Antisense oligonucleotides targeted to the human α folate receptor inhibit breast cancer cell growth and sensitize the cells to doxorubicin treatment

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Abstract
Folates are essential for cell survival and are required for numerous biochemical processes. The human α isofrom folate receptor (αhFR) has a very high affinity for folic acid and is considered an essential component in the cellular accumulation of folates and folate analogues used in chemotherapy. The expression of αhFR is not detected in most normal tissues. In contrast, high levels of the expression of αhFR have been reported in a variety of cancer cells. The significance of αhFR overexpression in malignant tissues has not been elucidated, but it is possible that it promotes cell proliferation not only by mediating folate uptake but also by generating other regulatory signals. The purpose of the present study was to evaluate αhFR as a potential target for the treatment of breast cancer. Initial studies were done in nasopharyngeal carcinoma (KB) cells, which express high levels of αhFR. In KB cells, antisense oligodeoxyribonucleotides (ODN) complementary to the αhFR gene sequences were found to reduce newly synthesized αhFR protein up to 60%. To examine the effect of αhFR antisense ODNs in a panel of cultured human breast cancer cell lines, we used a tumor cell–targeted, transferrin-liposome–mediated delivery system. The data show that αhFR antisense ODNs induced a dose-dependent decrease in cell survival. Finally, we determined that αhFR antisense ODNs sensitized MDA-MB-435 breast cancer cells by 5-fold to treatment with doxorubicin. The data support the application of αhFR antisense ODNs as a potential anticancer agent in combination with doxorubicin. [Mol Cancer Ther 2004;3(12):1505–12]

Introduction
The human α class folate receptor (αhFR) is one member of a multigene family of surface glycoproteins with a very high affinity for folic acid and reduced folates (Ka = 0.1–20 nmol/L; refs. 1–4). Folates are essential for cell survival. They are required for numerous biochemical processes, including DNA and RNA synthesis and trans-methylation reactions (1–4). Internalization of folates by αhFR involves receptor-mediated endocytosis (5). Consequently, αhFR is considered an essential component in the cellular accumulation of folates and folate analogues used in chemotherapy.

αhFR is expressed in some (6) but cannot be detected in most normal tissues. In contrast, high levels of αhFR expression have been reported in cancer cells. These include ovarian, breast, brain, lung, and colorectal cancers (7, 8). The relevance of αhFR overexpression in malignant tissues remains unclear. It is possible that elevated levels of αhFR induce cell proliferation not only by mediating folate uptake but also by generating other regulatory signals. αhFRs represent a class of cell surface proteins that are inserted into the membrane through a tail called the glycosylphosphatidylinositol (GPI) anchor (9–11). Glycosylphosphatidylinositol-linked proteins are enriched in membrane clusters termed lipid rafts, in which various components of the cell signaling machinery are concentrated (12, 13). In the ovarian carcinoma cell line IGROV1, Miotti et al. (14) showed that αhFR distributes in low-density membrane microdomains and that the receptor coprecipitates with the Src family tyrosine kinase lyn and the Goα3 heterotrimeric G-protein subunit. An in vitro kinase assay revealed that both of these signaling molecules become phosphorylated in αhFR immunoprecipitates (14). Furthermore, transfection and expression of αhFR in murine NIH/3T3 fibroblast cells, which do not endogenously express αhFR, increased cell growth in vitro and in vivo (15). The data suggest that αhFR participates in a macromolecular complex that generates intracellular signals potentially involved in modulating cell survival/proliferation processes.

The data presented above suggested the feasibility of targeting the αhFR for the treatment of cancers with elevated αhFR. To explore this idea, we designed αhFR antisense oligodeoxyribonucleotides (ODN). We have shown previously that ligands such as folate and transferrin can be effective to target liposomal delivery...
systems to cancer cells \textit{in vitro} and \textit{in vivo} (16, 17). To avoid the complication of activating the ahFR, we used the transferrin-liposome–mediated gene delivery system to examine the effect of ahFR antisense ODNs in cultured human breast cancer cell lines. We found that ahFR antisense ODNs reduced ahFR levels, and this reduction correlated with decreased cell survival. Furthermore, we show that treatment of MDA-MD-435 breast cancer cells with ahFR antisense ODNs sensitized them to doxorubicin. The data support the potential use of ahFR antisense ODNs as an anticancer agent in combination with doxorubicin.

