47).

Vascular endothelial growth factor
The vascular endothelial growth factor (VEGF) family of growth factors is known to induce angiogenesis, and members of this family are required for cancer progression and metastasis. A DNA plasmid expressing siRNA for vascular endothelial growth factor C (VEGFC) was complexed with CaCO\(_3\) nanoparticles and used to treat colon carcinoma cells in vivo. Mice xenografted with colon carcinoma cells were treated with the CaCO\(_3\)–DNA complex by direct injection into the tumour mass and showed an average tumour size of nearly half the untreated or saline-treated tumours. The treatment also significantly inhibited lymph-node metastasis and lymphangiogenesis (Ref. 48).

Osteopontin
Osteopontin (OPN) is overexpressed in a majority of gastric cancers and is associated with pathogenesis. Transfection of a vector expressing siRNA for OPN into human gastric cancer cells significantly inhibited growth and invasiveness of those cells in vitro. It also decreased expression of several other proteins that are important in breast and melanoma cell migration, growth and invasion. Nude mice with gastric cancer xenografts that received intratumoural injections of a poly(ethylene imine) (PEI)–OPN vector complex had an average tumour size that was about a third of untreated tumours. Half of the treated mice survived until the end of the study (60 days), whereas no untreated mice survived to day 40 (Ref. 49).

MYC
The MYC proto-oncogene family is thought to contain central regulators of cell growth, and deregulation of expression is associated with several types of cancer. Elevated expression of MYC is commonly seen in colon cancer. Nude mice with colon cancer xenograft tumours that received intratumoural injections of siRNA targeting MYC showed a 40% reduction in MYC expression and experienced reduced rates of tumour growth, exhibiting an average tumour size that was half of that observed in untreated mice. The treated mice had large areas of cytoreduction that inhibited tumour cell proliferation (Ref. 50).

Eukaryotic initiation factor 4E
Eukaryotic initiation factor 4E (EIF4E) plays an important role in protein translation, which is upregulated in breast cancer. A vector using the survivin promoter was created to provide

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shRNA-mediated knockdown of EIF4E. Human breast carcinoma cells transfected with this vector showed a reduction in EIF4E mRNA and protein expression, as well as downregulation of expression of VEGF, basic fibroblast growth factor (FGF2) and cyclin D1 (CCND1), which are all proteins associated with the progression of cancer. Nude mice with breast cancer xenografts treated with the vector showed significantly smaller tumours, averaging 233.5 mm\(^3\) compared with 397.7 mm\(^3\) for mice treated with a control plasmid. Mice with treated xenografts also showed enhanced chemosensitivity to cisplatin, seen by decreased tumour size in mice treated with cisplatin and the shRNA plasmid, which was 134.5 mm\(^3\), compared with 208.9 mm\(^3\) in mice treated with cisplatin and a control plasmid (Ref. 51).

Epithelial cellular adhesion molecule

Epithelial cellular adhesion molecule (EPCAM) is overexpressed in gastric cancer, with increased lymph-node metastasis in patients with higher levels of EPCAM expression. Nude mice received xenografts from two gastric cancer cell lines (AGS and SGC7901) for in vivo study. The tumour volume for both types of xenograft was about half the size in mice treated with EPCAM-siRNA vectors delivered by Lipofectamine 2000 compared with untreated mice. EPCAM-siRNA significantly reduced the expression of cyclin D1, causing cell cycle arrest at the G1 phase (Ref. 52).

Urokinase-type plasminogen activator

Urokinase-type plasminogen activator receptor (u-PAR; PLAUR) is overexpressed in many types of malignant tumours. A retroviral vector expressing siRNA for PLAUR was injected intratumourally into nude mice with oral squamous cell carcinoma xenografts; 30 days after treatment, the treated mice had an average tumour size of 1382 mm\(^3\), whereas the control group treated with saline had an average tumour size of 4181 mm\(^3\). The number of apoptotic cells increased in the treated xenografts, with the treated group showing an average of 3.2 compared with 2.7 for the control group. Proliferation-related Ki-67 (MKI67) was inhibited by the treatment, and protein expression levels of u-PAR, matrix metalloproteinases MMP2 and MMP9, VEGFC, VEGFD, and the VEGF receptor VEGFR-3 (FLT4) were significantly reduced in treated tumours (Ref. 53).

