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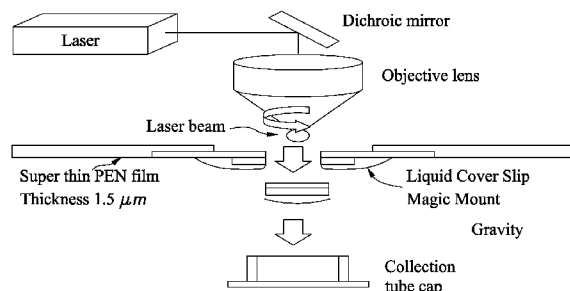
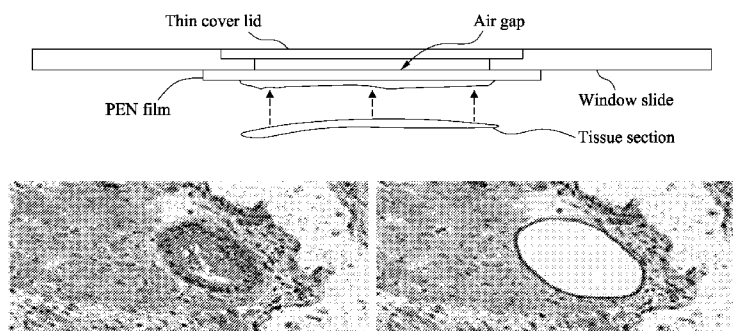
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[Continued on next page]

(54) Title: BIOMARKERS AND MORPHOLOGY BASED APTAMER SELECTION OF SAME

**FIG. 2F**

(57) Abstract: Several embodiments disclosed herein relate to methods for developing a panel of aptamers that are tailored specifically to the biomarkers of the targeted cells of a particular patient. In some embodiments, laser microdissection is used to isolate highly pure cell populations from a heterogeneous tissue section, cytological preparation, or live cell culture via direct visualization of the cells.



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BIOMARKERS AND MORPHOLOGY BASED APTAMER SELECTION OF SAME

RELATED CASES

[0001] This application claims the benefit of United States Provisional Application No. 62/030,544, filed July 29, 2014, the entirety of which is incorporated by reference herein.

STATEMENT REGARDING FEDERALLY SPONSORED R&D

[0002] This invention was made with U.S. Government support under Grant No. 275200800020C awarded by the National Institutes of Health. The government has certain rights in the invention.

BACKGROUND

Field

[0003] Disclosed are embodiments relating generally to the field of nucleic acids, and more particularly to aptamers capable of binding to target tissues, for example, cancerous cells. The aptamers are useful for therapeutics and in diagnostics. Several embodiments relate to materials and methods for the generation of aptamers and identification of tissue biomarkers.

SUMMARY

[0004] With the continued advancement in targeted therapeutics and diagnostics, there is a need for the rapid, efficient and accurate development of molecules to target specific cells within a tissue or collection of cells. There are provided herein, to that end, methods and systems for developing pools of aptamers that are highly specific for a target moiety. There are also provide compositions of aptamers, selected using the evolutionary methods disclosed herein that are used for diagnosis of disease, identification of biomarkers and/or for cell-specific delivery of therapeutics.

[0005] There is provided, in several embodiments, a method for selecting aptamers specific for a diseased target tissue, comprising obtaining a library comprising putative diseased tissue-specific aptamers, incubating the library with a first tissue sample comprising a first region of tissue affected with a disease and a second region of the tissue not affected with the disease, washing the first tissue sample to remove aptamers that are not

bound to the first or second region of tissue, microscopically identifying at least one cell of the first tissue sample within the first region and capturing the at least one cell using laser microdissection (LMD), eluting the aptamers bound to the at least one captured cell, amplifying the eluted aptamers using polymerase chain reaction to generate a first next-generation library of aptamers, incubating the first next-generation library with a second tissue sample comprising a first region of tissue affected with a disease and a second region of the tissue not affected with the disease, washing the second tissue sample to remove aptamers that are not bound to the first or second region of tissue, microscopically identifying at least one cell of the second tissue sample within the first region and capturing the at least one cell using LMD, eluting the aptamers bound to the at least one captured cell from the second tissue sample, amplifying the eluted aptamers using polymerase chain reaction to generate a second next-generation library of aptamers and repeating the incubation, washing, LMD capturing, elution and amplification steps a plurality of additional times to generate a pool of enriched aptamers that are specific for the diseased target tissue.

[0006] In several embodiments, a first incubation is for a first period of time and employs a first set of conditions of a particular stringency, while the incubation of the first next-generation library with the second tissue sample employs a second set of conditions that are more stringent than the first set of conditions. Alternatively, there need not be a change in stringency between repeated screenings. In some embodiments, each subsequent round of screening employs an incubation condition that is more stringent (in at least one characteristic) than a prior incubation. Advantageously, in several embodiments, the increasing stringency enhances the specificity of the aptamers that are eventually generated for a target of interest.

[0007] In several embodiments, the method additional includes sequencing the pool of enriched aptamers to determine their sequence and relative frequency within the pool. Depending on the embodiment, one or more of a variety of sequencing approaches may be used. In several embodiments, the sequencing comprises next generation sequencing (NGS). Further, in several embodiments, the methods optionally include grouping the pool of enriched aptamers based on comparison of their sequences (e.g., a cluster analysis) and/or

predicting the secondary structure of the sequences (e.g., to predict putative binding interactions with a target marker).

[0008] In several embodiments, the methods may also include confirming the binding of at least one aptamer from the pool of enriched aptamers against an additional sample of diseased target tissue. This confirmatory step is optional in some embodiments, but can serve to advantageously confirm the efficacy of an aptamer under in vitro or in vivo conditions, rather than solely relying on frequency predictions or three-dimensional modeling. Depending on the embodiment, binding can be confirmed by one or more of an in vitro biochemical assay, a functional cell based assay, a dot blot assay, identifying colocalization of a candidate aptamer with an antibody known to bind to a putative target, ELISA, radiolabeled binding assay, protein pull-down assay, and western blot. Cell-free model systems may also be used, in several embodiments.

[0009] Additionally, several embodiments also include amplifying at least one candidate aptamer from the pool of enriched aptamers, labeling or immobilizing the at least one candidate aptamer such that the at least one aptamer can be specifically identified and isolated, mixing the at least one labeled or immobilized aptamer with a lysate derived from the diseased target tissue under conditions that allow the formation of an aptamer-target complex, recovering the aptamer-target complex, dissociating the aptamer and the target, and retrieving the target, and sequencing the target from the aptamer-target complex to identify the identity of the target. These additional aspects, in several embodiments, allow the identification of the specific target of an aptamer (e.g., a biomarker that the aptamer is specific for). In several embodiments, the biomarker can be used in a diagnostic context (e.g., an aptamer directed against the biomarker and labeled with a reporter agent can signal the presence of the biomarker in a tissue sample, such as a biopsy to identify cancer tissue). Depending on the embodiment, the target can be performed in various ways. In several embodiments, sequencing of the target is performed by electrospray ionization (ESI) and tandem mass spectrometry-based sequencing. In several embodiments, the sequencing of the target is performed by matrix-assisted laser desorption/ionization (MALDI) and tandem mass spectrometry-based sequencing. In still additional embodiments, the sequencing of the target

is performed by enzymatically digesting the target and identifying resultant peptides by mass fingerprinting and/or tandem mass spectrometry.

[0010] In several embodiments, the diseased tissue comprises a tissue affected by cancer, acute disease, chronic disease, acute injury, or indirectly affected by cancer, acute disease, chronic disease, or acute injury. In several embodiments, the library comprises thio-modified aptamers. However, in several embodiments, non-modified aptamers make up all or a portion of the library. Additionally, in several embodiments, other modifications to the aptamers may be made to enhance their stability, improve specificity, enhance amplification efficiency, or improve detectability of the aptamers.

[0011] In several embodiments, the evolved pool of candidate aptamers is at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, or at least 96-99% specific for a diseased target tissue.

[0012] In one embodiment, a biomarker can be identified by selecting a pool of enriched aptamers according by evolutionarily screening an aptamer library and capturing tissue to which the aptamer library binds, amplifying at least one candidate aptamer from the pool of enriched aptamers, labeling or immobilizing the at least one candidate aptamer such that the at least one aptamer can be specifically identified and isolated, mixing the at least one labeled or immobilized aptamer with a lysate derived from the diseased target tissue under conditions that allow the formation of an aptamer-target complex, recovering the aptamer-target complex, sequencing the target from the aptamer-target complex to identify the identity of the target, thereby identifying a biomarker. As discussed above, the sequencing of the target can be accomplished by any of a variety of protein sequencing methodologies.

[0013] Also provided for herein are methods for identifying a biomarker comprising, obtaining a library comprising putative diseased tissue-specific aptamers, incubating the library with a first tissue sample comprising a first region of tissue affected with a disease and a second region of the tissue not affected with the disease, removing aptamers that are not bound to the first or second region of tissue, isolating at least one cell of the first tissue sample within the first region, amplifying aptamers that were bound to the isolate cell to generate a first next-generation library of aptamers, repeating the incubation, removing, isolating, and amplification steps a plurality of additional times to generate a pool

of enriched aptamers that are specific for the diseased target tissue, amplifying a candidate aptamer from the pool of enriched aptamers, labeling or immobilizing the amplified candidate aptamer, exposing the labeled amplified aptamer to a lysate of diseased tissue, the diseased tissue comprising target markers, isolating complexes of labeled amplified aptamer and target markers; and sequencing the target marker.

[0014] In several embodiments, the candidate aptamer is labeled with a fluorophore (e.g., Cy3, green fluorescent protein), a radiolabel (e.g., ^3H , I_{125} , etc.), a chromophore (DAB), a magnetic particle (SPIO), or antigen (streptavidin/biotin). In several embodiments, the candidate aptamer is labeled with streptavidin. In several embodiments, the candidate aptamer is labeled with superparamagnetic iron oxide particles. In several embodiments, the candidate aptamer is immobilized on a solid support, such as, for example a tissue culture plate or a population of agarose beads (optionally for use in a column).

[0015] Depending on the embodiment, the biomarker is expressed on a tissue affected by cancer, acute disease, chronic disease, acute injury, or indirectly affected by cancer, acute disease, chronic disease, or acute injury.

[0016] There are also provided methods for selecting aptamers specific for a diseased target tissue, comprising receiving a first and a second sample of a target tissue, wherein the first sample is from a region of the target tissue affected with a disease and a the second sample is from a region of the target tissue not affected with the disease, wherein the first and the second sample are collected using laser microdissection, obtaining a library comprising putative diseased tissue-specific aptamers, screening the library against the first sample to identify aptamers that bind to the diseased tissue, removing aptamers that are unbound, thereby generating a pool of candidate aptamers, screening the pool of candidate aptamers against the second tissue sample to identify candidate aptamers that bind to the normal tissue, collecting aptamers that do not bind to the normal tissue, thereby generating a pool of screened candidate aptamers, amplifying the pool of screened candidate aptamers, repeating the screening against the first and second tissue samples a plurality of times to enrich the pool of screened candidate aptamers, thereby generating a pool of enriched aptamers that are specific for the diseased target tissue. In several embodiments, the diseased tissue is cancerous. In several embodiments, the library comprises thio-modified aptamers

(though non-modified aptamers may also be used). In several embodiments, the pool of enriched aptamers is at least 80% specific for the diseased target tissue. In several embodiments, the laser microdissection is used to dissect tissue regions morphologically identified as diseased or normal. In several embodiments, the method also includes sequencing the pool of enriched aptamers. Still further, the method optionally includes confirming the tissue specificity of the pool of enriched aptamers by identifying aptamers that co-localize with tissue specific antigens identified by antibodies.

[0017] There is also provided a method for identifying a biomarker specific for a diseased tissue of a subject, comprising receiving a first and a second sample of a target tissue, wherein the first sample is from a region of the target tissue affected with a disease and a the second sample is from a region of the target tissue not affected with the disease, obtaining a library comprising putative diseased tissue-specific aptamers and screening the library against the first sample to identify aptamers that bind to the diseased tissue, removing aptamers that are unbound, thereby generating a pool of candidate aptamers, screening the pool of candidate aptamers against the second tissue sample to identify candidate aptamers that bind to the normal tissue, collecting aptamers that do not bind to the normal tissue, thereby generating a pool of screened candidate aptamers, amplifying the pool of screened candidate aptamers, sequencing the pool of screened candidate aptamers; and aligning the sequences of the screened candidate aptamers against known proteins to determine the biomarker that the aptamer is directed to, thereby identifying the biomarker specific to the diseased tissue of the subject.

[0018] There are also provided methods of treating a subject with a disease comprising, obtaining first and second sample of tissue from the subject, wherein the first sample is from a diseased region of tissue, and the second sample is from a region of the tissue not affected with the disease, ordering an enriched pool of diseased-tissue specific aptamers, wherein the enriched pool of diseased-tissue specific aptamers is at least 80% specific for the diseased tissue, conjugating at least one therapeutic agent to the pool of diseased-tissue specific aptamers, thereby generating therapeutic aptamers, and administering the therapeutic aptamers to the subject, wherein the therapeutic aptamers deliver the conjugated therapeutic agent specifically to the diseased tissue. In several embodiments, the

delivery route is selected from intravenous, local, oral, intramuscular, systemic, and transdermal.

[0019] In several embodiments, there is a method for treating cancer (e.g., ovarian cancer), comprising administering an tissue-specific population of aptamers to a subject with a cancer (e.g., ovarian cancer), the aptamers conjugated to a therapeutic agent, wherein the aptamers were identified by screening both cancerous and non-cancerous tissue collected from the subject against a pool of aptamers to identify those aptamers that bind the cancerous tissue but not the non-cancerous tissue. In several embodiments, the cancer tissue is cancer vasculature, while in other embodiments the cancer tissue is cancer cells. In several embodiments wherein the cancer is ovarian cancer, the aptamer targets vimentin or vimentin-related pathways. In one embodiment, the aptamer comprises a polynucleotide with the sequence of SEQ ID No. 5, variants thereof, or functional equivalents thereof. Uses of aptamers selected according to the methods disclosed herein for the treatment of disease is also provided for, in several embodiments.

[0020] In several embodiments, there is provided a diagnostic agent comprising a tissue-specific aptamer evolved through the morphological-based selection methods disclosed above and conjugated to a reporter agent. The reporting agent can be, but is not limited to, a fluorophore, a chromophore, an enzyme cleavable reagent, or a radio-labeled nucleotide.

[0021] In one embodiment, there is provided an ovarian cancer specific aptamer encoded by the polynucleotide of any one of SEQ ID NOs. 1 to 46.

[0022] Also provided is a method of identifying high binding affinity aptamer sequences comprising generating a combinatorial DNA aptamer library, incubating the combinatorial DNA aptamer library with diseased cells and negatively selecting the combinatorial DNA aptamer library against normal cells to generate a plurality of aptamers bound to the diseased cells, the selection performed by dissecting regions of interest bound with aptamers based on a morphological assessment of the tissue, eluting and amplifying the plurality of aptamers bound to the diseased cells, identifying high affinity aptamer sequences by next generation sequencing, and identifying the targeted proteins by mass spectrometry. In several embodiments, the plurality of aptamers bound to the diseased cells are further incubated with diseased cells and negatively selected against normal cells, eluted, and

amplified. In several embodiments the morphological positive and negative selection are performed at least 10 times. In several embodiments, the DNA aptamer library has been modified with thio substitution of the phosphate backbone at the 5'dA position. In several embodiments, the dissecting is accomplished using image directed laser microdissection.

[0023] In one embodiment, the diseased cells are human ovarian cancer endothelial cells and the normal cells are human ovarian endothelial cells. In some such embodiments, the ovarian cancer endothelial cells are CD31⁺ and CD146⁺. In several embodiments, the aptamer shows specific binding to the vasculature of human ovarian cancer tissue. In several embodiments, the aptamer showing specific binding to the vasculature of human ovarian cancer tissue is a vimentin-specific sequence.

[0024] There is also provided a method of identifying high binding affinity aptamer sequences to treat ovarian cancer comprising generating a combinatorial DNA aptamer library wherein the combinatorial DNA aptamer library has been modified with thio substitution of the phosphate backbone at the 5'dA position, incubating the combinatorial DNA aptamer library with human ovarian cancer cells and dissecting regions of morphological interest bound with aptamers using laser microdissection based on a morphological assessment of the tissue, to generate a plurality of aptamers bound to the diseased cells, eluting and amplifying the plurality of aptamers bound to the diseased cells, identifying high affinity aptamer sequences by next generation sequencing, and identifying the targeted proteins by mass spectrometry.

[0025] Methods of treating a disease using aptamer sequences are also provided, comprising screening a DNA aptamer library against a diseased tissue and dissecting regions of interest bound with aptamers based on a morphological assessment of the tissue, identifying high affinity aptamer sequences by next generation sequencing; conjugating the high affinity aptamer sequences with a therapeutic agent, and administering the aptamer-therapeutic agent complex.

[0026] In one embodiment, a method of treating ovarian cancer using aptamer sequences is provided and comprises screening a DNA aptamer library modified with thio substitution of the phosphate backbone against ovarian tissue and dissecting regions of interest bound with aptamers using laser microdissection based on a morphological

assessment of the tissue, identifying high affinity aptamer sequences by next generation sequencing, conjugating the high affinity aptamer sequences with a therapeutic agent and administering the complex. In one embodiment, the high affinity aptamer sequence is encoded by SEQ ID NO. 5.

BRIEF DESCRIPTION OF THE DRAWINGS

[0027] Various embodiments are depicted in the accompanying drawings for illustrative purposes, and should in no way be interpreted as limiting the scope of the embodiments. Furthermore, various features of different disclosed embodiments can be combined to form additional embodiments, which are part of this disclosure.

[0028] FIG. 1 illustrates an embodiment of the method for morphologically-based aptamer selection directed to obtaining an aptamer population with high selectivity for a particular tissue such as a tumor tissue. In some embodiments, the method illustrated in FIG. 1 can be performed on an ovarian tumor.

[0029] FIGS. 2A-2D illustrate the laser microdissection of blood vessels of a human ovarian tumor (after exposure to an aptamer library) pre-cut (FIG. 2A) and post-cut (FIG. 2B) and tumor cells of human ovarian cancer pre-cut (FIG. 2C) and post-cut (FIG. 2D). Note that, in accordance with several embodiments disclosed herein the correspondence between the tissue identified morphologically/histochemically is corresponds with that removed by laser microdissection (LMD).

[0030] FIG. 2E illustrates the detection of polymerase chain reaction (“PCR”) amplified thioaptamers from laser microdissected blood vessels and tumor cells as shown in FIGS. 2A-2D.

[0031] FIG. 2F illustrates a schematic of the laser microdissection of target tissue and cells used in several embodiments disclosed herein.

[0032] FIG. 3A illustrates candidate aptamers sequences specific to ovarian cancer vessel generated according to embodiments disclosed herein, as well as the frequency of occurrence for each from the vessel samples collected.

[0033] FIG. 3B illustrates a cluster dendrogram depicting the similarity and frequency of the candidate aptamers from the vessel samples collected.

[0034] FIG. 3C depicts the sequences for several aptamers and their frequency of occurrence, after confirming binding to vessel tissue.

[0035] FIG. 4A illustrates candidate aptamer sequences specific to ovarian cancer tumor cells generated according to embodiments disclosed herein, as well as the frequency of occurrence for each from the tumor cell samples collected

[0036] FIG. 4B illustrates a cluster dendrogram depicting the similarity and frequency of the candidate aptamers from the tumor cell samples collected.

[0037] FIG. 4C depicts the sequences for several aptamers and their frequency of occurrence, after confirming binding to tumor cell tissue.

[0038] FIGS. 5A-5D depict the binding of candidate thioaptamers that are capable of binding to both ovarian tumor vessel (5A and 5B) and ovarian tumor cells (5C and 5D).