\textbf{Materials and Methods}

\textbf{Cell Lines and Cell Culture}

Human nasopharyngeal epidermoid carcinoma (KB cells) as well as MCF7, ZR-75-1, SK-BR-3, MDA-MB-231, MDA-MB-453, T47D, and MDA-MB-468 breast cancer cell lines were obtained from American Type Culture Collection (Rockville, MD). Human breast carcinoma cell line MDA-MB-435, normal mammary immortalized cell lines MCF10 and HS578T-BST, and normal mammary primary cells HMEC were provided by the Georgetown University Medical Center Tissue Culture Core Facility. Breast cancer cell lines were maintained in DMEM with folic acid and 10% fetal bovine serum. KB cells were maintained in DMEM without folic acid and 10% fetal bovine serum. The final folic acid concentration was 1 to 10 nmol/L (18). Cell culture medium and medium ingredients were purchased from Biofluids, Inc. (Rockville, MD).

\textbf{Oligonucleotides}

Unmodified ODNs (Table 1) were synthesized by using an automated synthesizer (Applied Biosystems, Foster City, CA). Purity of 21-mer sense and antisense ODNs was assessed by measurement of 260/280-nm absorbance ratios and by electrophoretic analysis on a 10% polyacrylamide gel. End-modified phosphorothioated ODNs were purchased from Ampligene Biotechnologies (Rockville, MD).

\textbf{Immunoprecipitation}

Immunoprecipitations were done as described previously (19). KB cells (10\(^6\) cells per 35-mm well) were plated in 3 mL DMEM without folic acid and with 10% fetal bovine serum. Cells were washed once with 2 mL DMEM (Select Amine, Life Technologies, Inc., Rockville, MD) containing no methionine or cysteine and then incubated for 1 hour in 2 mL of the same medium. Newly synthesized proteins were radiolabeled by the addition of 50 \(\mu\)Ci [\(\text{\textsuperscript{35}}\text{S}\)]methionine and 50 \(\mu\)Ci [\(\text{\textsuperscript{35}}\text{S}\)]cysteine in 1 mL of growth medium containing no methionine or cysteine. Cells were incubated in medium containing radiolabeled amino acids for 16 hours in the presence of unmodified ahFR sense and antisense ODNs at the indicated concentrations. After labeling, cells were scraped and lysed by repeated freezing and thawing. Membrane proteins were solubilized in 1 mL PBS containing 1% Triton X-100 (v/v). The protein concentrations were determined by a protein assay reagent (Pierce Chemical Co., Rockford, IL). Protein samples (10 \(\mu\)g) were immunoprecipitated using 50 \(\mu\)L of polyclonal rabbit anti-ahFR antiserum and protein A-Sepharose in 750 \(\mu\)L immunoprecipitation buffer [10 mmol/L Tris, 150 mmol/L NaCl, 2 mmol/L EDTA (pH 7.4), 1% Triton X-100, and 0.1% SDS] for 3 hours at 4°C (20). Immunoprecipitates were boiled for 5 minutes in the same buffered solution and pelleted at 2,500 \(\times\) g for 5 minutes. Supernatants were fractionated by 12% SDS-PAGE and visualized and quantitated by PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