Survivin

Survivin (BIRC5) is overexpressed in gastric cancer and its inhibition by siRNA has been assessed in vivo. Gastric cancer cells were treated with a plasmid expressing siRNA targeting survivin or a control plasmid, and subcutaneously injected into mice. Four weeks after injection, mice that received treated cancer cells showed an average tumour size of 831 mm\(^3\); the average tumour size in mice that received cells treated with a control plasmid was 2617 mm\(^3\); and mice that received untreated cells had an average tumour size of 2536 mm\(^3\). Thus the treatment resulted in significant tumour growth inhibition. The percentage of apoptotic cells was much higher in treated cells (27.63%) than in cells with a control plasmid (2.15%) or in untreated cells (2.31%) (Ref. 54).

Vimentin

Vimentin, an intermediate filament protein, often has expression patterns correlating with the advent of metastatic cancer cells, such as increased motility, invasive ability and poor prognosis. There is considerable sequence homology between vimentin and miR-17-3p, a member of the miRNA cluster 17-92, suggesting that expression of vimentin is regulated by miR-17-3p. It is thought that miR-17-3p is a tumour suppressor because its expression is low in tumourigenic and metastatic cell lines but is higher in less tumourigenic cell lines. The effect of an shRNA plasmid expressing miR-17-3p was tested in vivo by giving mice prostate cancer xenografts and treating half of them. After 31 days, mice receiving the plasmid expressing miR-17-3p had an average tumour size less than half of that seen in mice receiving no treatment. An examination of the tumours excised from the mice showed a negative correlation between miR-17-3p expression and vimentin expression, as well as a negative correlation between tumour growth and miR-17-3p expression (Ref. 55).

miR-221 and miR-222

miR-221 and miR-222 are highly homologous miRNAs and their upregulation has been described in several types of cancer. Their role in tumourigenesis is shown by their target mRNA, p27, a negative regulator of cell cycle
progression. Small synthetic RNAs with perfect complementarity to the specific miRNAs, known as antagomirs, can be used to silence the endogenous miRNAs. Mice were given a prostate cancer xenograft on each flank; one was treated with anti-miR-221 and anti-miR-222 antagomirs and the other was treated with a control antagomir. At 33 days, the average size of treated tumours was 197.2 mm$^3$, whereas the control tumours had an average size of 276.82 mm$^3$ (Ref. 56).

**Signal transducer and activator of transcription 3**

Signal transducer and activator of transcription 3 (STAT3) acts in a signalling pathway closely associated with the proliferation, differentiation and apoptosis of cells. Constant activation of STAT3 can promote carcinogenesis, and persistently activated STAT3 has been observed in HCC cells and tissues. Mice given HCC xenografts were treated with shRNA targeting STAT3, shRNA with no homology to human gene sequences, or saline (PBS) by intratumoural injection coupled with an electric impulse (electrotransfection). The average tumour size in mice treated with PBS was 0.67 g; in mice treated with the non-targeted shRNA the average tumour size was 0.6 g; and mice treated with shRNA targeting STAT3 had an average tumour size of 0.18 g. The protein levels of STAT3, phosphorylated STAT3, VEGF, survivin and MYC were downregulated in the treated mice, yet expression of caspase 3 (CASP3) and p53 (TP53) were upregulated (Ref. 57).