[0039] FIGS. 6A-6F depict the specific binding of candidate thioaptamers to ovarian tumor cells (6D, 6E, and 6F), not to ovarian tumor vessels (6D, 6E, and 6F).

[0040] FIGS. 7A-7I depict the enhanced specific binding of candidate thioaptamers to ovarian tumor vessel (7A, 7B, and 7C), and reduced binding to ovarian tumor cells (7D, 7E, and 7F). FIGS. 7G, 7H and 7I depict an absence of any substantial binding of any of the candidate aptamers to normal ovarian tissue, thereby confirming their specificity to tumor vessel.

[0041] FIGS. 8A-8F depict characterization of binding of various candidate aptamers. FIGS. 8A and 8D depict binding of an aptamer (TV1) to both vessel and tumor cells. FIGS. 8B and 8E depict binding of an aptamer (V3) specifically to vessel cells and not to tumor cells. FIGS. 8C and 8F depict binding of an aptamer specifically to tumor cells and not to vessels.

[0042] FIGS. 9A-9I depict the binding characterization of various aptamers. FIGS. 9A and 9D depict the binding of an aptamer (TV1) to both tumor vessels and tumor cells, while FIG. 9G shows no binding to normal ovarian tissue. FIG. 9B depicts the enhanced binding of thioaptamer (V3) to ovarian tumor vessel, reduced binding to ovarian tumor cells (9E), and no binding to normal ovarian tissue (9H). FIG. 9C depicts the limited binding of thioaptamer (T3) to ovarian tumor vessel, enhanced binding to ovarian tumor cells (9F), and no binding to normal ovarian tissue (9I).

[0043] FIG. 10 demonstrates confirmation of selected aptamers binding to endothelial cells. FIG. 10A depicts immunohistochemical staining of IGROV ovarian tumor cells with anti-CD44 antibodies. FIGS. 10B and 10C show selected T3 and T4 aptamers can bind to IGROV ovarian tumor cells. FIG. 10D depicts immunohistochemical staining of HMVEC (endothelial cells) with anti-CD31 antibodies. FIGS. 10E and 10F show selected V3 and V4 aptamers can bind to endothelial cells.

[0044] FIG. 11 provides a list of various proteins that interact with an aptamer selected by the methods disclosed herein and their putative function. Aptamers specific for any of these proteins and/or to vimentin, could be used to disrupt vimentin-related pathways and provide a therapeutic effect.

[0045] FIGS. 12A-12B depict vimentin expression on ovarian tumor (12A) and normal ovarian tissues (12B). Note the elevated expression of vimentin, as detected by vimentin antibody on human ovarian tumor tissue (12A) in contrast to low levels of vimentin expression on normal ovary tissues (12B).

[0046] FIGS. 13A-13D depict the results of a competition binding assay between a vimentin polyclonal antibody and fluorescently labeled aptamer V5. FIGS. 13A and 13B show the binding of the aptamer V5 to IGROV cancerous ovarian cells. FIGS. 13C and 13D depict a significant reduction in aptamer fluorescence (indicative of reduced aptamer binding) when the IGROV cells were exposed to an anti-vimentin antibody.

[0047] FIGS. 14A-F illustrate the identification and validation of the thioaptamer targeting progeins. FIGS. 14A-B illustrate the expression of Annexin A2 on both human ovarian cancer vasculature (FIG. 14A) and tumor cells (FIG. 14B). FIG. 14C illustrates the detection by flow cytometry of 97% of Annexin A2 positive HMVEC cells. FIG. 14D-E illustrates the specific down-regulation of Annexin A2 levels as confirmed in time correspondence by real-time PCR (FIG. 14D) and western blot (FIG. 14E) when HMVEC cells were either mock transfected or transfected with Annexin A2-specific siRNA. FIG. 14F illustrates the knockdown of Annexin A2 which resulted in reduced binding of Endo28 (red) and Endo31 thioaptamers (red) on HMVEC cells, thus indicating that Endo28 and Endo31 are annexin-directed.

DETAILED DESCRIPTION

[0048] The following discussion is presented to enable a person skilled in the art to make and use one or more of the described embodiments. The general principles described herein may be applied to embodiments and applications other than those detailed below without departing from the spirit and scope of the disclosure. Indeed, the described embodiments are not intended to be limited to the particular embodiments shown, but are to be accorded the widest scope consistent with the principles and features disclosed or suggested herein.

[0049] Personalized biomarker discoveries are becoming an essential part of individualized diagnosis and therapy. Antibody–drug conjugates are powerful new treatment options for solid tumors, and immunomodulatory antibodies have also recently achieved remarkable clinical success. However, current antibody biomarker imaging agents are limited by an antibody’s immunogenicity, stability, reusability, and ability to be modified with imaging labels or immobilization tags. An attractive alternative to antibody-based therapeutics and diagnostics are aptamer-based therapeutics and diagnostics, which are disclosed more fully herein. Systems for selecting specific aptamers with desired binding characteristics are disclosed, as well as the use of aptamers to identify tissue-specific biomarkers of interest.

General

[0050] Aptamers are nucleic acid molecules having specific binding affinity to molecules (e.g., cell surface markers, proteins, drugs, etc.) through interactions other than classic Watson-Crick base pairing. Aptamers are similar to antibodies, to some extent, and ideally selected aptamers exhibit high binding affinity (e.g., dissociation constants in nano- to picomolar range) and high selectivity towards their targets. Despite being significantly smaller in molecular weight compared to antibodies (which provides aptamers with some advantages in the therapeutic fields), aptamers are able to form complex tertiary, folded structures. This enables aptamers to recognize and bind specifically to protein targets and also to discriminate between subtle molecular differences within the target. In several embodiments disclosed herein, design or modification of aptamer sequences allows

enhancement of these features of aptamers, thereby allowing very specific therapeutics to be developed and used.

[0051] Aptamers are also capable of modulating a certain target's activity, e.g., through binding, aptamers may block their target's ability to function. Aptamers according to embodiments disclosed herein range from about 5 kilodaltons (kDa) in size to about 25 kDa (including about 5 to about 10 kDa, about 10 to about 15 kDa, about 15 to about 20 kDa, about 20 to about 25 kDa, and ranges in between those listed, including endpoints). Aptamers according to several embodiments are between about 20 and about 200 nucleotides in length, e.g., about 20 to about 25 nucleotides, about 25 to about 30 nucleotides, about 30 to about 35 nucleotides, about 35 to about 40 nucleotides, about 40 to about 45 nucleotides, about 45 to about 50 nucleotides, about 50 to about 70 nucleotides, about 70 to about 90 nucleotides, about 90 to about 110 nucleotides, about 110 to about 130 nucleotides, about 130 to about 150 nucleotides, about 150 to about 175 nucleotides, about 175 to about 200 nucleotides, and any size therebetween, including endpoints. In several embodiments, aptamers are between about 30 and 45 nucleotides. Aptamers selected according to methods disclosed herein may be capable of target binding with nanomolar to sub-nanomolar affinity and may be capable of discriminating between closely related targets (e.g., do not bind, or bind with substantially less affinity, other proteins from the same gene family). Advantageously, aptamers are capable of using the same types of binding interactions (e.g., hydrogen bonding, electrostatic complementarity, hydrophobic contacts, steric exclusion) that drive affinity and specificity in antibody-antigen complexes.

[0052] When selected according to the processes disclosed herein, the resultant aptamers have desirable characteristics for use as therapeutics and/or diagnostics. These include, among others, high specificity and affinity, enhanced biological efficacy, and desirable pharmacokinetic properties. Advantageously, aptamers may also have advantages over antibodies and other protein biologics, for example, rapid and accurate *in vitro* production, lower toxicity and immunogenicity, easier administration, and improved stability.

[0053] Because aptamers are produced by an *in vitro* process, promising aptamers can be rapidly generated and screened. Moreover, *in vitro* selection, such as by the processes disclosed herein and discussed in more detail below, allows the specificity and affinity of the

aptamer to be tightly controlled. Advantageously, this results in the ability to develop aptamers that bind very specifically to targets, such as biomarkers on cancer cells (that are not present on normal cells) even if these markers are not particularly immunogenic.

[0054] Aptamers are particularly useful as therapeutics because of their limited immunogenicity (and low toxicity). In contrast, the efficacy of many monoclonal antibodies can be severely limited by immune response to antibodies themselves. However, aptamers result in fairly low antibody generation, perhaps due to limited presentation by T-cells via the immune system's reduced propensity to recognize nucleic acid fragments. Thus, as disclosed herein, several embodiments provide for aptamers as therapeutic targeting agents (or therapeutic agents themselves) that are characterized by limited induction of immune responses.

[0055] Administration of aptamers is less demanding than that of antibody therapeutics, in many embodiments. Many antibody therapeutics are administered by intravenous infusion (typically over 2-4 hours), while aptamers can be administered by, for example, subcutaneous injection (though some embodiments employ direct injection, intramuscular injection, systemic delivery, or other modes). With higher solubility and lower molecular weight than antibodies, more administration options can be employed with aptamers. In addition, the small size of aptamers allows penetration into target binding sites where steric hindrance would prevent access by antibodies or antibody fragments.

[0056] As mentioned above, and discussed in more detail below, the *in vitro* generation and screening of aptamers results in ready scalability at reduced costs. Difficulties in scaling production are currently limiting the availability of some biologics and the capital cost of a large-scale protein production prohibitive.

[0057] Further, aptamers are chemically sturdy. They are intrinsically adapted to regain activity following exposure to factors such as heat and denaturants and can be stored for extended periods (e.g. greater than 1 year) at room temperature as lyophilized powders.

[0058] As mentioned above, in treating a number of disorders, aptamers can fold into tertiary conformations and bind to their targets through shape complementarity at the aptamer-target interface. An aptamer can bind to a protein and can modulate protein functions by interfering with protein interaction with natural partners. In addition to binding

to small organic and inorganic molecules, aptamers have the unique ability to recognize and bind to large targets, such as proteins, whole cells or even organs.

[0059] Aptamers, while similar to antibodies in some respects, also confer a number of beneficial advantages. Like antibodies, aptamers can gain entrance to target cells via receptor-mediated endocytosis upon binding to cell surface ligands. However, aptamers can penetrate into tumor cores much more efficiently than antibodies due to their ~20-25-fold smaller sizes compared with full sized monoclonal antibodies. Thus, in several embodiments, aptamer-based therapeutics derived from the aptamers identified by methods disclosed herein are particularly effective against tumors (both solid and suspension, e.g., leukemia).

[0060] Aptamers can also be produced in a more cost-effective way as compared to many other peptide or cell specific therapies. For example, as will be discussed below, the *in vitro* generation of aptamers via the methods disclosed herein confers a low-cost advantage over the long and arduous development process of antibodies. As well, once aptamers are selected, they can be chemically synthesized instead of being produced in animals or cultured mammalian cells, as is required with antibodies. This can therefore simplify the production of therapeutic grade materials and provide a key advantage for commercial development.

[0061] Aptamers can also be effectively used to simultaneously detect thousands of proteins in multiplex discovery platforms, where antibodies often fail due to cross-reactivity problems. Through chemical modification, vast ranges of additional functional groups can be added at any desired position in the oligonucleotide sequence of the aptamer. An aptamer can therefore be combined with other functional groups to provide the best features of small molecule drugs, proteins and antibodies.

Aptamer modifications

[0062] In employing aptamers in the presently disclosed method, several characteristics of aptamers are optionally considered, and when considered, can enhance the efficacy of the resultant aptamers (and related therapeutics). As aptamers function *in vivo* through the blood plasma, modifications to the aptamer can be configured to address issues such as enzyme degradation and short blood residence time.

[0063] First, as aptamers are polynucleotides, they can be naturally susceptible to enzymes degradation by exo- and/or endo-nucleases, leading to a reduced *in vivo* circulatory half-life. In some embodiments, chemical modifications of the oligonucleotides can be made to increase resistance for degradation by nucleases while also increasing the stability of the aptamer without compromising the binding affinity and specificity towards their targets. In some embodiments, chemical modifications can be made to the phosphate backbone, such as sugars and/or the bases, end-capping at the 3' or 5' termini and locked nucleic acids. In other embodiments, a sulfur substitution of the phosphate backbone (for both DNA and RNA) or a modification of the 2' position of the ribose sugar (for RNA) can be made. These modifications can further minimize the susceptibility to endonuclease and exonuclease attack.

[0064] Short blood residence time can be another challenge faced when using aptamers, which the presently disclosed methods address for several *in vivo* aptamer applications. Because most aptamers have a size smaller than the renal filtration threshold of approximately 40 kDa, the aptamers can be quickly removed from the circulation by renal filtration. Therefore, to achieve the desired serum half-life, in some embodiments, the presently disclosed aptamers can be conjugated with a terminal polyethylene glycol (PEG). In several embodiments, the conjugation to PEG is advantageous because the longer half-life allows a longer duration of use in diagnostic contexts. Also, for therapeutics, the longer half-life can reduce dosing intervals and be balanced with reduction/minimization of toxicity.

[0065] In some embodiments, the aptamer can be modified by substituting one or both of the non-bridging phosphoryl oxygens in the oligonucleotide phosphate backbone with sulfur to form thio-substituted aptamers, called thioaptamers (TAs). TAs are attractive choices for the presently disclosed aptamer application for several reasons. First, the sulfur substitutions of the phosphodiester backbone can render oligonucleotides more stable in cellular and plasma environments because of their enhanced nuclease resistance. Second, TAs have higher affinities towards proteins than do unmodified aptamers. Based on molecular dynamics and theoretical calculations, the increased affinity of TAs may be attributed to the decreased interaction of solvated cations with the sulfur atoms, which act as softer Lewis bases on the polyanionic backbone. Third, thioaptamers are easy to synthesize

by chemical or enzymatic methods, and their sequences can be amplified and read out by polymerase chain reaction methods.

[0066] In some embodiments, the modifications to the aptamer following the selection of the aptamers may alter their 3-D structure. For example, the alteration of the 3-D structure can lead to the lost or altered binding affinity and specificity of the aptamer. To address this concern, in some embodiments, random aptamer pools containing modified nucleotides can be used during the selection methods disclosed herein.

[0067] Another potential concern with aptamer modification is the decrease in the ability of aptamers to interact with cells as a result of the repulsion of nucleic acids by negatively-charged cell membranes. However, in some embodiments, this can be addressed by increasing the binding affinity and specificity of aptamers toward their cell surface receptors to trigger receptor-mediated endocytosis.

[0068] In several embodiments, variants of the aptamers may also be used, to at least the same efficacy as those derived from the morphological selection methods disclosed herein. In several embodiments, the variants provide unexpectedly increased efficacy. In several embodiments, the enhanced efficacy is due to one or more of improved stability, reduced steric hindrance, improved interaction with the target cell(s), and the like. In several embodiments, aptamer fragments, variants (e.g., modified forms), derivatives, or functional equivalents retain the ability to specifically bind or otherwise interact with target cells.

[0069] In several embodiments, a "form of the aptamer" shall be given its ordinary meaning and shall refer to aptamers that have a significant homology with an aptamer derived from the methods disclosed herein and still retains an ability to interact specifically with target cells. A "functionally equivalent aptamer" shall be understood an aptamer that substantially shares at least one major functional property with the aptamers disclosed herein.

[0070] In specific embodiments, variants of aptamers that are useful in treatment or diagnosis include those (i) having a nucleotide sequence at least 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 98% or 99% identical to the aptamers disclosed herein and those discoverable by the methods disclosed herein, (ii) having a nucleic acid sequence encoding a polypeptide that is at least 40%, 45%, 50%, 55%, 60%, 65%, 70%,

75%, 80%, 85%, 90%, 95%, 98% or 99%) identical the amino acid sequence of the polypeptides encoded by the aptamers disclosed herein and those discoverable by the methods disclosed herein; (iii) a nucleic acid sequence that contains 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or more nucleic acid base mutations (e.g., additions, deletions and/or substitutions) relative to the aptamers disclosed herein and those discoverable by the methods disclosed herein; (iv) a nucleic acid sequence that hybridizes under high, moderate or typical stringency to the targets that the aptamers the aptamers disclosed herein and those discoverable by the methods disclosed herein are capable of hybridizing to, or (v) an aptamer comprising one or more conservative substitutions to the aptamers disclosed herein and those discoverable by the methods disclosed herein (and their encoded polypeptides), such as for example based on the degeneracy of the codons of the genetic code.

[0071] In certain embodiments, variants of aptamers that are useful in treatment or diagnosis include those that retain at least 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 98% or 99% (and any range in between, including endpoints) of a function of the aptamers disclosed herein and those discoverable by the methods disclosed herein.

Next-Generation Aptamers

[0072] As discussed above, aptamers are chemically synthesizable, and more easily selected than antibodies. However, aptamers must achieve their selectivity through a more limited repertoire of functional groups (e.g., the sugar phosphate backbone and four bases) in contrast to antibodies that have 20 amino acids with a full range of chemical substituents (e.g. positively-charged, sulfhydryl, hydrophobic sidechains, etc.). Furthermore, aptamers are polyanions and it can therefore be difficult to select an aptamer targeted to very acidic proteins because there are no cationic groups to neutralize the anionic surfaces on the protein. To address these limitations, the presently disclosed aptamers can be chemically modified by adding various functional groups to the oligonucleotide bases. For example, the problems of cross-reactivity and non-specific absorption to chip surfaces can be largely eliminated by modifying the aptamer using DNA SOMAmers (Slow Off-rate Modified

Aptamers), DNA aptamers uniformly modified at the 5-position of dU residues, as capture reagents in a highly multiplexed assay platform.

Aptamer Selection Methods

[0073] The presently disclosed aptamers can be selected by *in vitro* methods through screening a large library of oligonucleotides against the target to find the highest affinity (e.g., the “tightest” binding) candidates. Several embodiments of the selection methods comprise one or more screening steps wherein a library of aptamers is screened against a tissue of interest, morphological identification of a cell (or cells) of interest that bind to aptamers from the library and collection of the cell (or cells) of interest that bind aptamers from the library (and discarding cells that are not of interest or do not bind the aptamers), amplification and re-screening (including morphological identification and physical separation), sequencing of the pool of aptamers, empirical testing of the aptamers to bind a target of interest, and identification of the identity of the target of interest. In several embodiments, all of these steps are performed. In some embodiments, a SELEX-type method can be preferentially used to select the disclosed library of aptamers. As will be discussed in more detail below, the embodiments of the methods involves iterative cycles of screening and PCR amplification at each round, as well as laser microdissection of cells of interest that bind the aptamers. In other embodiments, a bead-based method can be used to select the disclosed library of aptamers. The bead-based method, while less preferable for certain embodiments, can also be used to synthesize an oligonucleotide library on non-cleavable beads and the high-affinity binders are identified in a single-step screening. Additionally, in several embodiments, the bead-based method may also be combined with LMD for cell-type specific aptamer isolation, which is optionally followed by the evolutionary selection procedures employing morphological capture of tissue as are disclosed herein. As discussed in more detail below, the selection methods disclosed herein are advantageously enhanced by coupling them with morphological identification of target cells (e.g., tumor cells) and laser microdissection of those cells. Various aspects of these steps are discussed in more detail below.

The Evolutionary Enrichment of Aptamer Pools

[0074] Turning first to evolutionary selection methods, one such method is the SELEX (“Systematic Evolution of Ligands by Exponential Enrichment”) method. SELEX provides a method for the *in vitro* evolution of nucleic acid molecules with highly specific binding to target molecules. A SELEX process capitalizes on the fact that nucleic acids have sufficient capacity for forming a variety of two- and three-dimensional structures and sufficient chemical versatility available within their monomers to act as ligands (e.g., form specific binding pairs) with virtually any chemical compound, whether monomeric or polymeric. Molecules of any molecular weight, size, or composition can serve as targets.