\begin{table}[h]
\centering
\caption{ahFR antisense and sense ODN sequences and sequence location within the ahFR open reading frame}
\begin{tabular}{lll}
\hline
ODN & Sequence* & Target Site in ahFR Open Reading Frame \\
\hline
Antisense 1 & TGT TGT CAT CCG CTG AGC CAT & 21–1 \\
Sense 1 & ATG GCT CAG CGG ATG ACA ACA & 1–21 \\
Antisense 2 & AGG AGG TCA GCT GAG CAG CCA & 780–760 \\
Sense 2 & TGG CTG CTC AGC TGA CCT CCT & 760–780 \\
Antisense 3 & CTT GTG GTG GTT CCC GTT CAT & 132–112 \\
Sense 3 & ATG A AC C GC A A G AAC C A C C A C A G & 112–132 \\
Antisense 4 & GTA GGA AAC ATC CTT ATG GGC & 240–220 \\
Sense 4 & GCC CAT A A G GAT GTT TCC TAC & 220–240 \\
Antisense 5 & GAT CCC GGG CCC CAA GTT GGG & 351–313 \\
Sense 5 & C C C A A C G T G G G G C G C T G G & 331–313 \\
Antisense 6 & GCT CCT GCA GTT GTA GGA GGT & 462–442 \\
Sense 6 & A C C T C C T A C T G C C A G A C C & 442–462 \\
Antisense 7 & GCT CCT GCA GTT GTA GGA GGT & 462–442 \\
Sense 7 & A C C T C C T A C T G C A A G A C C & 442–462 \\
Antisense 8 & TTC ATT GCA CAG AAC AGT GGG & 553–573 \\
Sense 8 & C C C A C T G T G T G C A T G A A & 553–573 \\
Antisense 9 & CGC CAC CTC CTC ATT GGG GTT & 678–658 \\
\hline
\end{tabular}
\*Underlined oligonucleotides represent phosphorothioated-modified backbones.
\end{table}
RNase Protection Assay

The relative abundance of hFR transcripts in normal human tissue was determined with RNase protection assay as described previously (21). RNA was purchased from Clontech (Palo Alto, CA) and the integrity and quantity of RNA was verified by Northern analysis. The 5′ EcoRI-Hinfl restriction fragment from the KB4 cDNA clone was radiolabeled using the Promega (Madison, WI) in vitro transcription kit and used as the hFR riboprobe. Total RNA (25 µg), internal control RNA (2 µg), and hFR riboprobe (100,000 counts/min) in 30 µL of hybridization buffer [40 mmol/L PIPES (pH 6.4), 80% (v/v) formamide, 0.4 mol/L NaCl, and 1 mmol/L EDTA] was denatured at 85°C for 8 minutes and then reannealed at 45°C for 18 hours. The ssRNA was digested by the addition of 350 µL of buffer [10 mmol/L Tris-HCl (pH 7.5), 200 mmol/L NaCl, 100 mmol/L LiCl, and 2 mmol/L EDTA] containing RNase A (14 µg) and RNase T1 (0.7 µg) followed by incubation at 25°C for 15 minutes. The digestion was terminated by the addition of 10% SDS (w/v) and proteinase K (100 µg) and incubation at 37°C for 30 minutes. After extraction in an equal volume of phenol/chloroform/isooamyl alcohol (25:24:1, v/v/v), RNA was precipitated with ethanol and fractionated on a 6% sequencing wedge gel. The dried bands were visualized using a PhosphorImager (Molecular Dynamics).

Western Blot Analysis

Cellular lysates for Western analyses were prepared as described previously (22). Breast cancer and normal mammary cells were washed twice in room temperature PBS and harvested by scraping in cold lysis buffer containing PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 0.1 mg/mL phenylmethylsulfonylfluoride, 30 µg/mL aprotinin, and 1 mmol/L sodium orthovanadate. After 20 minutes in an ice bath, lysates were passed through a 21-gauge needle, incubated on ice an additional 30 minutes, and then centrifuged at 15,000 × g for 20 minutes at 4°C. Protein concentrations were determined using the Pierce Micro bicinchoninic acid protein assay reagent and the proteins were stored at −80°C.

Primary anti-human hFR polyclonal rabbit antibody was generated as described previously (20). To visualize the transferrin receptor in breast cancer cells, we used mouse anti-human transferrin receptor (Zymed Laboratories, Inc., San Francisco, CA). The anti-human glyceraldehyde-3-phosphate dehydrogenase rabbit polyclonal antibody was purchased from Trevigen (Gaithersburg, MD). The anti-actin (C-11) goat polyclonal antibody and all secondary horseradish peroxidase–conjugated antibodies were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA).