**miR-16**

miR-16 has lower levels of expression in prostate cancer and has the capacity to reduce the proliferation of prostate cancer cells (Ref. 58). To evaluate miR-16 as a therapy for bone-metastatic prostate cancer, synthetic miRNA was created. Mice were given prostate cancer xenografts that can form tumours in bone and treated intravenously with a complex of the miR-16 mimic and atelocollagen, a control miRNA mimic with atelocollagen, or atelocollagen alone. Tumour development was monitored in vivo by bioluminescent imaging. At the end of the experiment (day 28), mice in the control miRNA group and the atelocollagen-only group showed the presence of tumours in several places by increased luminescence, yet mice treated with the miR-16 mimic showed no increase in luminescence (Ref. 59).

**miR-26a**

Preclinical evidence supports the therapeutic potential of miR-26a expression in HCC (Ref. 60). miR-26a directly downregulates cyclins D2 (CCND2) and E2 (CCNE2) and induces a G1 arrest in human liver cancer cells. Expression of miR-26a inhibits tumour cell proliferation in vitro and significantly inhibits cancer progression in vivo. Delivery of miR-26a may provide therapeutic potential via targeting cyclins D2 and E2.

**CD147**

CD147 (BSG) is a highly conserved glycoprotein that is overexpressed on many epithelial cancer cells, especially malignancies of the pancreas (Ref. 61). It is required for the function and expression of monocarboxylate transporter 1 (MCT1, SLC16A1), MCT3 (SLC16A3) and MCT4 (SCL16A4), and has been linked to expression of lactate transporters. The effect of CD147 silencing was observed in vivo by giving mice xenografts of pancreatic cancer cells, or clones of those cells expressing shRNA targeting CD147. Half of each group was then treated with doxycycline, creating four treatment groups. There was no significant difference in tumour size between mice that did not receive doxycycline. However, mice with unchanged pancreatic cancer xenografts treated with doxycycline had an average tumour size of 89.7 mm$^3$, whereas mice with a xenograft from cloned pancreatic cancer cells expressing shRNA targeting CD147 and treated with doxycycline had an average tumour size of 39.7 mm$^3$ (Ref. 62).

**Erythropoietin**

Erythropoietin (EPO) is a glycoprotein hormone made in the kidney in response to hypoxia and acts through its receptor EPOR. Ovarian cancer cells showed high EPOR expression, but did not express EPO under normal or hypoxic conditions in vitro. In addition, exogenous EPO introduced to the ovarian cancer cells in vitro had no biological effect. Ovarian cancer cells transfected with shRNA targeting EPOR and a negative control vector were xenografted into mice. After seven weeks, mice with xenografts treated with the control plasmid had an average tumour size approximately ten times the average size of mice treated with the EPO silencing shRNA (Ref. 63).
size in mice with treated xenografts. The difference in tumour growth was predominantly due to decreased proliferation in treated cells because the rates of apoptosis were similar between the groups. This suggests there is an EPO-independent, EPOR-mediated mechanism of growth in some cancer cells (Ref. 63).

**Clinical trials**

There are currently numerous cancer therapy trials based on RNAi technology (Table 2). A clinical trial involving targeted delivery of siRNA by Calando Pharmaceuticals is in Phase I. The delivery system utilises a cyclodextrin-containing polymer, which self-assembles into a nanoparticle with nucleic acid, and this is the first targeted delivery of siRNA in humans. Conjugates of adamantane (AD) with polyethylene glycol (PEG) (AD-PEG) and AD-PEG-transferrin (TF) were added to the surface to provide steric stabilisation and targeted delivery of the nanoparticle, respectively. Transferrin is used to target cancer cells because many cancer types are known to overexpress the transferrin receptor. This system is used to deliver siRNA against ribonucleotide reductase subunit 2 (RRM2) intravenously (Ref. 64). Anti-RRM2 siRNA exhibits antiproliferative activity in several types of human cancer cells as well as mouse, rat and monkey cells due to the complete sequence homology between these organisms at the target site (Ref. 65). A dose-escalation study in monkeys showed no significant side effects until 27 mg/kg, which is equivalent to 20–100 times larger than doses showing efficacy in mice. The effect seen was an increase in blood urea nitrogen and creatinine, indicative of renal dysfunction (Ref. 66). The trial is intended to treat adults with solid tumours who are refractory to standard-of-care therapies and the first patient was treated in May 2008 (Ref. 64).