[0075] In some embodiments, the aptamers of the presently disclosed method can be selected using an evolutionary selection method as described herein. As will be described in more detail below, the sequences selected to bind to the target can be optionally minimized to determine the minimal sequence having the desired binding affinity. The selected sequences and/or the minimized sequences are optionally optimized by performing random or directed mutagenesis of the sequence to increase binding affinity or to determine which positions in the sequence are essential for binding activity. Additionally, selections can be performed with sequences incorporating modified nucleotides to stabilize the aptamer molecules against degradation *in vivo*.

[0076] In several embodiments, a large library or pool of single stranded oligonucleotides comprising randomized sequences can be first generated. In some embodiments, the oligonucleotides generated can be modified or unmodified DNA, RNA, or DNA/RNA hybrids. In other embodiments, the pool can comprise 100% random or partially random oligonucleotides.

[0077] In some embodiments, the partially random oligonucleotides can contain at least one fixed and/or conserved sequence incorporated within a randomized sequence. The partially random oligonucleotides can contain at least one fixed and/or conserved sequence at its 5' and/or 3' end which may be a sequence shared by all the molecules of the oligonucleotide pool. Fixed sequences are sequences such as hybridization sites for PCR primers, promoter sequences for RNA polymerases (e.g., T3, T4, T7, and SP6), restriction sites, or homopolymeric sequences, such as poly A or poly T tracts, catalytic cores, sites for

selective binding to affinity columns, and other sequences to facilitate cloning and/or sequencing. Conserved sequences are sequences, other than the previously described fixed sequences, shared by a number of aptamers that bind to the same target.

[0078] In some embodiments, the oligonucleotides of the pool can include a randomized sequence portion as well as fixed sequences necessary for efficient amplification. In some embodiments, the oligonucleotides of the starting pool can contain fixed 5' and 3' terminal sequences which flank an internal region of 30-50 random nucleotides.

[0079] The randomized nucleotides can be produced in a number of ways established in the art. In some embodiments, the randomized nucleotides can be produced using chemical synthesis. In another embodiment, the randomized nucleotides can be produced using size selection from randomly cleaved cellular nucleic acids. Sequence variation in test nucleic acids can also be introduced or increased by mutagenesis before or during the selection/amplification iterations.

[0080] The random sequence portion of the oligonucleotide can also be of variable length and composition. In some embodiments, the random sequence portion can comprise any of the following: ribonucleotides, deoxyribonucleotides, modified nucleotides, non-natural nucleotides, or nucleotide analogs. In some embodiments, random oligonucleotides can be synthesized from phosphodiester-linked nucleotides using solid phase oligonucleotide synthesis techniques. In other embodiments, random oligonucleotides can be synthesized using solution phase methods such as triester synthesis methods.

[0081] The methods of syntheses described above can yield a number of individual molecules that are sufficiently large (e.g. 10^{14} - 10^{15} molecules, or greater) with sufficiently large regions of random sequence in the sequence design to increase the likelihood that each synthesized molecule represents a unique sequence.

[0082] As described above, the starting library of oligonucleotides may be generated by automated chemical synthesis on a DNA synthesizer. In some embodiments, to synthesize randomized sequences, mixtures of all four nucleotides are added at each nucleotide addition step during the synthesis process, allowing for random incorporation of nucleotides. As stated above, in some embodiments, random oligonucleotides comprise entirely random sequences; however, in some embodiments, random oligonucleotides can

comprise stretches of nonrandom or partially random sequences. Partially random sequences can be created by adding the four nucleotides in different molar ratios at each addition step. Modified bases can also be included, in several embodiments.

[0083] Once the starting library of oligonucleotides is generated, the library can then be used to obtain aptamers that bind to the specific target site. In some embodiments, the generated aptamers are first contacted with the target under conditions favorable for binding. As will be described in more detail below, the target can be any diseased site such as, for example, cancerous tumor cells, or a population of such cells.

[0084] Next, the mixture of aptamers and cells of the target site are partitioned so as to separate the unbound aptamers from those aptamers which have bound specifically to target molecules. In some embodiments, the mixture of aptamers can be negatively selected (e.g., to reduce or eliminate aptamers that bind to undesired cells) against healthy target cells (e.g. non-cancerous cells of the target tissue). According to several embodiments disclosed herein, the purpose of evolutionary selection methods (including the morphology-based methods disclosed herein and discussed in more detail below) is to generate a number of aptamers that bind selectively to diseased target cells, by negatively selecting the aptamer library against healthy target cells, the ultimately selected pool of aptamers increases the likelihood that any subsequent treatment method will selectively target diseased cells.

[0085] Once the mixture of aptamers is partitioned to selectively include aptamers that specifically bind to target cells, the nucleic acid-target complexes can be dissociated to separate the aptamers from the attached target cell.

[0086] In some embodiments, the dissociated aptamers can then be amplified to yield a ligand-enriched mixture of aptamers. In some embodiments, the aforementioned steps of binding, partitioning, dissociating, and amplifying can be reiterated using the generated aptamers for as many cycles as is desired to yield highly specific, high affinity nucleic acid ligands to the target molecule. In some embodiments, 1-2, 2-3, 3-4, 4-5, 5-6, 6-7, 7-8, 8-9, 9-10, or 10 or more cycles can be performed. In other embodiments, selection/amplification can continue until no significant improvement in binding strength is achieved on repetition of the cycle. This method can be used to sample approximately 10^{14} different nucleic acid species. In some embodiments, greater numbers of nucleic acid species can be sampled, for

example, 10^{15} , 10^{16} , 10^{17} , or 10^{18} , depending on the embodiment and the amount of starting material needed/available. Generally, nucleic acid aptamer molecules are selected in a 5 to 20 cycle procedure. In one embodiment, heterogeneity is introduced only in the initial selection stages and does not occur throughout the replicating process.

[0087] Repeating the aforementioned steps can ensure that the selected aptamers do not include any aptamers that may have been unintentionally included as a result untargeted bonding (e.g. non-covalent bonds such as intermolecular forces) between the aptamers and the target site. As discussed in more detail below, several embodiments incorporate a positive selection step with laser microdissection of particular regions of interest to which candidate aptamers bind. This provides a manner of “physical negative selection” by which those aptamers that do not bind to cells of interest (e.g., tumor cells) are eliminated from further amplification/screening. Within a nucleic acid mixture containing a large number of possible sequences and structures, there is a wide range of binding affinities for a given target. A nucleic acid mixture comprising, for example, a 20 nucleotide randomized segment can have 4^{20} candidate possibilities. Those which have the higher affinity constants for the target are most likely to bind to the target. After partitioning, dissociation and amplification, a second nucleic acid mixture is generated, enriched for the higher binding affinity candidates. Additional rounds of selection progressively favor the best ligands until the resulting nucleic acid mixture is predominantly composed of only one or a few sequences. These can then be cloned, sequenced and individually tested for binding affinity as pure ligands or aptamers. Alternatively, in several embodiments, the nucleic acid mixture can be directly sequenced without cloning, for example, using NGS.

[0088] In embodiments where RNA aptamers are selected, the evolutionary methods method can further comprise the steps of reverse transcribing the nucleic acids dissociated from the nucleic acid-target complexes before the amplification step noted above. Thereafter, in embodiments where RNA aptamers are selected, the amplified nucleic acids can be transcribed before reiterating any of the aforementioned steps.

Laser Microdissection And Morphologically-Based Aptamer Selection

[0089] Figure 1 illustrates an embodiment of the methods disclosed herein directed to obtaining an aptamer population with high selectivity for a particular tissue such as a tumor tissue. The method is illustrated for ovarian tumor, though it shall be appreciated that the approaches disclosed herein can be applied to any tissue type (including specific tissues, specific cells within a tissue, or even specific sub-types of cells). An initial aptamer library may contain as many as 10^{14} aptamers which may be DNA or RNA. The library is then incubated with a tissue comprising cells of potential interest, for example a histological slide with tumor tissue. The incubation occurs under conditions configured to allow binding of the candidate aptamers within the library with target molecules within the tissue on the slide.

[0090] After incubation, the slide is washed to remove any aptamers from the library that fail to bind the tissue (e.g., they do not bind normal tissue and do not bind cancerous tissue, as an example). The slide is then subject to laser microdissection (LMD), which is a technique for isolating highly pure cell populations from a heterogeneous tissue section, cytological preparation, or live cell culture via direct visualization of the cells. LMD allows the exact cellular morphology, as well as the DNA, RNA and proteins of the procured cells, to remain intact and isolated with the cells of interest. Using LMD, both frozen and fixed tissues can be successfully dissected and used for DNA, RNA and protein analysis. In several embodiments, a user will select and cut out with a laser cells that are of potential interest (the cells collectively making up a region of interest). Depending on the embodiment the aptamer library may comprise a visualizeable marker (e.g., fluorescent tag or dye) so that the user can identify particular cells of interest having a high degree of aptamer binding. However, in several embodiments, visualization of the aptamers is not performed.

[0091] FIG. 2F illustrates a schematic of one embodiment of the laser microdissection of target tissues and cells according to the present disclosure. Once the cells are visualized, the cells can be excised using either an infrared (IR) capture system or an ultraviolet (UV) cutting system, or other wavelengths of electromagnetic radiation. In embodiments where an IR capture system is used, laser energy is used to cut a region of interest (ROI) within tissue (cells or groups of cells) bound to a thin, flat polymer within a

microscope slide, and the cut piece is then collected; unselected cells and tissues are left behind in the microscope slide (IR system). In other embodiments where an IR capture system is used, laser energy is transferred to a thermolabile polymer with the formation of a polymer-cell composite (alternate IR system). In other embodiments where a UV cutting system is used, the UV system performs a photo volatilization of the cells surrounding a selected area. In some embodiments, once the target cells are selected, the cells of interest can be removed from the heterogeneous tissue selection.

[0092] After dissection of the tissue, the aptamers are eluted from the captured tissue. These aptamers are then amplified (e.g., by polymerase chain reaction) to create a subsequent generation of the library (e.g., the library has evolved to include a greater percentage of aptamers that bind to a target region of interest).

[0093] Following the evolution, the next generation library is then exposed to another tissue sample on a microscope slide. In several embodiments, each round of binding of a next-generation library is performed under conditions of increasing stringency (e.g., higher temperature, different salt concentrations, etc.). Again the aptamers will (or will not) bind to a cell or cells of interest. After washing to remove unbound aptamers, the tissue on the slide is once again subject to LMD and cells of interest are collected. Conditions of increased stringency, where conditions remove weakly bound aptamers, can be used to increase specificity during the cycles. This cycle of exposure, collection, washing, amplification and re-exposure is carried out multiple times to generate a library of highly specific candidate aptamers. In several embodiments, 10 cycles are carried out. Other embodiments employ 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or more cycles. Laser microdissection of blood vessels and tumor cells is shown FIGs 2A-2D. The result of the procedure of Figure 1 is shown in Figures 3 and 4. As a result of the method, aptamers are detected with high specificity to vessel (Figure 3) and tumor (Figure 4) tissue. For example, TV1 in Figure 4 has a frequency of occurrence of 19,338. Figures 5-8 show staining of the tumor cells compared to a negative control (normal ovary, by way of example). This shows the specificity of the selected aptamers for the vessel and tumor tissue. This empirical testing is used to confirm the specific binding of the aptamers resulting from the evolutionary selection methods.

[0094] In some embodiments, the LMD can be performed using an inverted light microscope and a near-IR laser to facilitate the procurement of the desired cells. As discussed above, after direct visualization of the cells of interest, the user can use laser pulses to activate a thermoplastic polymer film that expands and surrounds the cells of interest. Next, this polymer-cell composite can be lifted from the slide, effectively microdissecting the cells of interest from the heterogeneous tissue section.

[0095] LMD is compatible with a variety of tissue types and the selective procurement of cells can be obtained with a great deal of precision, with laser cutting widths of approximately 1 micron or less. This method is also compatible with plant cells and microorganisms, in addition to animal cells.

[0096] In addition to the approach described above, positive and negative selection can be performed after one round of LMD. For example, LMD can first be used to capture cells of interest, with positive and negative screening steps being performed against an aptamer library sequentially (multiple times, if desired) to identify specific aptamers. Such an approach employs captured cells being converted to lysates (e.g., protein preparations) that are screened against the library and LMD is not necessarily employed at every generation of the library. For example, in one embodiment, the library is first contacted with normal ovary tissue. The aptamers which do not bind to the normal ovary tissue are then contacted with ovary tumor tissue. This reduces the number of aptamers from the original population. This number may be further reduced by repeated washes, elution and amplifications.

[0097] In still additional embodiments, a library of candidate aptamers is sequentially screened against a plurality of normal tissue sections. For example, a library may be incubated sequentially with 2, 3, 4, 5, 6, or more normal tissue sections (e.g., non-cancerous ovarian tissue). At each iteration, the wash is collected, the wash containing those aptamers that do not bind to the normal tissue. After panning against normal tissue, the resulting aptamers are incubated against a tissue of interest (e.g., a cancerous tissue). By removing a large percentage of those aptamers that bind to normal tissue, the remainder of the aptamers represents a smaller pool of aptamers that may be specific for the cells of

interest. After incubation, the cells of interest are removed by LMD and the candidate aptamers are eluted from the captured cells for further processing (see discussion below).

Bead-based Selection Method

[0098] In other embodiments, a bead-based selection method can be used to select the disclosed aptamers. In some contexts, bead-based selection method is less advantageous than a SELEX-type method, but can address the potential limitations posed by such methods. In particular, a bead-based selection method can be used to reduce the selection time and reduce any “amplificability bias” that may occur during the evolutionary procedure.

[0099] As will be discussed below, the bead-based selection method reverses the SELEX approach to binding and separation. In some embodiments, the first step involves synthesizing a combinatorial oligonucleotide library, using a split/pool method on noncleavable beads exposed to the target protein. A ‘split and pool’ synthesis method can be used to create a combinatorial library of oligonucleotides, on micron-size beads, with any type of backbone modification.

[0100] Next, in some embodiments, the bead-based library can be incubated with the fluorescently tagged protein, and the protein-bound aptamer beads can be manually picked under a fluorescence microscope or be sorted by high-throughput flow-cytometry. Alternatively, proteins can be labeled with biotin and the bound aptamer beads can be sorted by magnetic selection using streptavidin coated magnetic nanoparticles (Invitrogen).

[0101] Lastly, in some embodiments, the final step of the process involves reading-out by PCR amplification the sequences of the aptamers on the selected beads.

[0102] In some embodiments, the bead-based selection method can similarly be used in conjunction with LMD as discussed above.

Determination of Candidate Aptamer Sequences

[0103] After screening the initial aptamer library (whether labeled or not), in several embodiments the resultant pool of candidate aptamers is sequenced. In several embodiments, the sequencing is performed by next generation sequencing (NGS). In additional embodiments, Maxim-Gilbert sequencing and/or chain termination sequencing

(e.g., Sanger sequencing) can be used. In several embodiments, sequencing of the pool of candidate aptamers allows for further amplification and characterization of the members of the pool.

[0104] The candidate pools of aptamers, once amplified, are processed, in several embodiments, to generate a stochastic model for modeling of a randomly changing systems (e.g., a Markov model). Software algorithms are also optionally used to perform base calling and quality filtering.

[0105] Additionally, in several embodiments, the sequencing can also be used to determine the relative frequency of each individual sequence in the pool. The prevalence of each individual sequence can be identified in every candidate pool. Copy number and cluster association of the sequences can be used to identify those sequences with the greatest frequency of occurrence. In several embodiments, wherein multiple candidate pools are generated, the analysis optionally further includes determining the degree of homology among the different pools for the sequences with the highest occurrence. In several embodiments, sequence reads were preprocessed first by removing sequences that were greater than 80% homopolymeric. Lower homopolymeric thresholds can also be used in additional embodiments, such as, for example, greater than 75%, greater than 70%, greater than 65%, greater than 60%, greater than 55%, or greater than 50%. In several embodiments, sequences that do not contain any aptamer sequence are also removed. In several embodiments, there is a coordinate analysis of the results of a positive control aptamer, in that sequences containing more at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, or at least 15 contiguous bases matching the positive control aptamer sequence are removed. Based on an optimal alignment and statistical analysis, candidate aptamer sequences present in libraries at high frequency of occurrence and having high sequence homology among one another are selected. Optionally, in several embodiments, the selected sequences can be grouped based on their alignments (e.g., by Clustal W or a similar alignment program) and their secondary structures can be predicted (e.g., by M fold31, or a similar program). In some embodiments, this relative frequency and/or sequence homology is indicative of a higher probability of an aptamer with that sequence having higher specificity for the target tissue (as a result of the evolution of the

library, a more frequently occurring sequence, or variant of a sequence, suggests that the sequence was more enriched and bound the target tissue early in the evolutionary process, or that the target tissue has more complementary sites for that aptamer to bind). Sequencing conditions are established and readily derivable by one of ordinary skill in the art for application in the presently disclosed methods.

Empirical Testing of Candidate Aptamers

[0106] In several embodiments, the frequency and/or homology predictions disclosed herein are adequate to identify aptamers that specifically bind a target tissue. However, in several embodiments the complex nature of binding in an *in vivo* setting result in an aptamer with a theoretical lower binding specificity. Therefore, in several embodiments, the methods of aptamer selection and identification are optionally followed up with an empirical assay to confirm the specificity of binding. Various testing strategies can be used, depending on the embodiment. For example, an *in vitro* biochemical assay and/or a functional cell based assay and/or by binding in a dot blot assay can be used. In addition, in several embodiments, the empirical assay can be based on the colocalization of a candidate aptamer with an antibody known to bind to a putative target (e.g., an antibody directed to endothelial cells). Functional assays may also be used, depending on the embodiment. For example, in several embodiments, a candidate can be assessed for its ability to disrupt, or enhance a particular biochemical signaling pathway of interest. In several embodiments, binding assays can also be used. Colorimetric assays (e.g., ELISA) and/or radiolabeled binding assays can be used, depending on the nature and characteristics of a target of a candidate aptamer. In several embodiments, protein pull-down (e.g., assays akin to immunoprecipitation) are used. For example, an extract or lysate of target tissue comprising a known target is mixed with candidate aptamers. In some such embodiments, the candidate aptamers are immobilized and, once washed, can be eluted in conjunction with the bound target, which can then be identified by various means (e.g., size, gel mobility, mass-spec, protein sequencing, etc.).

Biomarker Identification

[0107] As discussed above, in several embodiments, the selection methods disclosed herein allow the identification of a candidate pool of aptamers (or in some embodiments, single specific aptamers) that specifically bind to a target of interest. In several embodiments, that target of interest is a marker that is specific to a cell in need of a treatment, for example a cancer cell, in that the expression of the marker is highly biased towards expression on the cell in need of treatment, as compared to normal cells. By way of example, in several embodiments, aptamers are selected that bind to a cancer marker present on a cancerous cell and not present on a non-cancerous cell. In several embodiments, there is a differential expression of a marker that allows an aptamer to bind with a high degree of preference to the cell expressing the marker. For example, in several embodiments the marker is expressed at a level of about 50% more, about 60% more, about 70% more, about 80% more, about 90% more, about 100% more, about 150% more, about 200% more, about 250% more, about 300% more, or greater amounts.

[0108] In several embodiments, the target of the aptamer may be known; however, in several embodiments the target is unknown. Thus, in several embodiments, identification of the target of the aptamer results in the identification of new biomarkers that are suitable for targeting with the aptamer conjugated to a therapeutic (e.g., chemotherapeutic agent) or diagnostic (e.g., fluorophore) agent.

[0109] Thus, in order to determine the identity of the target, in several embodiments, the target-aptamer complex is isolated and the target is identified by one or more various methods. In some embodiments, an affinity pull-down (akin to immunoprecipitation with an antibody) method is used to isolate the aptamer-target complex. In some embodiments, an aptamer confirmed to specifically bind a target is labeled (e.g., with a magnetic bead, such as a supra-paramagnetic iron oxide bead) or immobilized on a solid surface (e.g., a plate or sepharose bead).