Samples containing 20 µg of total cellular protein were electrophoresed on a 4% to 20% SDS-PAGE gradient gel and electroblotted onto a 0.2-µm nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA). To block nonspecific binding, the membrane was incubated at room temperature for 1 hour with 5% nonfat dry milk in 10 mmol/L Tris-HCl buffer (pH 8.0) containing 150 mmol/L NaCl and 0.05% Tween 20 (TBST). The blot was probed for 1 hour with primary antibody and washed thrice for 15 minutes each with TBST. The specific protein was detected using secondary horseradish peroxidase–conjugated immunoglobulin G. The membrane was probed with secondary antibody for 45 minutes and washed thrice for 15 minutes each with TBST. Proteins were visualized using the enhanced chemiluminescence Western blotting detection reagent and Hyperfilm enhanced chemiluminescence (Amersham, Piscataway, NJ).

Transfection and Cell Survival Assay

Transfection of hFR S6 and AS6 end-modified phosphorothioated ODNs into breast cancer cells was accomplished using a transferrin-mediated liposomal Be delivery system as described previously (16, 17). Briefly, 2 × 10⁴ cells were plated per well in a 96-well plate and washed twice with serum-free DMEM. Transferrin-liposome-ODN complex was prepared by mixing 50 nmol liposome B with 625 µg transferrin and then 5 nmol ODNs in 800 µL of serum-free DMEM at a final ODN concentration of 5 µmol/L and then added to cells at indicated concentrations for 5 hours. Transfections were terminated by the addition of an equal volume of DMEM containing 10% fetal bovine serum. Cell survival was determined after 48 hours by 2,3-bis[2-methoxy-4-nitro-5-sulphophenyl]-2H-tetrazolium-5-carboxanilide inner salt (XTT) assay according to the manufacturer’s instructions (Boehringer Mannheim, Indianapolis, IN). In the presence of an electron coupling reagent, XTT is converted into orange formazan by dehydrogenase in the mitochondria of living cells. The formazan absorbance, which correlates to the number of living cells, was measured at 450 nm using a microplate reader (Molecular Devices, Menlo Park, CA). The IC₅₀ was interpolated from the graph of the log of drug concentration versus the fraction of surviving cells.

Chemosensitization

For chemosensitization studies, MDA-MB-435 cells were plated and transfected with 500 nmol/L of hFR AS6 or S6 ODN as described above. Twenty-four hours after transfection, the medium was replaced with DMEM containing 10% fetal bovine serum and doxorubicin (Bedford Labs, Bedford, OH) at the indicated concentrations. Cell survival was determined by XTT assay, as described above, after 72 hours.

Results

Treatment with hFR Antisense ODNs Decreases Protein Synthesis and Cell Survival in KB Cells

Eight antisense ODNs were designed to target the hFR open reading frame (Fig. 1A) to determine whether decreased levels of hFR impact cell survival. For preliminary experiments, we used cultured human nasopharyngeal cells (KB cells), which express high levels of hFR. KB cells were incubated with hFR antisense ODNs (AS1, AS2, and AS6) at the indicated concentrations for 6 hours. Figure 1B shows a dose-dependent decrease in the levels of hFR protein up to 60% 2 days after the treatment.

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as determined by immunoprecipitation analysis. In contrast, aFR sense ODNs (S1, S2, and S6) had little effect on the levels of aFR in KB cells (Fig. 1C). The data correspond to survival analysis of KB cells, which decreased after 2 days of aFR antisense exposure (data not shown).

Expression of aFR in Normal Tissue and Cultured Human Breast Cancer Cells

The levels of aFR are reportedly higher in cancer compared with normal tissue (7, 8). We determined the levels of aFR transcripts in a panel of normal tissue using a RNase protection assay. Figure 2 shows that aFR transcripts are present in some tissues (i.e., the lung, kidney, breast, salivary gland, and placenta) but are not detected or are present at very low levels in most other normal tissue; in contrast, much higher levels are present in KB cells. The data correspond to survival analysis of KB cells, which decreased after 2 days of aFR antisense exposure (data not shown).