Another Phase I clinical trial is being conducted by Alnylam Pharmaceuticals on patients with advanced liver cancers or other solid tumours with liver involvement. The trial utilises two siRNAs in a lipid nanoparticle that uses stable nucleic-acid–lipid particle (SNALP) technology, which was developed by Tekmira Pharmaceuticals (Ref. 67). The siRNAs target kinesin spindle protein (KSP/ KIF11) and VEGF (http://www.medicalnewstoday.com/articles/145218.php). The antitumour effect of VEGF was discussed earlier, and the effects of KSP inhibition include cell-cycle arrest and induction of apoptosis (Ref. 68). A study by Tekmira Pharmaceuticals tested the therapeutic effect of KSP inhibition using the SNALP delivery method on mice with liver tumour xenografts. The median survival time of treated mice was 28 days, compared with a median survival time of 20 days in mice treated with a control SNALP formation (Ref. 69).

Silence Therapeutics has recently begun a Phase I study to address the safety, tolerability and pharmacokinetics of Atu027 (http://www.silence-therapeutics.com/index.php?option=com_content &task=view&id=132). It contains an siRNA lipoplex constructed to target protein kinase N3 (PKN3), and inhibition of this kinase in vitro in primary endothelial cells impairs tube formation in the extracellular matrix and cell migration. The complex has been tested in vivo by systemic infusion in mice, rats and nonhuman primates. Mice with prostate cancer xenografts treated with Atu027 showed an average tumour volume of less than half that seen in mice injected with a sucrose solution. Treated mice also showed half as many lymph-node metastases compared with the sucrose-treated control. The lymphatic vessel density in the tumour area was decreased in treated mice, yet the blood vessel density was not significantly affected. Atu027 was tested for toxicity in *Cynomolgus* monkeys at doses of 0.3, 1.0 and 3.0 mg siRNA/kg every fourth day. PKN3 gene expression levels were determined from lung tissue taken from the animals after the last dose and silencing was observed for all three doses at a significant level as compared with sucrose-treated animals. An additional study conducted at doses of 0.03 and 0.1 mg siRNA/kg determined that 0.3 mg siRNA/kg was the lowest active dose (Ref. 70).

**Concluding section**

The ability to detect pathways that malignant tissue depends on combined with the specific gene-knockdown ability of RNAi may change cancer treatment. The capability of providing personalised care to cancer patients allows therapy to be specifically tailored for each case. However, there is still work to be done for this technology to be readily available as a therapy. The target sequence on the mRNA of a targeted gene must be selected carefully because shifting an siRNA by only a few nucleotides can drastically affect its silencing function (Ref. 71).
A carefully chosen target sequence is imperative to limit off-target effects from harming the patient further. A targeted delivery system with minimal toxicity must be devised to specifically administer the nucleic acid treatment to malignant cells without harming healthy cells.

If these issues can be resolved, personalised RNAi therapy focusing on patient-cancer-specific targets should become standard cancer treatment, either alone or in combination with other treatments. However, many limitations need to be overcome for personalised RNAi therapy to become a reality. First of all, safe, robust and specific systemic delivery of the RNAi therapeutic needs to be demonstrated early (Phase I) during clinical trial. Next, sufficient knockdown of the target protein, ideally to >75% reduction compared with baseline, needs to be achieved (later Phase I to Phase II). Follow-up assessment of the malignant tissue would need to determine the possibility of tumour cell signalling adaptation; furthermore, possible off-target knockdown within nonmalignant tissue should be investigated to demonstrate safety (Phase II). Finally, correlation of target knockdown with tumour regression will need to be observed at a high enough frequency during Phase II to justify Phase III investigation.