[0110] Once the aptamers are labeled, immobilized or otherwise treated to be specifically recognized or isolatable, a lysate of a target tissue is mixed with the aptamers. After incubation, the conditions for which are discussed in more detail below, but are also readily determined by one of ordinary skill in the art based on the present disclosure, the

mixture is washed (to remove unbound lysate) and the complexes are collected. Collection varies, depending on the embodiment. For example, when magnetically labeled, exposure of the aptamer-target complex to a magnetic field (generated, for example, by soft magnets or bulk magnets) results in selective retention of the labeled aptamer-target complex. Variations of MACS (magnetic associated cell sorting) can also be used. In embodiments, employing a solid support, a wash buffer is passed over the solid support (e.g., a plate or column) and then an elution buffer is used to detach the aptamer-target complex from the solid support. Regardless of the method of capture, once isolated the aptamer target complex is then dissociated and the target is subject to identification (e.g., protein sequencing). In several embodiments, protein mass spectrometry is employed.

[0111] Depending on the embodiment, whole proteins may be sequenced, e.g., by one of electrospray ionization (ESI) or matrix-assisted laser desorption/ionization (MALDI) and tandem mass spectrometry-based sequencing. In some embodiments, intact proteins are ionized by either ESI or MALDI and then introduced to a mass analyzer (i.e. “Top-Down” protein sequencing). Alternatively, the target proteins are enzymatically digested (e.g., with trypsin, chymotrypsin, etc.) into smaller peptides which are then introduced into the mass spectrometer and identified by peptide mass fingerprinting or tandem mass spectrometry.

[0112] In several embodiments, the identity of the biomarker protein is unknown and its identity is determined by assembling the identity of the whole protein by assessing amino acids from peptide fragment masses of the target protein after using the aptamer to isolate the target protein(s). Several algorithmic approaches are available and can be used to identify peptides and proteins from tandem mass spectrometry (MS/MS) and yield complete results with respect to target protein identity.

Aptamer-guided Active Targeting Systems

[0113] As disclosed, in using aptamers as personalized biomarkers for individualized diagnosis and therapy, the rates of treatment response and overall survival in patients with diseases such as cancer can be improved. Aptamers selected using any of the methods disclosed above can form part of an aptamer-guided active targeting. The function of active targeting is to guide therapeutic agents with the aid of targeting ligands to diseased

cells. The disclosed aptamers can be configured to target and bind to various biomarkers on the target cell. As will be discussed below, the target biomarker can include, for example, tumor biomarkers, vimentin, and biomarkers associated with the enzymatic activity.

Tumor Biomarkers

[0114] In some embodiments, the disclosed aptamers can be selected to specifically bind to tumor biomarkers. As discussed below, the selected aptamer can be conjugated with drugs (e.g. chemotherapeutic agents) that allow the targeted delivery of toxins to the diseased cells while protecting normal cells from harm.

[0115] The selected aptamer in the presently disclosed methods will bind to a variety of different tumor biomarkers. In some embodiments, the aptamer can bind particularly to the extracellular domain (“ECD”) of the tumor biomarkers. In some embodiments, the disclosed aptamers can be configured to specifically bind to human tumor biomarkers, particularly the ECD of the human tumor biomarkers. In some embodiments, the aptamers can be configured to bind to a tumor biomarker that is a variant of human tumor biomarkers that performs a biological function that is essentially the same as a function of human tumor biomarkers. In some embodiments, the ECD of tumor biomarkers to which the aptamers of bind is a variant ECD of human tumor biomarkers that performs a biological function that is essentially the same as a function of the ECD of human tumor biomarkers. In some embodiments, the biological function of tumor biomarkers, ECD of tumor biomarkers or a variant thereof, to which aptamers derived from the presently disclosed aptamers methods are obtained, is the associated enzymatic activity. In some embodiments, the variant of human ECD of tumor biomarkers has substantially the same structure and substantially the same ability to bind aptamers generated by the methods disclosed herein as that of human ECD of tumor biomarkers. In some embodiments, the presently disclosed aptamer can bind to the ECD of tumor biomarkers, or a variant thereof, that comprises an amino acid sequence which is at least 80%, particularly at least 90% identical to sequences generated by the presently disclosed methods. In some embodiments, the targeted tumor biomarker can include, but is not be limited to, biomarkers such as AFP, BCR-ABL, BRCA1/BRCA2, BRAF V600E, CA-125, CA19.9, CEA, EGFR, HER-2, KIT, PSMA, PSA, S100, vimentin, annexin (including

among others Annexin, type I, Annexin, type II, Annexin, type III, Annexin, type IV, Annexin, type V, Annexin, type VI, Alpha giardin, Annexin, type X, Annexin, type VIII, Annexin, type XXXI, Annexin, type fungal XIV, Annexin, type plant, Annexin, type XIII, Annexin, type VII, Annexin like protein, and Annexin XI), as well as other cellular targets. For example, in one embodiment, the ovarian tumor tissue is screened using the methods disclosed herein. In several such embodiments, an aptamer at least 80%, particularly at least 90% identical to SEQ ID NO. 5 is able to bind specifically to tumor tissue. In some embodiments, an aptamer encoded by SEQ ID NO. 5 (or the amino acid sequence encoded by SEQ ID NO. 5) binds specifically to ovarian tumor tissue and can therefore be used as a diagnostic or therapeutic targeting agent.

Vimentin

[0116] In some embodiments, the active targeting approach can select an aptamer that is configured to target vimentin. Vimentin is a type III intermediate filament protein that is expressed in mesenchymal cells. Vimentin can therefore be used as a marker of cells undergoing an epithelial-to-mesenchymal transition during normal cell development and also metastatic progression. In some embodiments, the methylation of vimentin can be used to identify the presence of certain diseases such as: colon cancer, certain upper gastrointestinal pathologies such as Barrett's esophagus, esophageal adenocarcinoma, and intestinal type gastric cancer, breast cancer, and rheumatoid arthritis. FIG. 11 provides a table showing the different interactors of vimentin and their possible functions.

[0117] In some embodiments, an aptamer can be selected to target vimentin in order to induce apoptosis in cancer cells. Vimentin can be present in many different neoplasms but is particularly expressed in those originated from mesenchymal cells. For example, Sarcomas such as: fibrosarcoma, malignant fibrous histiocyoma, angiosarcoma, and leio- and rhabdomyosarcoma, as well as lymphomas, malignant melanoma and schwannoma, are virtually always vimentin positive. In other embodiments, other cancer related cells that express vimentin include: mesoderm derived carcinomas (e.g. renal cell carcinoma, adrenal cortical carcinoma and adenocarcinomas from endometrium and ovary), thyroid carcinomas, and any low differentiated or sarcomatoid carcinoma.

Enzymatic Systems

[0118] In other embodiments the aptamers can specifically bind to a gene in the cytochrome P450 system. Genetic mutations or polymorphisms (genetic variants) of CYP are known to exist among patients. Depending on the CYP phenotype encoded by a particular patient's genes, the metabolism of certain drugs may vary significantly. Therefore, by binding to a gene in an enzymatic system, aptamers can be used to improve an individual's metabolic functions.

Aptamer-Therapeutic Agent Delivery Systems

[0119] Once the target aptamer has been selected, the aptamer can be beneficially conjugated with drugs (e.g. chemotherapeutic agents) to increase the drugs delivered to targeted cells (e.g. tumor) while minimizing the exposure of non-target sites to chemotherapy agents.

[0120] In some embodiments, aptamer-toxin conjugates are a potential therapy for a range of indications that can be directed at the treatment of cancer. In other embodiments, the aptamer can be configured to target specific metabolizers or specific proteins expressed on cells. One method of treatment is through the surface modification of drug carriers by tumor specific recognition molecules.

[0121] The goal of surface modification of drug carriers by aptamers, is to enhance specific drug accumulation, internalization and retention in tumors through specific ligand-mediated interactions which can increase the therapeutic index. Therefore, effective delivery of drugs to tumors can be achieved through active targeting utilizing tumor-specific aptamers binding to their targets present on the surface of tumor cells.

[0122] Aptamer-guided active targeting enables the increased delivery of therapeutic agents to tumors, with a higher effective concentration, as well as a reduction in toxicity and side effects by minimizing the exposure of normal tissues to the therapeutic agent. An aptamer can be preferential to the use of antibodies for a number of reason. As an active targeting ligand, antibodies suffer from immunogenicity as even humanized antibodies may elicit immune responses in patients. In contrast, being nucleic acids, aptamers are generally non-immunogenic or low-immunogenic.

[0123] The chemical synthesis of aptamers confers additional advantages to aptamers, such as low batch-to-batch variation, and ease of scalability of production for large scale manufacturing with minimal risk of contamination of microorganisms and endotoxins. Furthermore, the chemical synthesis of aptamers provides more control over the nature of the conjugate. For example, the stoichiometry (ratio of toxins per aptamer) and site of attachment can be precisely defined. Different linker chemistries can be readily tested. As well, the reversibility of aptamer folding means that the loss of activity during conjugation is unlikely and provides more flexibility in adjusting conjugation conditions to maximize yields.

[0124] The use of conjugated aptamer-toxins is additionally beneficial as the small size of an aptamer can allow for better tumor penetration. This can be contrasted against the poor penetration of larger antibody-toxin conjugates that is often cited as a factor limiting the efficacy of conjugate approaches.

[0125] An additional beneficial aspect of aptamer-toxin conjugates is that aptamer half-life/metabolism can be easily tuned to match properties of toxin payload, thereby optimizing the ability to deliver toxin to the tumor while minimizing systemic exposure. In some embodiments, the appropriate modifications to the aptamer backbone and/or addition of high molecular weight PEGs can make it possible to match the half-life of the aptamer to the intrinsic half-life of the conjugated toxin/linker. This can minimize the systemic exposure to non-functional toxin-bearing metabolites (expected if $t_{1/2}(\text{aptamer}) \ll t_{1/2}(\text{toxin})$) and reduce the likelihood that persisting unconjugated aptamer will functionally block uptake of conjugated aptamer (expected if $t_{1/2}(\text{aptamer}) \gg t_{1/2}(\text{toxin})$).

[0126] Lastly, treatment with aptamer-toxin conjugates has relatively low material requirements. Because dosing levels can be limited by the toxicity intrinsic to the cytotoxic payload, a single course of treatment may entail relatively small (<100 mg) quantities of aptamer.

Tumor Cell-Targeting Aptamers

[0127] In some embodiments of the invention, the aptamer used in the aptamer-drug conjugate can be selected for the ability to specifically recognize a marker that is

expressed preferentially on the surface of tumor cells, but is relatively deficient from all normal tissues. Suitable target tumor markers can include, but are not limited to: PSMA, PSCA, E-selectin, EphB2 (and other representative ephrins), Cripto-1, TENB2 (also known as TEMFF2), ERBB2 receptor (HER2), MUC1, CD44v6, CD6, CD19, CD20, CD22, CD23, CD25, CD30, CD33, CD56, IL-2 receptor, HLA-DR10 β subunit, EGFRvIII, MN antigen (also known as CA IX or G250 antigen), Caveolin-1, Nucleolin, vimentin and/or the annexins.

[0128] In some embodiments, aptamers that are specific for a given tumor cell marker, such as those listed above, can be generated using the aptamer selection methods, as described above. Aptamers can be generated, depending on the embodiment, both to isolated, purified tumor cell surface proteins (e.g. tenascin C, MUC1, PSMA, CD44) and to tumor cells cultured in vitro (e.g. U251 (glioblastoma cell line), YPEN-1 (transformed prostate endothelial cell line)). In some embodiments, the extracellular portion of an identified tumor marker protein can be recombinantly expressed, purified, and treated as a soluble protein for screening purposes. In cases where soluble protein domains cannot readily be produced, direct selection for binding to transformed cells (optionally negatively selecting against normal cell binding) can yield aptamers that bind to tumor-specific markers.

[0129] Once the aptamer sequence has been identified through application of the using the aptamer selection methods disclosed herein, the aptamer can be optimized for both large-scale synthesis and in vivo applications through a progressive set of modifications. In some embodiments, these modifications can include, for example, 5'- and 3'-terminal and internal deletions to reduce the size of the aptamer, doped reselection for sequence modifications that increase the affinity or efficiency of target binding, introduction of stabilizing base-pair changes that increase the stability of helical elements in the aptamer, site-specific modifications of the 2'-ribose (e.g. 2'-hydroxyl $\rightarrow$ 2'-O-methyl substitutions) and phosphate (e.g. phosphodiester $\rightarrow$ phosphorothioate substitutions) positions to both increase thermodynamic stability and to block nuclease attack in vivo, and adding 5'- and/or 3'-caps (e.g. inverted 3'-deoxythymidine) to block attack by exonucleases. In some embodiments, aptamers generated through this process can be 15-40 nucleotides long and exhibit serum half-lives greater than 10 hours.

[0130] In some embodiments, to facilitate synthesis of the aptamer conjugate, reactive nucleophilic or electrophilic attachment points are introduced, for example, by directed solid phase synthesis or by post-synthesis modifications. In some embodiments, a free amine is introduced at either the 5'- or 3'-end of the aptamer by incorporating the appropriate amino-modifier phosphoramidite at the end or beginning of solid phase synthesis respectively. This amine can serve directly as a nucleophilic attachment point, or alternatively, this amine can be further converted into an electrophilic attachment point. Multiple amines may be introduced at the 5'-end of the aptamer through solid phase synthesis in which a 5'-symmetric doubler is incorporated one or more times and followed with a terminal reaction with the 5'-amino modifier described above.

Conjugation with Cytotoxins

[0131] In some embodiments, the therapeutic aptamer-drug conjugates of the invention are used in the targeted killing of tumor cells through aptamer-mediated delivery of cytotoxins by targeting a marker that readily internalizes or recycles into the tumor cell.

[0132] In some embodiments, drugs can be attached to the linker such that their pharmacological activity is preserved in the conjugate or such that in vivo metabolism of the conjugate leads to release of pharmacologically active drug fragments. Example potent cytotoxins which are suitable for conjugation include: Calcichearnicins (e.g. NAc- γ -DMH, NAc- γ -NHS), Maytansinoids (e.g. Maytansine, May-NHS), Vinca alkaloids (e.g. DAVH, DAVS), Cryptophycins (e.g. Cryptophycin-52, Cryp-NH₂), Tubulysins (e.g. TUB). The following modified cytotoxics can be used to construct aptamer-linker-drug conjugates in various embodiments.

[0133] In some embodiments, the aptamer can be conjugated with calicheamicins. For example, NAc- γ -DMH can be conjugated to aldehyde bearing linkers, or, alternatively, can be converted to an N-hydroxysuccinimide-bearing amine-reactive form (NAc- γ -NHS) to be conjugated to amine-bearing aptamers.

[0134] In additional embodiments, the aptamer can be conjugated with maytansinoids. Conjugatable forms of maytansinoids can be accessible through re-esterification of maytansinol which itself may be produced through reduction of maytansine

or ansamitocin P-3 using one of several reducing agents (including lithium aluminum hydride, lithium trimethoxyaluminum hydride, lithium triethoxyaluminum hydride, lithium tripropoxyaluminum hydride, and the corresponding sodium salts). In some embodiments, maytansinol may be converted to an amine-reactive form by reaction with a disulfide-containing carboxylic acid in the presence of carbodiimide (e.g. dicyclohexylcarbodiimide) and catalytic amounts of zinc chloride, reduction of the disulfide using a thiol-specific reagent (e.g. dithiothreitol) followed by HPLC purification to yield a thiol-bearing maytansinoid, and reaction with a bifunctional thiol- and amine-reactive crosslinking agent (e.g. N-succinimidyl 4-(2-pyridyldithio) pentanoate).

[0135] In some embodiments, aptamers can be conjugated with vinca alkaloids. Vinca alkaloids such as vinblastine can be conjugated directly to aldehyde-bearing linkers following conversion to a hydrazide form. For example, in some embodiments the disclosed aptamers can be conjugated with vinblastine sulfate after it has been converted desacetylvinblastine 3-carboxhydrazide. In other embodiments, amine-reactive forms of vinblastine may be generated in situ by initially converting vinblastine sulfate to the desacetyl form, reacting the resulting free base with approximately 2-fold excess succinic anhydride to generate the hemisuccinate, and reacting the isobutyl chloroformate to form the reactive mixed anhydride.

[0136] In additional embodiments, a cryptophycin can be conjugated to the presently disclosed aptamers. Cryptophycin is a naturally occurring, highly potent tubulin inhibitor. In some embodiments, the cryptophycin used is cryptophycin-52 (LY355703).

[0137] In some embodiments, the disclosed aptamers can be conjugated to tubulysins. Tubulysins are a class of highly potent tubulin inhibitors. As linear peptides of modified amino acids, they are amenable to chemical synthesis and conjugation using relatively standard peptide chemistries (e.g. in situ carboxylate activation via carbodiimides).

[0138] The aforementioned list of potential cytotoxins is not intended to be limiting. A number of other highly potent cytotoxic agents have been identified and characterized, many of which may additionally be suitable for the formation of aptamer-linker-drug conjugates. These can include, for example, modified variants of dolastatin-10, dolastatin-15, auristatin E, rhizoxin, epothilone B, epothilone D, taxoids.

Attachment Of A Drug To The Aptamer

[0139] As described above, aptamers can be conjugated to a drug. In several embodiments, the aforementioned therapeutic aptamer-drug conjugates have the following general formula: (aptamer)_n-linker-(drug)_m, where *n* is between 1 and 10 and *m* is between 0 and 20, particularly where *n* is between 1 and 10 and *m* is between 1 and 20. In particular embodiments directed to ovarian cancer, the aptamer can be selected from the group consisting of: SEQ ID NOS. 1 to 46, and any sequences sharing up to 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% homology with those sequences.

[0140] In some embodiments, the drug is conjugated to the 3'-end of the aptamer, while in other embodiments, the drug is conjugated to the 5'-end of the aptamer. In some embodiments, the drug can be encapsulated in nanoparticle forms, including but not limited to liposomes, dendrimers, and comb polymers. In other embodiments, the cytotoxic moiety is a small molecule, including without limitation, vinblastine hydrazide, calicheamicin, vinca alkaloid, a cryptophycin, a tubulysin, dolastatin-10, dolastatin-15, auristatin E, rhizoxin, epothilone B, epithilone D, taxoids, maytansinoids and any variants and derivatives thereof. In yet another embodiment, the drug is a protein toxin, including without limitation, diphtheria toxin, ricin, abrin, gelonin, and Pseudomonas exotoxin A.

[0141] The linker portion of the conjugate for the presently disclosed aptamers can present a plurality (i.e., 2 or more) of nucleophilic and/or electrophilic moieties that serve as the reactive attachment points for aptamers and drugs. In some embodiments, nucleophilic moieties can include free amines, hydrazides, or thiols. In other embodiments, electrophilic moieties can include activated carboxylates (e.g. activated esters or mixed anhydrides), activated thiols (e.g. thiopyridines), maleimides, or aldehydes.

[0142] In some embodiments, the aptamers of the invention which are conjugated to a cytotoxic moiety are also conjugated to a high molecular weight, non-immunogenic compound that serves as a linker. In a preferred embodiment, the high molecular weight, non-immunogenic compound is a polyethylene glycol moiety (PEG). By using a high molecular weight linker, renal clearance of the conjugate can be minimized, even in the eventuality that aptamers connected to the conjugate are removed (e.g. as a result of nuclease degradation in vivo). Preventing renal elimination can increase the in vivo half-life of the

drug conjugate and also prevent toxic concentrations of drug from accumulating in the kidneys, a particular concern with high potency cytotoxin conjugates.

[0143] In some embodiments of the PEG-aptamer-cytotoxin of the invention, the PEG moiety is conjugated to the 5' end of the aptamer, and the cytotoxic moiety is conjugated to the 3' end, while in other embodiments, the PEG moiety is conjugated to the 3' end of the aptamer and the cytotoxic moiety is conjugated to the 5' end. While in some embodiments, the aptamer is linked to the cytotoxin by the PEG moiety.