Impact of aFR Antisense ODNs on Survival of Cultured Human Breast Cancer Cells

To determine the effect of aFR antisense ODNs on the survival of cultured breast cancer cells, we transfected end-modified phosphorothioated AS6 ODNs and S6 ODNs (Table 1) at various concentrations via the transferrin-liposome Be–mediated gene delivery system. As shown in Fig. 4A, increased amounts of transfected AS6 ODNs correlated with decreased cell survival as determined by XTT assay. The IC50 values (μM/L) for the treated breast

Figure 1. A, schematic representation of eight antisense ODNs targeted to various regions of the aFR open reading frame. Immunoprecipitation analysis of aFR protein levels in KB cells treated with aFR antisense ODNs (B) or sense ODNs (C). KB cells (10^6 cells per 35-mm well) were incubated with unmodified ODNs for 6 hours in serum-free medium. Relative amounts of aFR protein were determined 36 hours after transfection using a polyclonal rabbit aFR antibody. Immunoprecipitates were fractionated by 12% SDS-PAGE and visualized and quantitated by a PhosphorImager (Molecular Dynamics).

Figure 2. RNase protection assay of aFR transcript levels in normal tissue. Normal human tissue RNA was purchased from Clontech. Total tissue RNAs (25 μg) were used for RNase protection assay exactly as described previously (20). The RNA species was precipitated and fractionated on a 6% (w/v) sequencing wedge gel. The dried gel was visualized using a PhosphorImager (Molecular Dynamics).
cancer cell lines were 0.633 (T47D), 0.600 (MDA-MB-231), and 0.416 (MDA-MB-435). In contrast to these data, cell lines treated with S6 ODNs had significantly less effect on the survival of model breast cancer cell lines (Fig. 4B).

Effect of a hFR ODNs on the Levels of a hFR Protein in MDA-MB-435 Breast Cancer Cells

Antisense ODNs are believed to abrogate protein function by direct hybridization of the ODNs to exposed regions of the targeted mRNA. The resulting RNA-DNA is thought to preclude the translation of the mature, functional protein, thereby diminishing the overall cellular target protein levels (30, 31). To examine if the a hFR ODN effect on cell survival was correlated to decreased levels of a hFR protein, we transfected MDA-MB-435 breast cancer cells with end-modified phosphorothioated AS6 and S6 ODN, as shown in Fig. 5, and did Western analyses. We observed a significant decrease in the levels of a hFR after treatment with 0.25 and 0.50 μmol/L AS6 ODNs for 24 or 48 hours. However, the a hFR levels of the S6 ODN–treated samples remained relatively indistinguishable from the untreated cells or cells treated with liposome alone. These results strongly suggest that the cellular levels of a hFR protein are an important component of cell survival.

Sensitization of MDA-MB-435 Breast Cancer Cells to Doxorubicin after Treatment with a hFR Antisense ODN

To determine whether a hFR antisense ODN sensitizes breast cancer cells to chemotherapy, we transfected, using transferrin-liposome Be, 500 nmol/L of end-modified phosphorothioated AS6 ODN or S6 ODN into MDA-MB-435 cells and treated them with varying concentrations of doxorubicin 24 hours after transfection. Cell survival was determined after 72 hours by XTT assay and the degree of sensitization was calculated by determining the ratio of IC50 values of the combined treatment relative to that of the S6 ODN and untreated controls. In Fig. 6, chemosensitization of MDA-MB-435 cells by AS6 ODN was 5-fold (IC50S6/IC50AS6 = 5) relative to the sense control and 10-fold (IC50Untreated/IC50AS6 = 10) relative to the untreated control.

Discussion

a hFR is one member of a multigene family of folate binding proteins. a hFR is considered an important component in the maintenance of cellular folate homeostasis. a hFR binds folates with high affinity (Kd = 0.1–20 nmol/L) and internalizes the vitamin by a process involving receptor-mediated endocytosis (1–4).

We and others (6–8) have reported that a hFR levels are higher in cancer cells than in normal tissue (Fig. 2). For example, a hFR is elevated in >90% of human epithelial cancer cell lines were 0.633 (T47D), 0.600 (MDA-MB-231), and 0.416 (MDA-MB-435). In contrast to these data, cell lines treated with S6 ODNs had significantly less effect on the survival of model breast cancer cell lines (Fig. 4B).