Not addressed in this review, but of critical importance, is the necessity of relevant targeting delivery vehicles that are safe and effectively deliver RNAi to the malignant cell sites. Moreover, confidence in the target through appropriate bioinformatic assessment and high-throughput proteogenomic determination will be required for expanded clinical use.

Table 2. Cancer therapy in clinical trials based on RNAi

<table>
<thead>
<tr>
<th>Company</th>
<th>RNAi agent name</th>
<th>Disease</th>
<th>Target</th>
<th>Trial Phase</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calando</td>
<td>CALAA-01</td>
<td>Solid tumours</td>
<td>RRM2</td>
<td>Phase I</td>
<td>72, 73</td>
</tr>
<tr>
<td>Alnylam</td>
<td>ALN-VSP</td>
<td>Liver cancers and solid tumours</td>
<td>KSP, VEGF</td>
<td>Phase I</td>
<td>72&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Silence Therapeutics</td>
<td>Atu027</td>
<td>Lung cancers</td>
<td>PKN3</td>
<td>Phase I</td>
<td>72&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Benitec/City of Hope</td>
<td>pHIV7-shl-TAR-CCR5RZ</td>
<td>AIDS lymphoma</td>
<td>HIV, TAR, CCR5</td>
<td>Phase I</td>
<td>72</td>
</tr>
<tr>
<td>University of Duisburg-Essen</td>
<td>BCR-ABL siRNA</td>
<td>Chronic myeloid leukaemia</td>
<td>bcr-abl</td>
<td>Single patient</td>
<td>74</td>
</tr>
<tr>
<td>Cequent Pharmaceuticals</td>
<td>CEQ508</td>
<td>Familial adenomatous polyposis</td>
<td>β-Catenin</td>
<td>Phase I</td>
<td>&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Gradalis, Inc.</td>
<td>FANG</td>
<td>Advanced cancer</td>
<td>Furin</td>
<td>Phase I</td>
<td>75</td>
</tr>
<tr>
<td>Hadassah Medical Organization</td>
<td>SV40/BCR-ABL</td>
<td>Chronic myeloid leukaemia</td>
<td>bcr-abl</td>
<td>Phase I</td>
<td>&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Duke University Hospital</td>
<td>siRNA immunotherapy</td>
<td>Metastatic melanoma</td>
<td>Proteasome</td>
<td>Phase I</td>
<td>&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>http://www.medicalnewstoday.com/articles/145218.php
<sup>b</sup>http://www.silence-therapeutics.com/index.php?option=com_content&amp;task=view&amp;id=132
<sup>c</sup>http://www.cequentpharma.com/cequentFDA-IND12-10-09-final.pdf
<sup>d</sup>http://clinicaltrials.gov/

Abbreviations: AIDS, acquired immune deficiency syndrome; CCR5, C-C motif cytokine receptor 5; HIV, human immunodeficiency virus; KSP, kinesin spindle protein; PKN3, protein kinase N3; RRM2, M2 subunit of ribonucleotide reductase; TAR, trans-activating response region.
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Further reading, resources and contacts

Hannon, G.J. (2002) RNA interference. Nature 418, 244-251
This article describes RNA interference and explains its significance as a means to manipulate gene expression experimentally.
This article discusses the importance of identifying dysregulated pathways of the cancer phenotype as the basis for prediction of molecular intervention.
This seminal article first described specific RNA interference in C. elegans.
This paper summarises the advances in the last decade in the field of post-transcriptional gene silencing using RNA interference approaches and provides relevant comparisons with other oligonucleotide-based approaches, with a specific focus on oncology applications.
Potential use of RNA interference in cancer therapy

Feasibility of RNA interference pathways

Table 1. Examples of in vivo use of RNAi.
Table 2. Cancer therapy in clinical trials based on RNAi.

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