[0144] In other embodiments, instead of using PEG chains of a high molecular weight (e.g. 20,000 Da), multiple medium (e.g. 2,000 Da) molecular weight PEG chains can instead be used. In some embodiments, the reactive attachment points for the aptamers and drugs may be introduced either into the core used to anchor the PEG chains or introduced at the free ends of the PEG chains.

[0145] In the presently disclosed method, several different types of core molecules can be used to anchor the aforementioned PEG chain attachment. Examples can include simple small molecules bearing multiple nucleophiles or electrophiles (e.g. erythritol, sorbitol, lysine), linear oligomers or polymers (e.g. oligolysine, dextrans), or singly-reactive molecules with the capacity to self-assemble into higher order structures (e.g. phospholipids with the capacity to form micelles or liposomes).

Pharmaceuticals Containing Aptamer Molecules

[0146] The aforementioned aptamers, in several embodiments, are pharmaceutically acceptable and can be administered *in vivo*. In some embodiments, pharmaceutical compositions containing the aforementioned aptamer molecules that bind to a biomarker and/or are conjugated to a cytotoxic moiety can be suitable for internal use. In some embodiments, the aptamer molecules make up all, or a portion of an effective amount of a pharmacologically active compound of the invention, alone or in combination, with one or more pharmaceutically acceptable carriers. The compounds can include very low, if any toxicity. In some embodiments, binding of the aptamer or aptamer-toxin conjugate results in the stabilization or reduction in size of a tumor *in vivo*.

[0147] In some embodiments, the aptamers, the aptamer-toxin conjugates, or their pharmaceutically acceptable salts, are administered in amounts which will be sufficient to exert their desired biological activity. In some embodiments, the desired biological activity is the binding of the aptamer to the target biomarker and delivery of a toxic payload to a specific cell type.

[0148] In some embodiments, the disclosed aptamer composition of the invention can be used in combination with other treatments for cancer related disorders. This “combination therapy” (or “co-therapy”) includes the administration of an aptamer composition of the invention and at least a second agent as part of a specific treatment regimen intended to provide the beneficial effect from the co-action of these therapeutic agents. The beneficial effect of the combination can include, but is not limited to, pharmacokinetic or pharmacodynamic co-action resulting from the combination of therapeutic agents. Administration of these therapeutic agents in combination typically is carried out over a defined time period (usually minutes, hours, days or weeks depending upon the combination selected). The aptamer composition may contain, for example, more than one aptamer. In some embodiments, an aptamer composition containing one or more compounds of the invention, is administered in combination with another useful composition such as a cytotoxic, cytostatic, or chemotherapeutic agent such as an alkylating agent, anti-metabolite, mitotic inhibitor or cytotoxic antibiotic. In general, the currently available dosage forms of the known therapeutic agents for use in such combinations will be suitable.

[0149] In some embodiments, the co-therapy can encompass the administration of two or more of these therapeutic agents as part of separate monotherapy regimens that incidentally and arbitrarily result in the combinations of the present invention. The co-therapy can be provide for administration of these therapeutic agents in a sequential manner, wherein each therapeutic agent is administered at a different time. In some embodiments, the aforementioned therapeutic agents can be administered in a substantially simultaneous manner. In some embodiments, the substantially simultaneous administration can be accomplished, for example, by administering to the subject a single capsule having a fixed ratio of each therapeutic agent or in multiple, single capsules for each of the therapeutic agents.

[0150] The therapeutic agents can be administered through a variety of routes. These routes can include, but is not limited to, topical routes, oral routes, intravenous routes, intramuscular routes, intraperitoneally, systemic, local, transdermal, intraarterial, and direct absorption through mucous membrane tissues (the aptamers disclosed herein can also be delivered by any of these routes). In embodiments where more than one therapeutic agents are used, the plurality of therapeutic agents can be administered by the same route or by different routes. For example, a first therapeutic agent of the combination selected may be administered by injection while the other therapeutic agents of the combination may be administered topically.

[0151] In some embodiments, the use of co-therapy in the presently disclosed method include the administration of the therapeutic agents as described above in further combination with other biologically active ingredients. Where the co-therapy further comprises a non-drug treatment, the non-drug treatment may be conducted at any suitable time so long as a beneficial effect from the co-action of the combination of the therapeutic agents and non-drug treatment is achieved. For example, in some embodiments, the beneficial effect is still achieved when the non-drug treatment is temporally removed from the administration of the therapeutic agents, perhaps by days or even weeks.

[0152] In embodiments where a therapeutic or pharmacological composition is used with the presently disclosed method, the therapeutic or pharmacological composition can comprise an effective amount of the active component(s) of the therapy, dissolved or dispersed in a pharmaceutically acceptable medium. Pharmaceutically acceptable media or carriers can include any of the following: solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. Supplementary active ingredients can also be incorporated into the therapeutic compositions of the present invention.

[0153] The aforementioned pharmaceutical or pharmacological compositions can be prepared in one of a variety of ways. In some embodiments, the compositions can be prepared in any of the following ways: as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection; as tablets or other solids for oral administration; as time release capsules; or in any other form

currently used (e.g. including eye drops, creams, lotions, salves, inhalants). The use of sterile formulations, such as saline-based washes, by surgeons, physicians or health care workers to treat a particular area in the operating field may also be particularly useful. Compositions may also be delivered via microdevice, microparticle or sponge.

[0154] In embodiments where therapeutics are used, the aforementioned therapeutics can be administered in a manner compatible with the dosage formulation, and in such amount as is pharmacologically effective. In some embodiments, the formulations can be easily administered in a variety of dosage forms, such as injectable solutions. In other embodiments, drug release capsules and the like can also be employed.

[0155] When desired or necessary, suitable binders, lubricants, disintegrating agents, and coloring agents can also be incorporated into the mixture. For example, suitable binders that can be used can include, but is not limited to: starch, magnesium aluminum silicate, starch paste, gelatin, methylcellulose, sodium carboxymethylcellulose and/or polyvinylpyrrolidone, natural sugars such as glucose or beta-lactose, corn sweeteners, natural and synthetic gums such as acacia, tragacanth or sodium alginate, polyethylene glycol, and waxes.

[0156] In other embodiments, lubricants that can be used can include, but is not limited to sodium oleate, sodium stearate, magnesium stearate, sodium benzoate, sodium acetate, sodium chloride, silica, talcum, stearic acid, its magnesium or calcium salt and/or polyethyleneglycol, and the like.

[0157] In additional embodiments, disintegrating agents that can be included are, without limitation, starch, methyl cellulose, agar, bentonite, xanthan gum starches, agar, alginic acid or its sodium salt, or effervescent mixtures, and the like.

[0158] Finally, in some embodiments, diluents can be used. The diluents can include, but are not limited to, lactose, dextrose, sucrose, mannitol, sorbitol, cellulose and/or glycine.

[0159] In some embodiments, the disclosed compounds can also be administered in such oral dosage forms as timed release and sustained release tablets or capsules, pills, powders, granules, elixirs, tinctures, suspensions, syrups and emulsions. Suppositories are advantageously prepared from fatty emulsions or suspensions.

[0160] The pharmaceutical compositions may be sterilized and/or contain adjuvants, such as preserving, stabilizing, wetting or emulsifying agents, solution promoters, salts for regulating the osmotic pressure and/or buffers. In addition, they may also contain other therapeutically valuable substances. In some embodiments, the compositions can be prepared according to conventional mixing, granulating, or coating methods, and typically contain about 0.1% to 75%, preferably about 1% to 50%, of the active ingredient.

[0161] Liquid, particularly injectable compositions can, for example, be prepared by dissolving, dispersing, etc. In embodiments where the presently disclosed aptamers are used, the active compound can be dissolved in or mixed with a pharmaceutically pure solvent such as, for example, water, saline, aqueous dextrose, glycerol, ethanol, and the like, to thereby form the injectable solution or suspension. Additionally, solid forms suitable for dissolving in liquid prior to injection can be formulated.

[0162] The presently disclosed compounds can also be administered in intravenous (both bolus and infusion), intraperitoneal, subcutaneous or intramuscular form, all using forms well known to those of ordinary skill in the pharmaceutical arts. Injectables can be prepared in conventional forms, either as liquid solutions or suspensions.

[0163] Parenteral injectable administration can be generally used for subcutaneous, intramuscular or intravenous injections and infusions. Additionally, parenteral administration can be employed in the implantation of a slow-release or sustained-released systems, which assures that a constant level of dosage is maintained.

[0164] Furthermore, compounds to be used with the presently disclosed aptamers can be administered in intranasal form via topical use of suitable intranasal vehicles, inhalants, or via transdermal routes (e.g. transdermal skin patches). In embodiments where a transdermal delivery system is used, the dosage administration will be continuous rather than intermittent throughout the dosage regimen. Other preferred topical preparations include creams, ointments, lotions, aerosol sprays and gels, wherein the concentration of active ingredient would typically range from 0.01% to 15%, w/w or w/v.

[0165] Solid compositions can also be used to deliver the disclosed aptamers. In embodiments where solid compositions are considered, excipients can be chosen from, but is not limited to, any of the following: pharmaceutical grades of mannitol, lactose, starch,

magnesium stearate, sodium saccharin, talcum, cellulose, glucose, sucrose, and, magnesium carbonate. The active compound defined above, may be also formulated as suppositories. In some embodiments, polyalkylene glycols, for example, propylene glycol, can be used as the carrier. In some embodiments, suppositories can be prepared from fatty emulsions or suspensions.

[0166] The disclosed compounds can also be administered in the form of liposome delivery systems, such as small unilamellar vesicles, large unilamellar vesicles and multilamellar vesicles. Liposomes can be formed from a variety of phospholipids, containing cholesterol, stearylamine or phosphatidylcholines. In some embodiments, a film of lipid components is hydrated with an aqueous solution of drug to a form lipid layer encapsulating the drug. For example, the aptamer molecules described herein can be provided as a complex with a lipophilic compound or non-immunogenic, high molecular weight compound constructed using methods known in the art. Additionally, liposomes can include aptamers on their surface for targeting and carrying cytotoxic agents internally to mediate cell killing.

[0167] The compounds of the present invention may also be coupled with soluble polymers as targetable drug carriers. Such polymers can include polyvinylpyrrolidone, pyran copolymer, polyhydroxypropyl-methacrylamide-phenol, polyhydroxyethylaspanamidephenol, or polyethyleneoxidepolylysine substituted with palmitoyl residues. Furthermore, the compounds of the present invention may be coupled to a class of biodegradable polymers useful in achieving controlled release of a drug, for example, polylactic acid, polyepsilon caprolactone, polyhydroxy butyric acid, polyorthoesters, polyacetals, polydihydropyrans, polycyanoacrylates and cross-linked or amphipathic block copolymers of hydrogels.

[0168] In some embodiments the pharmaceutical composition to be administered can also contain minor amounts of non-toxic auxiliary substances such as wetting or emulsifying agents, pH buffering agents, and other substances such as for example, sodium acetate, and triethanolamine oleate.

Aptamer Applications

[0169] As discussed above, aptamers are useful in diagnostic and therapeutic applications (including biomarker discovery) and can provide various applications for the

diagnosis of diseases and detection of small molecules. Additionally, in several embodiments, the selection methods disclosed herein can be used to specifically identify biomarkers that are specific for a tissue type, a cell type, or even for a cell sub-type.

[0170] In several embodiments, the methods are used to generate patient-specific therapies (e.g., aptamer-conjugated compositions) to treat a variety of cancers, including but not limited to acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), adrenocortical carcinoma, kaposi sarcoma, lymphoma, gastrointestinal cancer, appendix cancer, central nervous system cancer, basal cell carcinoma, bile duct cancer, bladder cancer, bone cancer, brain tumors (including but not limited to astrocytomas, spinal cord tumors, brain stem glioma, craniopharyngioma, ependymblastoma, ependymoma, medulloblastoma, medulloepithelioma, breast cancer, bronchial tumors, burkitt lymphoma, cervical cancer, colon cancer, chronic lymphocytic leukemia (CLL), chronic myelogenous leukemia (CML), chronic myeloproliferative disorders, ductal carcinoma, endometrial cancer, esophageal cancer, gastric cancer, Hodgkin lymphoma, non-Hodgkin lymphoma hairy cell leukemia, renal cell cancer, leukemia, oral cancer, liver cancer, lung cancer (including but not limited to, non-small cell lung cancer, (NSCLC) and small cell lung cancer), pancreatic cancer, bowel cancer, lymphoma, melanoma, ocular cancer, ovarian cancer, pancreatic cancer, prostate cancer, pituitary cancer, uterine cancer, and vaginal cancer.

[0171] In other embodiments, the methods are used to generate patient-specific therapies (e.g., aptamer-conjugated compositions) to treat complement-related disorders such as acute ischemic diseases (myocardial infarction, stroke, ischemic/reperfusion injury); acute inflammatory diseases (infectious disease, septicemia, shock, acute/hyperacute transplant rejection); chronic inflammatory and/or immune-mediated diseases (allergy, asthma, rheumatoid arthritis, and other rheumatological diseases, multiple sclerosis and other neurological diseases, psoriasis and other dermatological diseases, myasthenia gravis, systemic lupus erythematosus (SLE), subacute/chronic transplant rejection, glomerulonephritis and other renal diseases). In some embodiments, the methods are used to generate patient-specific therapies (e.g., aptamer-conjugated compositions) to treat complement activation associated with dialysis or circumstances in which blood is passed over and/or through synthetic tubing and/or foreign material. In some embodiments, the

methods are used to generate patient-specific therapies to treat disorders selected from the group consisting of: myocardial injury relating to CABG surgery, myocardial injury relating to balloon angioplasty, myocardial injury relating to restenosis, complement protein mediated complications relating to CABG surgery, complement protein mediated complications relating to percutaneous coronary intervention, paroxysmal nocturnal hemoglobinuria, acute transplant rejection, hyperacute transplant rejection, subacute transplant rejection, and chronic transplant rejection.

[0172] In other embodiments, the methods are used to generate patient-specific therapies (e.g., aptamer-conjugated compositions) to treat auto-immune diseases such as myocarditis, postmyocardial infarction syndrome, postpericardiotomy syndrome, subacute bacterial endocarditis, anti-glomerular basement membrane nephritis, interstitial cystitis, lupus nephritis, autoimmune hepatitis, primary biliary cirrhosis, primary sclerosing cholangitis, antisynthetase syndrome, alopecia areata, autoimmune angioedema, autoimmune progesterone dermatitis, autoimmune urticarial, bullous pemphigoid, cicatricial pemphigoid, dermatitis herpetiformis, discoid lupus erythematosus, epidermolysis bullosa acquisita, erythema nodosum, gestational pemphigoid, hidradenitis suppurativa, lichen planus, lichen sclerosus, linear iga disease, morphea, pemphigus vulgaris, pityriasis lichenoides et varioliformis acuta, mucha-habermann disease, psoriasis, systemic scleroderma, vitiligo, addison's disease, autoimmune polyendocrine syndrome, autoimmune polyendocrine syndrome type 2, autoimmune polyendocrine syndrome type 3, autoimmune pancreatitis, diabetes mellitus type 1, autoimmune thyroiditis, ord's thyroiditis, graves' disease, autoimmune oophoritis, endometriosis, autoimmune orchitis, sjogren's syndrome, autoimmune enteropathy, celiac disease, crohn's disease, microscopic colitis, ulcerative colitis, antiphospholipid syndrome, aplastic anemia, autoimmune hemolytic anemia, autoimmune lymphoproliferative syndrome, autoimmune neutropenia, autoimmune thrombocytopenic purpura, cold agglutinin disease, essential mixed cryoglobulinemia, evans syndrome, igg4-related systemic disease, paroxysmal nocturnal hemoglobinuria, pernicious anemia, pure red cell aplasia, thrombocytopenia, adiposis dolorosa, adult-onset still's disease, ankylosing spondylitis, crest syndrome, drug-induced lupus, enthesitis-related arthritis, eosinophilic fasciitis, felty syndrome, juvenile arthritis, lyme disease (chronic), mixed

connective tissue disease, palindromic rheumatism, parry romberg syndrome, parsonage-turner syndrome, psoriatic arthritis, reactive arthritis, relapsing polychondritis, retroperitoneal fibrosis, rheumatic fever, rheumatoid arthritis, sarcoidosis, schnitzler syndrome, systemic lupus erythematosus, undifferentiated connective tissue disease, dermatomyositis, fibromyalgia, inclusion body myositis, myositis, myasthenia gravis, neuromyotonia, paraneoplastic cerebellar degeneration, polymyositis, acute disseminated encephalomyelitis, acute motor axonal neuropathy, anti-n-methyl-d-aspartate receptor encephalitis, balo concentric sclerosis, Bickerstaff's encephalitis, chronic inflammatory demyelinating polyneuropathy, Guillain-Barré syndrome, Hashimoto's encephalopathy, idiopathic inflammatory demyelinating diseases, Lambert-Eaton myasthenic syndrome, multiple sclerosis, pediatric autoimmune neuropsychiatric disorder associated with streptococcus, progressive inflammatory neuropathy, restless leg syndrome, stiff person syndrome, Sydenham chorea, transverse myelitis, autoimmune retinopathy, autoimmune uveitis, Cogan syndrome, graves ophthalmopathy, intermediate uveitis, ligneous conjunctivitis, mooren's ulcer, neuromyelitis optica, opsoclonus myoclonus syndrome, optic neuritis, scleritis, Susac's syndrome, sympathetic ophthalmia, tolosa-hunt syndrome, autoimmune inner ear disease, Ménière's disease, anti-neutrophil cytoplasmic antibody-associated vasculitis, Behçet's disease, Churg-Strauss syndrome, giant cell arteritis, Henoch-Schonlein purpura, Kawasaki's disease, leukocytoclastic vasculitis, lupus vasculitis, rheumatoid vasculitis, microscopic polyangiitis, polyarteritis nodosa, polymyalgia rheumatic, urticarial vasculitis, and other vasculitides.

[0173] In several embodiments, the methods disclosed herein can be used to develop patient-specific therapies (e.g., aptamer-conjugated compositions) to treat chronic diseases, including but not limited to neurological impairments or neurodegenerative disorders (e.g., Alzheimer's disease, Parkinson's disease, Huntington's disease, epilepsy, dopaminergic impairment, dementia resulting from other causes such as AIDS, multiple sclerosis, amyotrophic lateral sclerosis, cerebral ischemia, physical trauma any other acute injury or insult producing neurodegeneration), immune deficiencies, repopulation of bone marrow (e.g., after bone marrow ablation or transplantation), arthritis, auto-immune disorders, inflammatory bowel disease, cancer, diabetes, muscle weakness (e.g., muscular

dystrophy, amyotrophic lateral sclerosis, and the like), progressive blindness (e.g. macular degeneration), and progressive hearing loss.

[0174] In several embodiments, the methods are used to generate patient-specific therapies (e.g., aptamer-conjugated compositions) treat a variety of cancers, including but not limited to acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), adrenocortical carcinoma, Kaposi's sarcoma, lymphoma, gastrointestinal cancer, appendix cancer, central nervous system cancer, basal cell carcinoma, bile duct cancer, bladder cancer, bone cancer, brain tumors (including but not limited to astrocytomas, spinal cord tumors, brain stem glioma, craniopharyngioma, ependymblastoma, ependymoma, medulloblastoma, medulloepithelioma, breast cancer, bronchial tumors, burkitt lymphoma, cervical cancer, colon cancer, chronic lymphocytic leukemia (CLL), chronic myelogenous leukemia (CML), chronic myeloproliferative disorders, ductal carcinoma, endometrial cancer, esophageal cancer, gastric cancer, Hodgkin lymphoma, non-Hodgkin lymphoma hairy cell leukemia, renal cell cancer, leukemia, oral cancer, liver cancer, lung cancer (including but not limited to, non-small cell lung cancer, (NSCLC) and small cell lung cancer), pancreatic cancer, bowel cancer, lymphoma, melanoma, ocular cancer, ovarian cancer, pancreatic cancer, prostate cancer, pituitary cancer, uterine cancer, and vaginal cancer.