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We and others (6–8) have reported that a hFR levels are higher in cancer cells than in normal tissue (Fig. 2). For example, a hFR is elevated in >90% of human epithelial
ovarian malignancies as compared with normal epithelial ovarian cells (32). Other cancers with elevated a FR relative to their normal counterpart include breast, brain, lung, and colorectal cancers (7, 8). Consequently, several strategies have been developed for the targeted delivery of drugs to a FR-positive tumor cells (33). Covalent attachment of therapeutic agents to a FR-targeted monoclonal antibodies has been evaluated for imaging and immunotherapy and have shown significant targeting efficacy in patients with ovarian cancer (34). Alternatively, folate-derivatized anticancer treatments have been successfully applied in vitro for a FR-specific delivery (35). Of interest, low molecular weight radiopharmaceuticals based on folate conjugates have shown favorable pharmacokinetic properties and tumor selectivity in a FR-positive animal tumor models (36, 37). Unlike previous reports that have used a FR as a drug delivery vehicle, the present study is the first account to target a FR using gene-specific antisense ODN.

The purpose of elevated a FR in cancer is unclear. It has been proposed that a FR has a role in promoting cell proliferation independent of folate internalization. Bottero et al. (15) showed that transfection of a FR into non-expressing NIH/3T3 cells increased growth in vitro and in vivo. The data presented in this report agree with these observations. We show that reducing the levels of a FR, by a FR-targeted antisense ODNs (Fig. 1A), inhibited cell survival in cultured human breast cancer and KB cells after 36 hours (Fig. 1B). Decreased cell number is not likely to be attributable to folate starvation, a process that occurs over the order of weeks. The literature suggests that a FR may be involved in cell processes that are unrelated to folate internalization. For example, a FR, similar to other glycosylphosphatidylinositol-linked proteins (12, 13), may have a role in cell signaling processes leading to cell growth and proliferation (14). Further studies are required to delineate the mechanism(s) by which a FR ODN mediates death of cancer cells.

In the present study, we were interested in testing a FR antisense ODNs as a potential anticancer therapeutic agent for the treatment for breast cancer. We evaluated the levels of a FR in breast cancer and normal mammary cells. As shown in Fig. 3, a FR was present in all tested cells; however, unlike other disease models, the levels of a FR were not significantly elevated in the cultured breast cancer cells compared with normal mammary cells. In an attempt to achieve breast cancer cell–specific targeting, we used a transferrin receptor–mediated delivery system. In Fig. 3, the transferrin receptor is more highly expressed in breast cancer cell lines than in the immortalized and primary normal mammary cells. The data show the feasibility of delivering a FR antisense ODNs via a transferrin targeting liposomal delivery system even where a FR is not overexpressed.

The mechanism by which a FR antisense ODNs selectively decreased a FR protein in MDA-MB-435 breast cancer cells (Fig. 5) is not entirely clear. In general, antisense ODNs are believed to abrogate protein function by direct hybridization of the ODNs to exposed regions of the targeted mRNA. The resulting RNA-DNA is thought to preclude the translation of the mature, functional protein, thereby diminishing the overall cellular target protein levels. The antisense-mediated decrease of protein production is hypothesized to occur by (a) RNase H digestion of the RNA-DNA duplex or (b) physical disruption of the translation machinery (30, 31). Nonantisense mechanisms may also be involved in selective protein targeting by ODN. For example, aptamers are a class of small nucleic acid ligand agonists...
that exhibit exquisite specificity for proteins (38, 39). Additional studies are required to understand the mechanism of ahFR antisense ODN–mediated selective decrease of ahFR protein in cancer cells.

The current study suggests an application of ahFR antisense ODNs for the treatment of ahFR-positive cancers, particularly ovarian cancer. ahFR has been characterized as a marker for ovarian carcinoma because it is expressed in 90% of epithelial ovarian carcinomas and is absent in normal ovarian epithelial cells (40). Furthermore, ahFR appears very early in the transformation process and even increases with tumor progression (32). The pathology of ovarian carcinogenesis has been well documented. In the early stages, the spread of the disease typically involves the opposite ovary, the uterus, and the fallopian tubes (41). In the more advanced stages, metastases occur at the peritoneal surfaces of the bladder, the rectosigmoid, or the pelvic peritoneum (42). The confinement of ovarian metastatic spread to the i.p. cavity has allowed for treatment strategies involving i.p. therapy (43). Wide expression of ahFR in ovarian carcinoma and its association with malignant transformation and cell survival processes as well as local disease metastasis support i.p. administration of ahFR-targeted antisense molecules for the treatment of ovarian cancer.

References


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