[0175] In several embodiments, the methods are used to generate patient-specific therapies (e.g., aptamer-conjugated compositions) to treat target tissues that are infected, for example with one or more bacteria, viruses, fungi, and/or parasites. In several embodiments, the therapeutic cell populations that have had one or more subpopulations removed are used to treat tissues with infections of bacterial origin (e.g., infectious bacteria is selected the group of genera consisting of *Bordetella*, *Borrelia*, *Brucella*, *Campylobacter*, *Chlamydia* and *Chlamydophila*, *Clostridium*, *Corynebacterium*, *Enterococcus*, *Escherichia*, *Francisella*, *Haemophilus*, *Helicobacter*, *Legionella*, *Leptospira*, *Listeria*, *Mycobacterium*, *Mycoplasma*, *Neisseria*, *Pseudomonas*, *Rickettsia*, *Salmonella*, *Shigella*, *Staphylococcus*, *Streptococcus*, *Treponema*, *Vibrio*, and *Yersinia*, and mutants or combinations thereof).

[0176] In several embodiments, the methods are used to generate patient-specific therapies (e.g., aptamer-conjugated compositions) treat a variety to treat viral infections, such as those caused by one or more viruses selected from the group consisting of adenovirus,

Coxsackievirus, Epstein-Barr virus, hepatitis a virus, hepatitis b virus, hepatitis c virus, herpes simplex virus, type 1, herpes simplex virus, type 2, cytomegalovirus, ebola virus, human herpesvirus, type 8, HIV, influenza virus, measles virus, mumps virus, human papillomavirus, parainfluenza virus, poliovirus, rabies virus, respiratory syncytial virus, rubella virus, and varicella-zoster virus.

[0177] It shall also be appreciated that the methods for aptamer selection disclosed herein can also be used to evaluate and identify biomarkers (e.g., the “antigen” with which a particular aptamer interacts). In several embodiments, the selection methods can be used to identify biomarkers (including patient-specific biomarkers) for any of the diseases or conditions disclosed herein.

Example 1

Overview

[0178] Ovarian cancer has the highest mortality of all cancers of the female reproductive system. As ovarian cancer lacks early symptoms and effective screening tests, it is difficult to diagnose at its early stage (I/II) until it spreads beyond the ovary and advances to later stages (III/IV). However, as is described above, several embodiments of the methods herein allow for the selection of aptamers that are highly selective for tumor tissue, thereby allowing a very specific delivery of a therapeutic agent to the tumor cells (or other cells that directly or indirectly support the tumor, such as vascular cells).

[0179] As well, angiogenesis-dependent tumor growth can pose a challenge in cancer treatment. New blood vessels are formed through an angiogenesis process and contribute to tumor progression and metastasis. Tumor blood vessels differ from their normal counterparts with relatively large, heterogeneous nuclei, cytogenetically abnormality and high sensitivity to epidermal growth factor. Tumor vasculature is often unevenly distributed and chaotic with uneven thickness and a more permeable basement membrane. These physical distinctions indicate that some tumor endothelial cells are likely to express different surface markers compared to normal endothelial cells. Preventing and inhibiting angiogenesis by targeting tumor endothelial cells represents a promising strategy for cancer treatment. The methods of aptamer selection and isolation disclosed herein enable identification of tumor

tissue or tumor vascular, precise selection of these tissues and removal from normal cells, followed by a screening and enrichment protocol to allow select aptamers specific for the tumor tissue or tumor vasculature cells.

[0180] A cell-based selection method, cell-SELEX (systematic evolution of ligands by exponential enrichment), which is described above, can be used to rapidly identify aptamers specific to a cell type of interest. Cell-SELEX is an evolutionary approach, and allows the selection of aptamers even without prior knowledge of specific targets. Advantageously, according to several embodiments, cell-SELEX can be used to generate aptamers for multiple targets in parallel and discover new biomarkers that are targetable. In live cells, all cell-surface molecules are presented in their natural surroundings, in their native conformations, and with their natural populations of post-translational modifications. Thus, this method, which is employed (optionally in conjunction with laser microdissection) identifies aptamers that react with targets as the targets exist on cells, rather than in an in vitro setting.

[0181] To overcome the limitation that cell-SELEX can only use cell lines or isolated cells as targets, several embodiments of the invention disclosed herein are related to the development of a morphologically-based aptamer tissue selection (sometimes referred to as Morph-X-Select) method. Advantageously, this approach enables use of tissue sections from individual patients and identification of high binding affinity aptamer sequences and their associated tumor biomarkers in a systematic and accurate way. Unlike traditional aptamer tissue selection using whole tissue sections, several embodiments of the present methods employ image directed laser microdissection (LMD) to dissect only Regions of Interest (ROIs) bound with TAs based on morphological assessment of the tissue.

[0182] We used primary endothelial cells isolated from fresh patient ovarian tumor to create a true molecular profile of the native target cells and to avoid any changes of binding affinity during cell culture. According to several embodiments, a combinatorial DNA aptamer library was generated, with thiol substitution of the phosphate backbone (thioaptamer) at the 5'-side of many of the dA positions to increase the binding affinity with targeting cells. The monothio substitutions which also enhance nuclease resistance can be introduced into the aptamer library by including thio-substituted-dNTP(α S) into the

enzymatic Taq amplification step during PCR (Nutiu, R 2005, Wilson, D. S. 1999, Yang, X. 2006, Thiviyanathan, V. 2007, A. P. Mann 2010, A. P. Mann 2011).

[0183] These thioaptamers were then incubated with purified human ovarian cancer endothelial cells (CD31⁺ and CD146⁺) isolated from ten human ovarian tumors. As is used in several embodiments, the thioaptamer-based SELEX selection strategy included selection of a panel of ovarian cancer endothelial cell-specific thioaptamers that only interacted with tumor endothelial cells but not with normal endothelial cells under counter-selection. Consequently, thioaptamer candidates exclusively binding to the tumor endothelium cells are enriched. Cell-SELEX was used to screen the thioaptamers against the isolated tumor cells. As disclosed above, such evolutionary rounds of selection (whether morphologically based or not) can be repeated 2 times, 3 times, 4 times, 5 times, 6 times, 7 times, 8 times, 9 times, 10 times or more. Two candidate thioaptamers, Endo-28 and Endo-31 were tested for their ability to specifically recognize endothelial cells and showed specific binding to both Human Microvascular Endothelial Cells (HMVEC) and vasculature of human ovarian cancer tissue. Thus, according to some embodiments, aptamers encoding Endo-28 and/or Endo-31 are used to specifically target endothelial cells and/or ovarian cancer tissue and optionally deliver a therapeutic agent to that tissue. A random thioaptamer sequence, R4, was used as a negative control for all analysis. In a protein-pull down experiment, Annexin A2, one of the targeted proteins, was pulled-down from the endo-thioaptamer. As such, this experiment demonstrates the ability to successfully identify thioaptamer sequences that bind specifically to ovarian tumor endothelial cells. According to several embodiments, candidate aptamers are further characterized to ensure their specificity and binding characteristics. Similarly, morphologically-based aptamer selection, as is disclosed herein, was used to identify candidate thioaptamers that specifically bind to human ovarian tissue and human microvascular endothelial cells. As discussed in more detail below, morphologically-based aptamer selection methods disclosed herein led to the characterization of a promising thioaptamer, (referred to as V5), which is a Vimentin-specific sequence that shows specific binding to tumor vasculature of human ovarian tissue and human microvascular endothelial cells.

[0184] Enriched pools of aptamer candidates generated by the methods disclosed herein provide many advantages in terms of their specificity for tumor cells, the significant reduction in off-target binding, and the reduced risk of adverse side effects from mis-targeting of therapeutic agents conjugated to the aptamers. In effect, the thioaptamers developed are those that bind specifically to tumor endothelium and identified potential targeting protein expressed on those tumor endothelial cells.

[0185] The methods disclosed herein, when practiced according to the embodiments discussed, allow the selection of tissue specific TAs in a rapid, cost-effective, and more representative way from large TA libraries. These methods provide a way to select high specific interested regions and the ability to identify specific aptamers and aptamer associated target protein as potential biomarker for individual patients. Thus, the approaches disclosed herein are believed to contribute in a significant and unexpectedly effective manner to improve aptamers-based diagnostics and therapies.

Material and Methods

[0186] DNA thioaptamer library: The random ssDNA library with a 30 nucleotide random region flanked by PCR primers was chemically synthesized using standard phosphorimidite chemistry, and the oligo strands were purified by HPLC under reverse-phase conditions. The synthesis of the DNA thioaptamer (TA) combinatorial library was performed according to established methods. The library was PCR amplified with α S-dATP and with other normal dNTPs, and sense and anti-sense primers labelled with 5'-biotin. The ssDNA was isolated by treating the PCR products with streptavidin coated magnetic beads and alkaline denaturation. The ssDNA TA library was taken in binding buffer (PBS/5mM MgCl₂) and heated at 95 °C for 5 minutes and then cooled on ice to form the secondary folds. Other conditions may be used, according to some embodiments. The library was purified by 10000 MWCO spin columns (Millipore) and confirmed by gel electrophoresis (15% polyacrylamide).

[0187] Morph-X-Select thioaptamer tissue selection: Five epithelial ovarian cancer samples and five normal ovary tissues were collected from surgical cases at The University of Texas M.D. Anderson Cancer Center and used for thioaptamer tissue selection. It shall be appreciated that other cancer types can also be used in the methods disclosed

herein. All tumor samples in the study were phenotyped (i.e. by morphologically based diagnoses) by the Dept. of Pathology and Laboratory Medicine at MD Anderson Cancer Center. Control samples (e.g., non-tumor tissue, such as fresh ovarian tumor or normal ovarian tissue) will be embedded in OCT compound prior to cryostat sectioning. Cryosections were cut at 5 to 10 microns in thickness and mounted on special window slides suitable for laser microdissection (ION LMD II, JungWoo F&B Corp., Korea), and stained with Hematoxylin and Eosin (H&E). The tissue sections were then covered with a “liquid” cover-slip solution (MagicMount) which can improve tissue morphology and stabilize the biomolecules of tissue. Based on cell morphology and pathological diagnosis, regions of interests (ROI) were defined and drawn directly on a touch screen and been cut by a laser beam and collected in a collection cap. FIG. 2 illustrates an example of the LMD of pre-cut and post-cut of blood vessels (top row, 2A is pre-cut vessel, 2B is post-cut vessel) and tumor cells (bottom row; 2C is pre-cut tumor cells, 2D is post-cut tumor cells). FIG. 2E illustrates the PCR amplification of the eluted TAs from the LMD blood vessels and tumor cells of FIG 2A-2D. Tumor cells were positively selected for each patient ovarian tumor tissue after multiple rounds of negative selection with normal ovary tissues. The TAs bound to LMD dissected tumor cells were eluted, PCR-amplified and enriched by 10 rounds selections with tumor tissue from the same patient. By increasing washes after binding and shortening incubation time with targeted tissue, the TA pool binding to ovarian cancer tissue was iteratively enriched. A non-limiting schematic of the selection procedure is illustrated in FIG. 1. After 10 rounds of selection, the enriched TAs libraries were eluted from the ovarian tumor tissue and PCR amplified.

[0188] Sequence analysis: After eluting the TA library from the ovarian cancer tissue, the TA pools were amplified by PCR and 10 ng of the purified products was used for fragment library construction (Life Technologies) (other template amounts can be used, depending on the embodiment, e.g., 10 ng, 20ng, 50 ng, 100 ng, etc.). After template preparation and enrichment with Ion OneTouch™ systems, Ion Sphere™ particles were loaded onto the Ion semiconductor chip and sequenced on the Ion Personal Genome Machine® (PGM™) System. After sequencing, the data was processed using APTAligner© software (Lu, E) which builds Markov models for each library and performs base calling and

quality filtering. The prevalence of each individual sequence was identified in every analyzed pool and in various pools from the same selection round. Based on copy number and cluster association, sequences were identified with high frequency of occurrence and high homology among different pools. Sequence reads were preprocessed first by removing sequences that were > 80% homopolymeric. Sequences that did not contain any aptamer sequence were also removed. After analyzing the results of the positive control aptamer, further analysis was performed with a data set in which all sequences containing 13 contiguous bases matching the positive control aptamer sequence were removed. Based on the optimal alignment and statistical analysis, TA sequences with high frequency of occurrence and high sequence homology were selected. Using PGM sequencing and Aptaligner© data analysis, several thioaptamer sequences were successfully identified from a thioaptamer library targeting ovarian cancer vasculature. The selected sequences from cycle 10 were grouped by Clustal W program and their secondary structures were analyzed by M fold31 program. As discussed above, the process from this example can readily be applied by one of ordinary skill in the art to identify aptamers sequences that are specific for other cancerous tissues (or related vasculature).

[0189] Selection of tumor endothelial cell or tumor cell specific binding thioaptamers: After sequence analysis, 10 sequences showing the highest frequency of occurrence throughout each pool were selected as thioaptamer candidates, 10 sequences showing the highest frequency of occurrence throughout each pool (tumor vasculature or tumor cells) were selected as thioaptamer candidates. The panel of selected thioaptamers was chemically synthesized, labeled with Cy-3 dye and tested for specific bindings to tumor endothelial cell or tumor cells. Sections of frozen human ovarian tumor were first incubated with universal blocking buffer (Pierce Biotechnology) for 30 min at room temperature, and then incubated with 50 nM thioaptamers for 30 min at room temperature and followed by washing, fixing and nuclei counter staining. Other conditions are readily derived from these for use with other cancerous tissues or aptamer candidate pools. The extent of thioaptamer binding to the tissue was assessed by fluorescence microscopic analysis (Nikon TE2000-E). The relative binding affinity of thioaptamers was determined by the fluoresce intensity detected on the tumor vasculature or tumor cells. CD31 and CD44 antibodies (eBiosciences)

were used as positive control for specific binding of endothelial cells and tumor cells. Hoechst 33342 (Thermo Fisher) was used for counter staining of nuclei. To confirm specific binding of selected TAs to ovarian tumor tissue, a random TA sequence (R4) was used as a negative control for all binding assays. Based on the binding affinity of the tested thioaptamers to human ovarian tumor vasculature or tumor cells, several thioaptamers showing high affinity and specific recognition against tumor vessels to tumor cells were selected for further analysis.

[0190] Affinity pull-downs and mass spectrometry: The associated target protein of selected TAs can be pulled down by crosslinking biotinylated TAs with cells (human endothelial cells, HMVEC or human ovarian cancer cells, IGROV) which has been validated with high binding affinity with selected TAs, and analyzed by mass spectrometry (MS). The protein targets can initially identified by adding the TAs to the cells, crosslinking a 5'-biotin-labeled TA to the target cell surface protein; pull down the TA-protein complex by streptavidin magnetic beads, and trypsinizing the protein targets for MS identification. Standard software packages for using MS/MS fragmentation data on an Orbitrap Fusion™ Tribrid™ Mass Spectrometer have been used for protein identification in the Center for Proteomics and Systems Biology in the Institute of Molecular Medicine at UTHealth. After comparing the protein lists from different selected TAs with nonspecific random control R4, potential target protein has been identified and expression of the identified target protein has been further validated by antibody detection and thioaptamer competition binding assays with antibodies against the target protein. Proteins were excluded from the resulting list of selected TAs if they presented in the random control R4 sample and had the same or higher score than in selected TA samples. Vimentin was selected as a potential target protein with high abundance to V5, but low abundance to control R4 in a ratio of 43:1. Table 2 below illustrates the mass spectrometric results from the protein pull down experiments utilizing bead-based affinity ligand V5 and control R4 discussed above:

Table 2 - Mass spectrometric results from protein pull down experiments utilizing bead-based affinity ligand V5 and control R4.

V5					
Score	Mass	Matches	Sequences	emPAI	Protein
2141	53676	51 (48)	10 (9)	1.13	VIM Vimentin
1250	227646	38 (34)	18 (15)	0.33	MYH9 Isoform 1 of Myosin-9
474	50548	14 (12)	6 (6)	0.62	TUBA1C Tubulin alpha-1C chain
397	11360	14 (14)	3 (3)	1.79	HIST2H4B;HIST1H4B Histone H4
348	42052	15 (13)	6 (6)	0.78	ACTB Actin, cytoplasmic 1
297	74380	10 (10)	5 (5)	0.32	LMNA Isoform A of Prelamin-A/C
					PTRF Isoform 1 of Polymerase I and transcript release factor
253	43450	6 (6)	3 (3)	0.45	
244	66170	6 (6)	3 (3)	0.2	KRT1 Keratin, type II cytoskeletal 1
					HIST1H2AE;HIST1H2AB; Histone H2A type 1-B/E
240	14127	9 (9)	2 (2)	1.3	AHNAK Neuroblast differentiation-associated protein AHNAK
222	629213	15 (13)	9 (8)	0.05	
217	103563	5 (5)	3 (3)	0.13	ACTN1 Isoform 1 of Alpha-actinin-1
120	50255	4 (4)	2 (2)	0.18	TUBB2C Tubulin beta-2C chain
					LUC7L2 cDNA FLJ55988, highly similar to RNA-binding protein Luc7-like 2
117	54761	2 (2)	1 (1)	0.08	
115	24304	3 (3)	1 (1)	0.18	RPL13 60S ribosomal protein L13
110	293407	2 (2)	1 (1)	0.01	FLNC Isoform 1 of Filamin-C
102	73881	3 (3)	2 (2)	0.12	ALB Uncharacterized protein
.....					
R4					
Score	Mass	Matches	Sequences	emPAI	Protein
127	66170	2 (2)	1 (1)	0.06	KRT1 Keratin, type II cytoskeletal 1
104	74380	2 (2)	1 (1)	0.06	LMNA Isoform A of Prelamin-A/C
94	73881	2 (2)	1 (1)	0.06	ALB Uncharacterized protein
86	50451	2 (2)	1 (1)	0.08	EEF1A1 Elongation factor 1-alpha 1
75	24304	2 (2)	1 (1)	0.18	RPL13 60S ribosomal protein L13
58	9651	11 (7)	1 (1)	0.49	- Glutathione peroxidase 4
55	180689	5 (3)	3 (3)	0.07	AHNAK cDNA FLJ46846 fis, moderately similar to Neuroblast differentiation associated protein AHNAK (Fragment)
					VIM Vimentin
50	53676	3 (3)	1 (1)	0.08	
45	59252	80 (17)	1 (1)	0.07	DAK Bifunctional ATP-dependent dihydroxyacetone kinase/FAD-AMP lyase (cyclizing)
					TUBA4A Tubulin alpha-4A chain
39	50634	1 (1)	1 (1)	0.08	
.....					

Results

[0191] After sequencing data analysis, a large number of TA sequences were identified and tested. The top 20 sequences from LMD dissected tumor vasculature and tumor cells were selected for synthesis and further tissue and cell binding assays. FIGS. 3A-C illustrate aptamers detected with high specificity to ovarian cancer vessel sequences. FIGS. 4A-C illustrate aptamers detected with high specificity to ovarian tumor cell sequences. We have identified several high binding affinity sequences from our established thioaptamer solution library applied with ovarian cancer tissues. Some sequences (such as TV1) have been identified from both groups of LMD dissected tumor vasculature and tumor cells, and show binding to ovarian tumor vessels and tumor cells. Sequences identified from LMD dissected vasculature (V3) or tumor cells (T3) show enhanced binding to only tumor vessels or tumor cells respectively. FIGS. 5-8 illustrate the staining of the tumor cells compared to the negative control of a normal ovary. The figures show the specificity of the selected aptamers for the vessel and tumor tissue. FIG. 9 illustrates the TAs identified by LMD and NGS analysis that show specific binding to ovarian tumor vessels (V3), tumor cells (T3), or both vessel and tumor cells (TV1), but not to normal ovarian tissue. For reference, the red coloration illustrates a Cy3 labeled TA, the green coloration illustrates the CD31 antibody labeled vessels, and the blue illustrates cell nuclei. FIG. 10 illustrates the specific binding of LMD and NGS identified sequences that have been confirmed by human HMVEC (see V3, V4) or IGROV (see T3, T4) cells. For reference, the red coloration identifies the TA or CD44 antibody, the green coloration indicates CD31 antibody, and the blue illustrates cell nuclei.

[0192] To validate vimentin as a target protein of selected V5, expression of vimentin on human ovarian cancer tissues, multiple cell lines (IGROV and HMVEC) were evaluated using a vimentin antibody immunofluorescence assay. High levels of vimentin expression were detected on human ovarian tumor tissues and vasculature (FIG. 12A) in contrast to low level of vimentin expression on normal ovary tissues (FIG. 12B). Expression of vimentin was also detected in IGROV and HMVEC cells. An aptamer-antibody competition binding was performed to determine the binding identity of V5 (FIG. 13A-13D). Significantly reduced fluorescence intensity of IGROV cells was observed with cy3 labeled

V5 after incubation with vimentin antibody (e.g., compare 13A/13B to 13C/13D). It may indicate V5 can bind the same sites of vimentin protein as the vimentin antibody binds to.

[0193] Using the same pull-down method, Annexin A2 was identified as a target protein (of aptamers Endo28 and Endo31) in our solution thioaptamer library selections against isolated human ovarian cancer endothelial cells. Table 3 below illustrates the mass spectrometric results from protein pull down experiments utilizing bead-based affinity ligand endo28 (A), endo31 (B) and control random 4 (R4) (C). Proteins with scores less than 150 are not shown except for R4 pulldown.

Table 3 – Mass spectrometric results from protein pull down experiments utilizing bead-based affinity ligand endo28 (A), endo31 (B) and control random 4 (R4) (C).

A						
Protein	Score	Mass	Matches	Sequences	emPAI	Brief Description
ANXA2_HUMAN	1651	38580	67 (54)	10 (7)	11.12054	Annexin A2
H2A1B_HUMAN	633	14127	31 (25)	3 (2)	11.03565	Histone H2A type 1-B/E
H4_HUMAN	570	11360	41 (33)	2 (2)	8.319185	Histone H4
ACTG_HUMAN	535	41766	29 (15)	5 (3)	2.886248	Actin, cytoplasmic 2
ALBU_HUMAN	482	69321	54 (35)	5 (3)	1.612903	Serum albumin
TBA1A_HUMAN	314	50104	23 (11)	4 (2)	1.528014	Tubulin alpha-1A chain
LMNA_HUMAN	211	74095	5 (4)	3 (2)	1.018676	Prelamin-A/C
RAP1A_HUMAN	206	20974	10 (3)	2 (1)	1.782683	Ras-related protein Rap-1A
TBB5_HUMAN	204	49639	9 (8)	4 (3)	2.37691	Tubulin beta chain
TBB3_HUMAN	107	50400	7 (7)	2 (2)	1.528014	Tubulin beta-3 chain
SERPH_HUMAN	170	46411	5 (5)	2 (2)	1.612903	Serpin H1
MOES_HUMAN	156	67778	78 (7)	6 (2)	1.103565	Moesin

B						
Proteins	Score	Mass	Matches	Sequences	emPAI	Brief Description
ANXA2_HUMAN	2165	38580	121 (88)	12 (9)	8.266309	Annexin
H4_HUMAN	1713	11360	86 (79)	2 (2)	4.37891	Histone H4
H2A1B_HUMAN	1263	14127	57 (49)	3 (2)	5.808758	Histone H2A type 1-B/E
H2A1A_HUMAN	579	14225	35 (28)	2 (2)	3.306524	Histone H2A type 1-A
MYH9_HUMAN	1174	226392	76 (38)	18 (9)	0.893655	Myosin-9
ACTB_HUMAN	939	41710	60 (35)	7 (6)	3.529937	Actin, cytoplasmic 1
ACTBL_HUMAN	578	41976	30 (16)	4 (3)	1.519214	Beta-actin-like protein 2
TBB5_HUMAN	925	49639	31 (29)	5 (4)	1.742627	Tubulin beta chain
TBB6_HUMAN	385	49825	17 (16)	3 (3)	1.251117	Tubulin beta-6 chain
LMNA_HUMAN	673	74095	21 (17)	6 (4)	1.117069	Prelamin-A/C
TBA1A_HUMAN	646	50104	29 (21)	5 (3)	1.251117	Tubulin alpha-1A chain
ACTN4_HUMAN	629	104788	21 (14)	7 (2)	0.357462	Alpha-actinin-4

C						
Protein	Score	Mass	Matches	Sequences	emPAI	Brief Description
IQCA1_HUMAN	38	95281	120 (7)	3 (1)	0.760456	IQ and AAA domain-containing protein 1
P3C2B_HUMAN	35	184651	10 (1)	5 (1)	0.380228	Phosphatidylinositol-4-phosphate 3-kinase C2 domain-containing subunit beta
NKTR_HUMAN	31	165577	44 (4)	7 (1)	0.570342	NK-tumor recognition protein
LRCC1_HUMAN	30	119522	33 (3)	7 (1)	0.570342	Leucine-rich repeat and coiled-coil domain-containing protein 1
PRKDC_HUMAN	29	468788	95 (4)	12 (1)	0.380228	DNA-dependent protein kinase catalytic subunit
ELOV4_HUMAN	28	36805	5 (1)	1 (1)	2.281369	Elongation of very long chain fatty acids protein 4
MAP4_HUMAN	27	120930	22 (1)	3 (1)	0.570342	Microtubule-associated protein 4
MARK1_HUMAN	26	88947	43 (1)	4 (1)	0.95057	Serine/threonine-protein kinase MARK1
MLH3_HUMAN	25	163607	5 (1)	2 (1)	0.570342	DNA mismatch repair protein Mlh3
BRWD1_HUMAN	24	262772	392 (2)	14 (1)	0.380228	Bromodomain and WD repeat-containing protein 1
RN220_HUMAN	24	62725	23 (1)	2 (1)	1.330798	E3 ubiquitin-protein ligase RNF220

[0194] Annexin A2 was identified as the top targeted protein in two different thioaptamer TA (Endo28 and Endo31) pull down experiments, was not pulled down when using a control, random thioaptamer (R4) experiment. It was also confirmed that Annexin A2 is overexpressed on human ovarian tumor vessels, tumor cells and HMVEC cells (FIGS.

14A-14C). The expression of Annexin A2 (green) was verified on both human ovarian cancer vasculature (FIG. 14A) and tumor cells (FIG. 14B). As shown in FIG. 14C, 97% of Annexin A2 positive HMVEC cells were detected by flow cytometry. A siRNA knockdown experiment of Annexin A2 led to reduced expression of Annexin A2 in both RNA and protein level (FIGS. 14D-E). Specific down-regulation of Annexin A2 levels was confirmed in time correspondence by real-time PCR (FIG. 14D) and western blot (FIG. 14E) when HMVEC cells were either mock transfected or transfected with Annexin A2-specific siRNA. Consistent with the siRNA Annexin A2 knockdown, binding of Endo28 and Endo31 to HMVEC cells were reduced significantly (FIG. 14F). As illustrated in FIG. 5F, the knockdown of Annexin A2 using siRNA resulted in reduced binding of Endo28 (red) and Endo31 thioaptamers (red) on HMVEC cells. All of these experiments further confirmed Annexin A2 is the specific target protein that thioaptamer Endo28 and Endo 31 bind to.

[0195] It clearly indicates that NGS analysis is a rapid, efficient way to identify representative high binding affinity TAs.

Example 2

Overview

[0196] A method will be developed that will enable the use of tissue sections from individual patients to identify high binding affinity aptamer sequences and their associated tumor biomarkers.

Materials and Methods

[0197] DNA thioaptamer library: A DNA thioaptamer combinatorial library will be synthesized in accordance with Example 1, using a tumor tissue of choice. The library will be synthesized using standard phosphoramidite chemistry. As well, the oligo strands will be purified by HPLC under reverse-phase conditions.

[0198] Morph-X-Select thioaptamer tissue selection: A plurality of tumor samples from a tumor tissue of choice will be collected along with a plurality of comparable healthy tissue. The plurality of tumor and healthy tissue will be excised and mounted on special window slides suitable for laser microdissection. Tumor cells will be selected after multiple rounds of negative selection with healthy tissue. The thioaptamers bound to LMD

dissected tumor cells will be eluted, PCR amplified, and enriched by a plurality of rounds of selections with tumor tissue from the same patient. The enriched thioaptamers library will then be eluted from the healthy tissue and amplified.

[0199] Sequence analysis: The thioaptamer pools will then amplified by PCR and a portion of the purified product will be used for fragment library construction. The fragments will be sequenced and then processed to build Markov models for each library. Additional quality filtering will be performed to remove sequences that are over 80% homopolymeric and did not contain any aptamer sequences. Thioaptamer sequences with higher frequency of occurrence and high sequence homology will be selected.

[0200] Selection of tumor endothelial cell or tumor cell specific binding thioaptamers: The extent of the binding of the thioaptamers will be then be assessed. The thioaptamers will be labeled with dye and tested for specific bindings to tumor cells. Sections of tumor tissue will first be incubated with universal blocking buffer and then incubated with the selected thioaptamers. The relative binding affinity will then be determined by the fluoresce intensity detected on the tumor cells.

[0201] Upon selection, the selected thioaptamers can optionally be conjugated with the appropriate chemical (e.g. cytotoxin) for treatment of the related target site.

[0202] It is contemplated that various combinations or subcombinations of the specific features and aspects of the embodiments disclosed above may be made and still fall within one or more of the inventions. Further, the disclosure herein of any particular feature, aspect, method, property, characteristic, quality, attribute, element, or the like in connection with an embodiment can be used in all other embodiments set forth herein. Accordingly, it should be understood that various features and aspects of the disclosed embodiments can be combined with or substituted for one another in order to form varying modes of the disclosed inventions. Thus, it is intended that the scope of the present inventions herein disclosed should not be limited by the particular disclosed embodiments described above. Moreover, while the invention is susceptible to various modifications, and alternative forms, specific examples thereof have been shown in the drawings and are herein described in detail. It should be understood, however, that the invention is not to be limited to the particular forms

or methods disclosed, but to the contrary, the invention is to cover all modifications, equivalents, and alternatives falling within the spirit and scope of the various embodiments described and the appended claims. Any methods disclosed herein need not be performed in the order recited. The methods disclosed herein include certain actions taken by a practitioner; however, they can also include any third-party instruction of those actions, either expressly or by implication. For example, actions such as “administering stem cells” include “instructing the administration of stem cells.” The ranges disclosed herein also encompass any and all overlap, sub-ranges, and combinations thereof. Language such as “up to,” “at least,” “greater than,” “less than,” “between,” and the like includes the number recited. Numbers preceded by a term such as “about” or “approximately” include the recited numbers. For example, “about 3 mm” includes “3 mm.”

WHAT IS CLAIMED IS:

1. A method for selecting aptamers specific for a diseased target tissue, comprising:
 - obtaining a library comprising putative diseased tissue-specific aptamers;
 - incubating the library with a first tissue sample comprising a first region of tissue affected with a disease and a second region of the tissue not affected with the disease,
 - wherein the incubation is for a first period of time and employs a first set of conditions;
 - washing the first tissue sample to remove aptamers that are not bound to the first or second region of tissue;
 - microscopically identifying at least one cell of the first tissue sample within the first region and capturing the at least one cell using laser microdissection (LMD);
 - eluting the aptamers bound to the at least one captured cell;
 - amplifying the eluted aptamers using polymerase chain reaction to generate a first next-generation library of aptamers;
 - incubating the first next-generation library with a second tissue sample comprising a first region of tissue affected with a disease and a second region of the tissue not affected with the disease,
 - wherein the incubation of the first next-generation library with the second tissue sample employs a second set of conditions that are more stringent than the first set of conditions;
 - washing the second tissue sample to remove aptamers that are not bound to the first or second region of tissue;
 - microscopically identifying at least one cell of the second tissue sample within the first region and capturing the at least one cell using LMD;
 - eluting the aptamers bound to the at least one captured cell from the second tissue sample;
 - amplifying the eluted aptamers using polymerase chain reaction to generate a second next-generation library of aptamers;

repeating the incubation, washing, LMD capturing, elution and amplification steps a plurality of additional times to generate a pool of enriched aptamers that are specific for the diseased target tissue.

2. The method of Claim 1, further comprising sequencing the pool of enriched aptamers to determine their sequence and relative frequency within the pool.

3. The method of Claim 2, wherein the sequencing comprises next generation sequencing (NGS).

4. The method of Claim 3, further comprising grouping the pool of enriched aptamers based on comparison of their sequences and predicting the secondary structure of the sequences.

5. The method Claim 1, further comprising confirming the binding at least one aptamer from the pool of enriched aptamers against an additional sample of diseased target tissue.

6. The method of Claim 5, wherein binding is confirmed by one or more of an in vitro biochemical assay, a functional cell based assay, a dot blot assay, identifying colocalization of a candidate aptamer with an antibody known to bind to a putative target, ELISA, radiolabeled binding assay, protein pull-down assay, and western blot.

7. The method of Claim 1, further comprising:

amplifying at least one candidate aptamer from the pool of enriched aptamers;
labeling or immobilizing the at least one candidate aptamer such that the at least one aptamer can be specifically identified and isolated;

mixing the at least one labeled or immobilized aptamer with a lysate derived from the diseased target tissue under conditions that allow the formation of an aptamer-target complex;

recovering the aptamer-target complex;

dissociating the aptamer and the target, and retrieving the target;

sequencing the target from the aptamer-target complex to identify the identity of the target, thereby identifying a biomarker.

8. The method of Claim 8, wherein the sequencing of the target is performed by electrospray ionization (ESI) and tandem mass spectrometry-based sequencing.

9. The method of Claim 8, wherein the sequencing of the target is performed by matrix-assisted laser desorption/ionization (MALDI) and tandem mass spectrometry-based sequencing.

10. The method of Claim 8, wherein the sequencing of the target is performed by enzymatically digesting the target and identifying resultant peptides by mass fingerprinting and/or tandem mass spectrometry.

11. A method according to any one of Claims 1 to 10, wherein the diseased tissue comprises a tissue affected by cancer, acute disease, chronic disease, acute injury, or indirectly affected by cancer, acute disease, chronic disease, or acute injury.

12. A method according to any one of Claims 1 to 11, wherein the library comprises thio-modified aptamers.

13. A method according to any one of Claims 1 to 12, wherein the pool of enriched aptamers is at least 80% specific for said diseased target tissue.

14. A method for identifying a biomarker comprising:

- selecting a pool of enriched aptamers according to the method of Claim 1;
- amplifying at least one candidate aptamer from the pool of enriched aptamers;
- labeling or immobilizing the at least one candidate aptamer such that the at least one aptamer can be specifically identified and isolated;
- mixing the at least one labeled or immobilized aptamer with a lysate derived from the diseased target tissue under conditions that allow the formation of an aptamer-target complex;

recovering the aptamer-target complex;
sequencing the target from the aptamer-target complex to identify the identity of the target, thereby identifying a biomarker.

15. A method for identifying a biomarker comprising:

obtaining a library comprising putative diseased tissue-specific aptamers;
incubating the library with a first tissue sample comprising a first region of tissue affected with a disease and a second region of the tissue not affected with the disease,

wherein the incubation employs a first set of conditions;

removing aptamers that are not bound to the first or second region of tissue;
isolating at least one cell of the first tissue sample within the first region using laser microdissection (LMD);

amplifying aptamers that were bound to the isolate cell to generate a first next-generation library of aptamers;

repeating the incubation, removing, isolating, and amplification steps a plurality of additional times to generate a pool of enriched aptamers that are specific for the diseased target tissue;

amplifying a candidate aptamer from the pool of enriched aptamers;
labeling or immobilizing the amplified candidate aptamer;
exposing the labeled amplified aptamer to a lysate of diseased tissue, the diseased tissue comprising target markers;
isolating complexes of labeled amplified aptamer and target markers; and
sequencing the target marker, thereby identifying a biomarker.

16. The method of Claim 15, wherein the candidate aptamer is labeled with a fluorophore, a radiolabel, a chromophore, a magnetic particle, or antigen.

17. The method of Claim 16, wherein the candidate aptamer is labeled with streptavidin.

18. The method of Claim 16, wherein the candidate aptamer is labeled with superparamagnetic iron oxide particles.

19. The method of Claim 15, wherein the candidate aptamer is immobilized on a solid support.

20. The method of Claim 15, wherein the solid support comprises agarose beads.

21. A method according to any one of Claims 15 to 20, wherein the biomarker is expressed on a tissue affected by cancer, acute disease, chronic disease, acute injury, or indirectly affected by cancer, acute disease, chronic disease, or acute injury.

22. A method for selecting aptamers specific for a diseased target tissue, comprising:

receiving a first and a second sample of a target tissue, wherein the first sample is from a region of the target tissue affected with a disease and a the second sample is from a region of the target tissue not affected with the disease,

wherein the first and the second sample are collected using laser microdissection;

obtaining a library comprising putative diseased tissue-specific aptamers;

screening said library against the first sample to identify aptamers that bind to said diseased tissue;

removing aptamers that are unbound, thereby generating a pool of candidate aptamers;

screening the pool of candidate aptamers against said second tissue sample to identify candidate aptamers that bind to said normal tissue;

collecting aptamers that do not bind to said normal tissue, thereby generating a pool of screened candidate aptamers;

amplifying said pool of screened candidate aptamers;

repeating the screening against the first and second tissue samples a plurality of times to enrich the pool of screened candidate aptamers, thereby generating a pool of enriched aptamers that are specific for the diseased target tissue.

23. The method of Claim 22, wherein the diseased tissue is cancerous.

24. A method according to Claim 22 or 23, wherein the library comprises thio-modified aptamers.

25. A method according to any one of Claims 22 to 24, wherein the pool of enriched aptamers is at least 80% specific for said diseased target tissue.

26. A method according to any one of Claims 22 to 25, wherein said laser microdissection is used to dissect tissue regions morphologically identified as diseased or normal.

27. A method according to any one of Claims 22 to 26, further comprising sequencing the pool of enriched aptamers.

28. A method according to any one of Claims 22 to 27, further comprising confirming the tissue specificity of the pool of enriched aptamers by identifying aptamers that co-localize with tissue specific antigens identified by antibodies.

29. A method for identifying a biomarker specific for a diseased tissue of a subject, comprising:

receiving a first and a second sample of a target tissue, wherein the first sample is from a region of the target tissue affected with a disease and a the second sample is from a region of the target tissue not affected with the disease,

obtaining a library comprising putative diseased tissue-specific aptamers and screening said library against the first sample to identify aptamers that bind to said diseased tissue;

removing aptamers that are unbound, thereby generating a pool of candidate aptamers;

screening the pool of candidate aptamers against said second tissue sample to identify candidate aptamers that bind to said normal tissue;

collecting aptamers that do not bind to said normal tissue, thereby generating a pool of screened candidate aptamers;
amplifying said pool of screened candidate aptamers;
sequencing the pool of screened candidate aptamers; and
aligning the sequences of the screened candidate aptamers against known proteins to determine the biomarker that the aptamer is directed to, thereby identifying the biomarker specific to the diseased tissue of the subject.

30. A method of treating a subject with a disease comprising:

obtaining first and second sample of tissue from the subject, wherein the first sample is from a diseased region of tissue, and the second sample is from a region of the tissue not affected with the disease;

ordering an enriched pool of diseased-tissue specific aptamers, wherein the enriched pool of diseased-tissue specific aptamers is at least 80% specific for the diseased tissue;

conjugating at least one therapeutic agent to the pool of diseased-tissue specific aptamers, thereby generating therapeutic aptamers; and

administering the therapeutic aptamers to the subject, wherein the therapeutic aptamers deliver the conjugated therapeutic agent specifically to the diseased tissue.

31. The method of Claim 30, wherein the delivery route is selected from intravenous, local, oral, intramuscular, systemic, and transdermal.

32. A method for treating ovarian cancer comprising:

administering an ovarian tissue-specific population of aptamers to a subject with ovarian cancer, said aptamers conjugated to a therapeutic agent, wherein the aptamers were identified by screening both cancerous and non-cancerous tissue collected from the subject against a pool of aptamers to identify those aptamers that bind the cancerous tissue but not the non-cancerous tissue.

33. The method of Claim 32, wherein the cancer tissue is cancer vasculature.

34. The method of Claim 32, wherein the cancer tissue is cancer cells.

35. The method of Claim 32, wherein the aptamer targets vimentin or vimentin-related pathways.

36. The method of Claim 11, wherein the aptamer comprises SEQ ID No. 5.

37. A diagnostic agent comprising a cancer tissue-specific aptamer conjugated to a reporter agent, the cancer tissue-specific aptamer generated by the method of any one of Claims 1 to 14.

38. The agent of Claim 16, wherein the reporter agent is a fluorophore, a chromophore, an enzyme cleavable reagent, or a radio-labeled nucleotide.

39. An ovarian cancer specific aptamer encoded by the polynucleotide of any one of SEQ ID NOs. 1 to 46.

40. A method of identifying high binding affinity aptamer sequences comprising:

generating a combinatorial DNA aptamer library;

dissecting regions of interest bound with aptamers based on a morphological assessment of the tissue;

incubating the combinatorial DNA aptamer library with diseased cells and negatively selecting the combinatorial DNA aptamer library against normal cells to generate a plurality of aptamers bound to the diseased cells;

eluting and amplifying the plurality of aptamers bound to the diseased cells;

identifying high affinity aptamer sequences by next generation sequencing;

and

identifying the targeted proteins by mass spectrometry.

41. The method of Claim 40, wherein the plurality of aptamers bound to the diseased cells are further incubated with diseased cells and negatively selected against normal cells, eluted, and amplified.

42. The method of Claim 41, wherein the steps are performed at least 10 times.

43. The method of Claim 40, wherein the DNA aptamer library has been modified with thio substitution of the phosphate backbone at the 5'dA position.

44. The method of Claim 40, wherein the dissecting is accomplished using image directed laser microdissection.

45. The method of Claim 40, wherein the diseased cells are human ovarian cancer endothelial cells and the normal cells are human ovarian endothelial cells.

46. The method of Claim 45, wherein the ovarian cancer endothelial cells are CD31+ and CD146+.

47. The method of Claim 40, wherein the aptamer showing specific binding to the vasculature of human ovarian cancer tissue are Endo-28 and Endo-31.

48. The method of Claim 40, wherein the aptamer showing specific binding to the vasculature of human ovarian cancer tissue is a vimentin-specific sequence.

49. A method of identifying high binding affinity aptamer sequences to treat ovarian cancer comprising:

- generating a combinatorial DNA aptamer library wherein the combinatorial DNA aptamer library has been modified with thio substitution of the phosphate backbone at the 5'dA position;

- dissecting regions of interest bound with aptamers using laser microdissection based on a morphological assessment of the tissue;

- incubating the combinatorial DNA aptamer library with purified human ovarian cancer endothelial cells and negatively selecting the combinatorial DNA aptamer library against normal human ovarian endothelial cells to generate a plurality of aptamers bound to the diseased cells;

- eluting and amplifying the plurality of aptamers bound to the diseased cells;

identifying high affinity aptamer sequences by next generation sequencing;
and
identifying the targeted proteins by mass spectrometry.

50. A method of treating a disease using aptamer sequences, comprising:
generating a combinatorial DNA aptamer library;
dissecting regions of interest bound with aptamers based on a morphological assessment of the tissue;
identifying high affinity aptamer sequences by next generation sequencing;
and
conjugating the high affinity aptamer sequences with a cytotoxin.

51. A method of treating ovarian cancer using aptamer sequences, comprising:
generating a combinatorial DNA aptamer library modified with thio substitution of the phosphate backbone;
dissecting regions of interest bound with aptamers using laser microdissection based on a morphological assessment of the tissue;
identifying high affinity aptamer sequences by next generation sequencing;
and
conjugating the high affinity aptamer sequences with a cytotoxin.

52. The method of Claim 51, wherein the high affinity aptamer sequence is comprises
SEQ ID NO. 5.

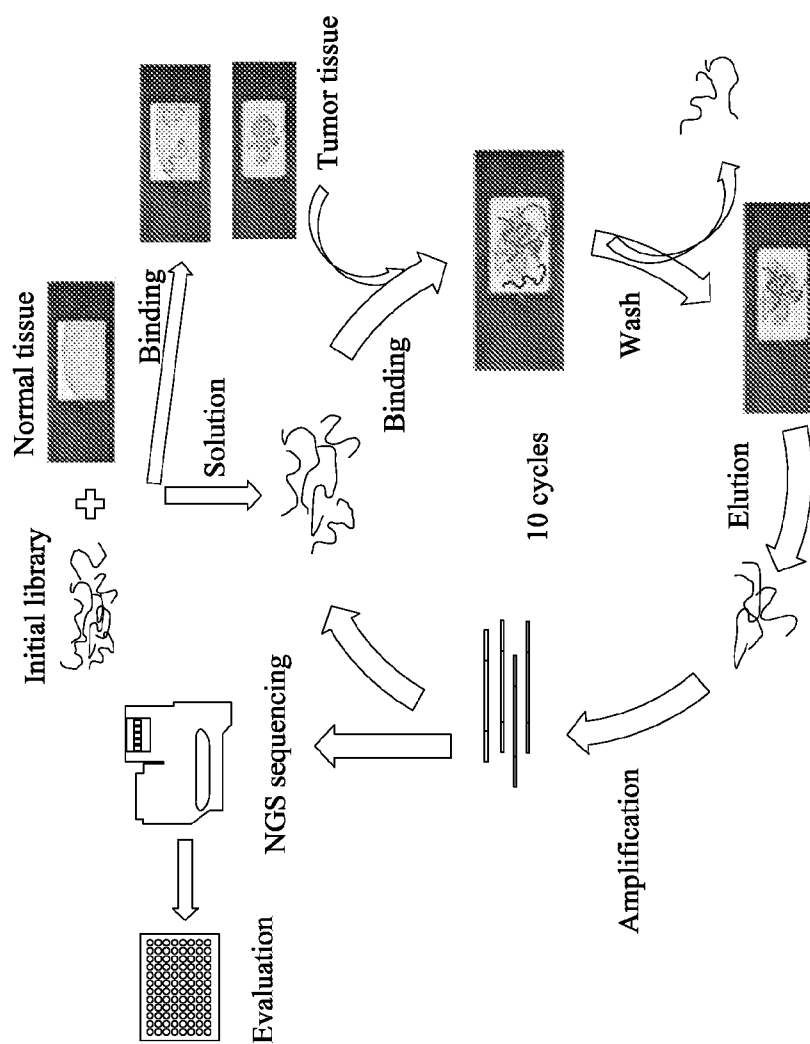
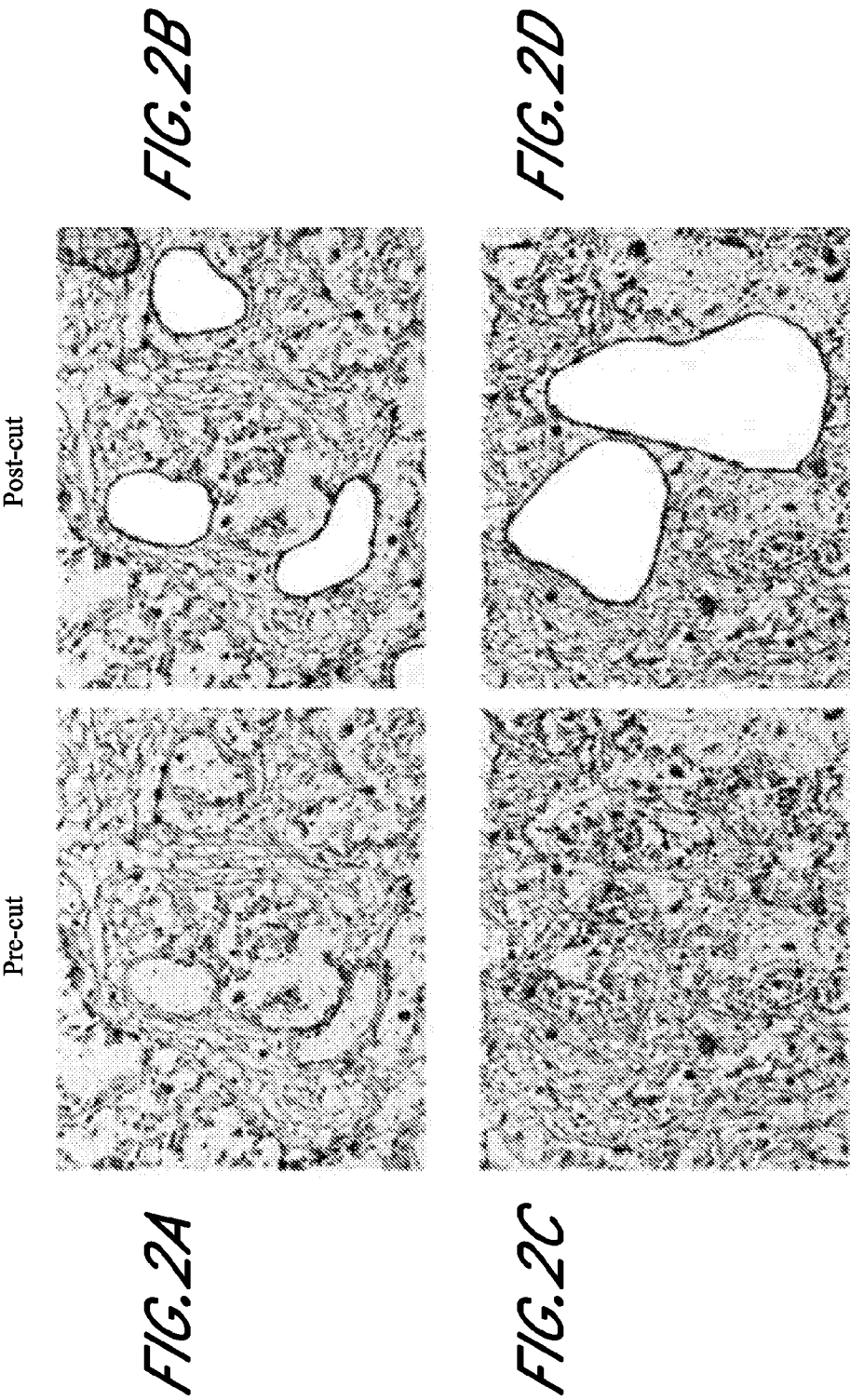


FIG. 1



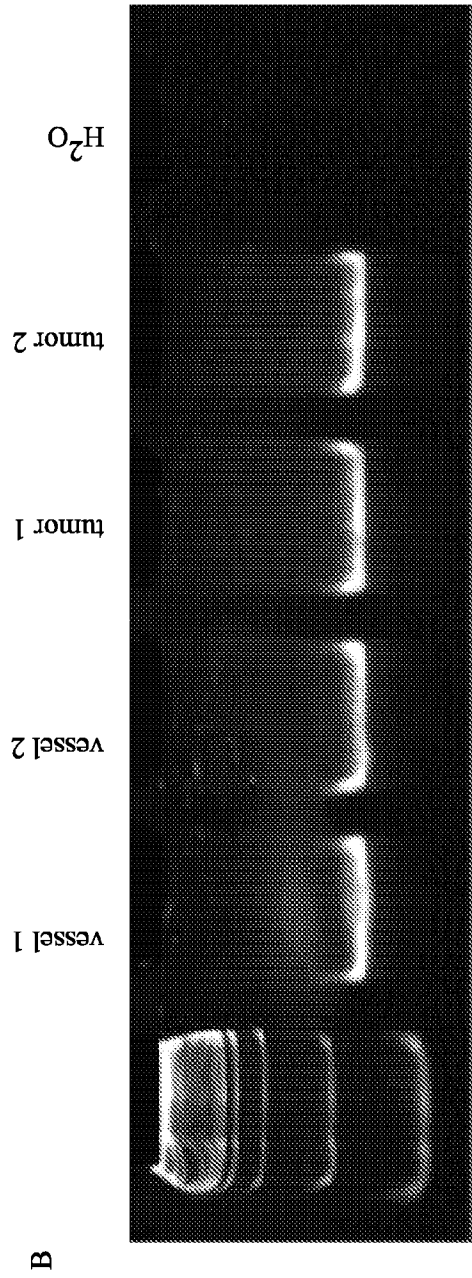
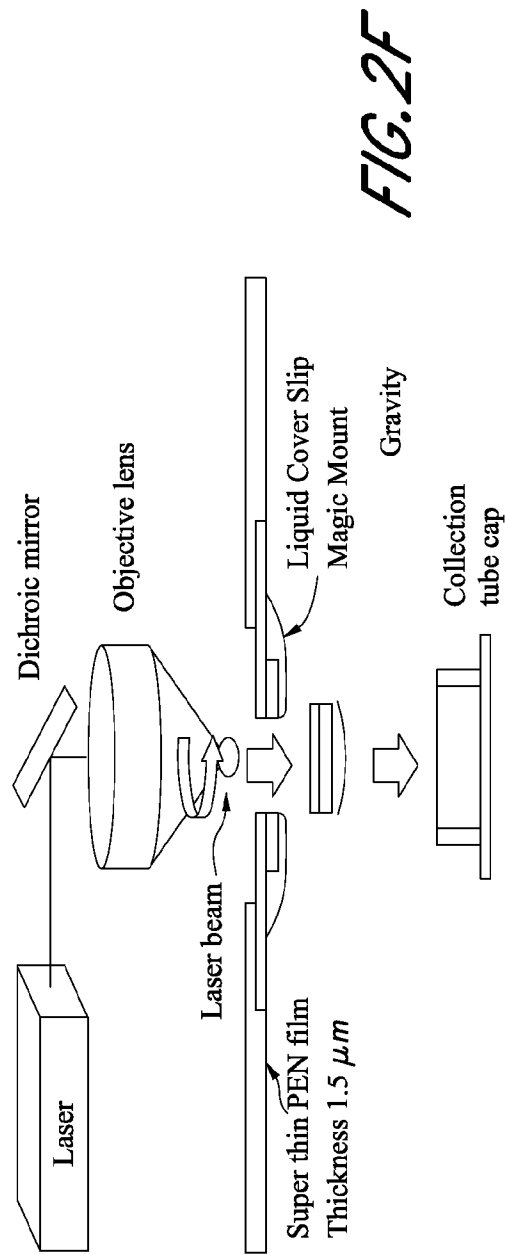
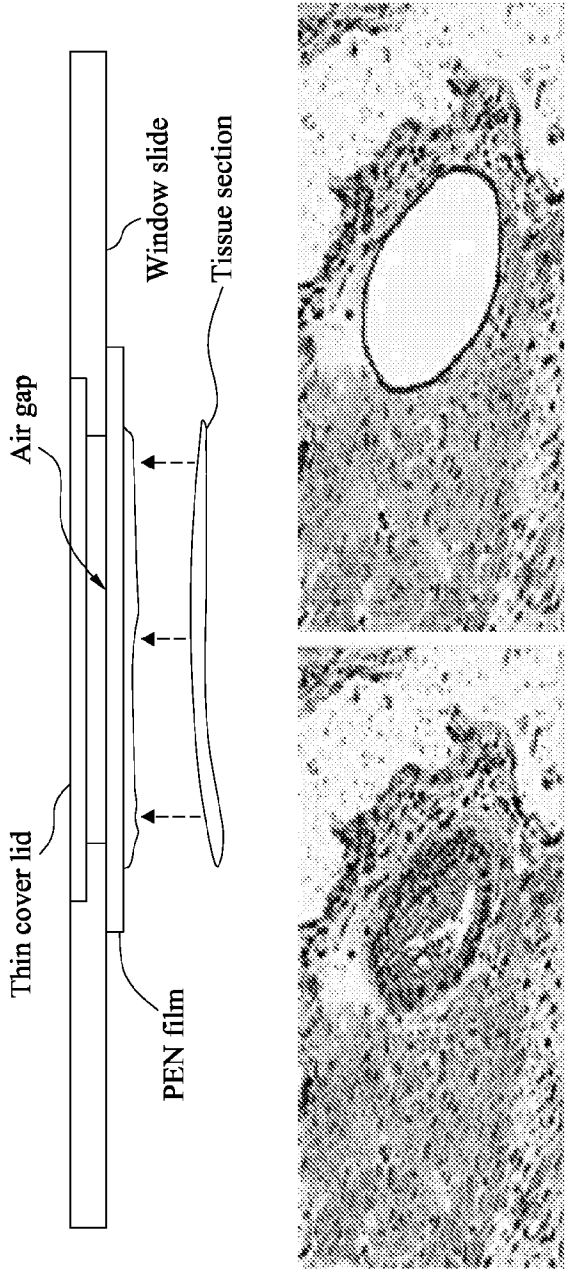


FIG. 2E



No.	Frequency of occurrence	Sequences
SEQ ID NO. 1	TV1 2160	TTTAACGATCCCAACATAGACCTGATTGTCA
SEQ ID NO. 2	TV2 9732	TATTTCCGGACCAGCTGGGTCTATGCAGG
SEQ ID NO. 3	V3 9158	ATCATCGATCACCTCTTCAAGAACATCCCT
SEQ ID NO. 4	V4 3846	GATCGCGGCAAAACTCAGGCTCAGCGGCA
SEQ ID NO. 5	V5 3684	CATAGACCCAGCTGGTCCGGAAAATAAGAT
SEQ ID NO. 6	V6 2321	TGGTGTAAACGCGGCTTTAATAATTTTA
SEQ ID NO. 7	V7 1957	AATGACAATCAGGTCTATGTTGGGATCGTTA
SEQ ID NO. 8	V8 1601	AAGCATCTGTTTAACGATCCCAACATAGAC
SEQ ID NO. 9	V9 1517	TTTAACGATCCAACATAGACCTGATTGTCA
SEQ ID NO. 10	V10 1394	CGCGAAGCATCTTATTTTCCGGACCAGCTGG
SEQ ID NO. 11	V11 990	TTTAACGATCCAACATAGACCTGATTGTCTAT
SEQ ID NO. 12	V12 968	TTTTAAGATCATCGATCACCTCTTCAAGAAC
SEQ ID NO. 13	V13 929	GAATGACAATCAGGTCTATGTTGGGATCGT
SEQ ID NO. 14	V14 927	CGCATGGGAGAGACCCACACTACCATCGGC
SEQ ID NO. 15	V15 880	ATCATCGATCACCTCTTCAAGAACATCCTGA
SEQ ID NO. 16	V16 854	CCGATGGTAGTGTGGGTCTCCCCATGCGA
SEQ ID NO. 17	V17 808	ATCGCGGCAAAAACTCAGGCTCAGCGGCA
SEQ ID NO. 18	V18 801	AGATCGCGGCAAAAACTCAGGCTCAGCGGCA
SEQ ID NO. 19	V19 672	AATGACAATCAGGTCTATGTTGGATCGTTA
SEQ ID NO. 20	V20 661	CAATCAGGTCTATGTTGGGATCGTTAAACA
		

FIG. 3A

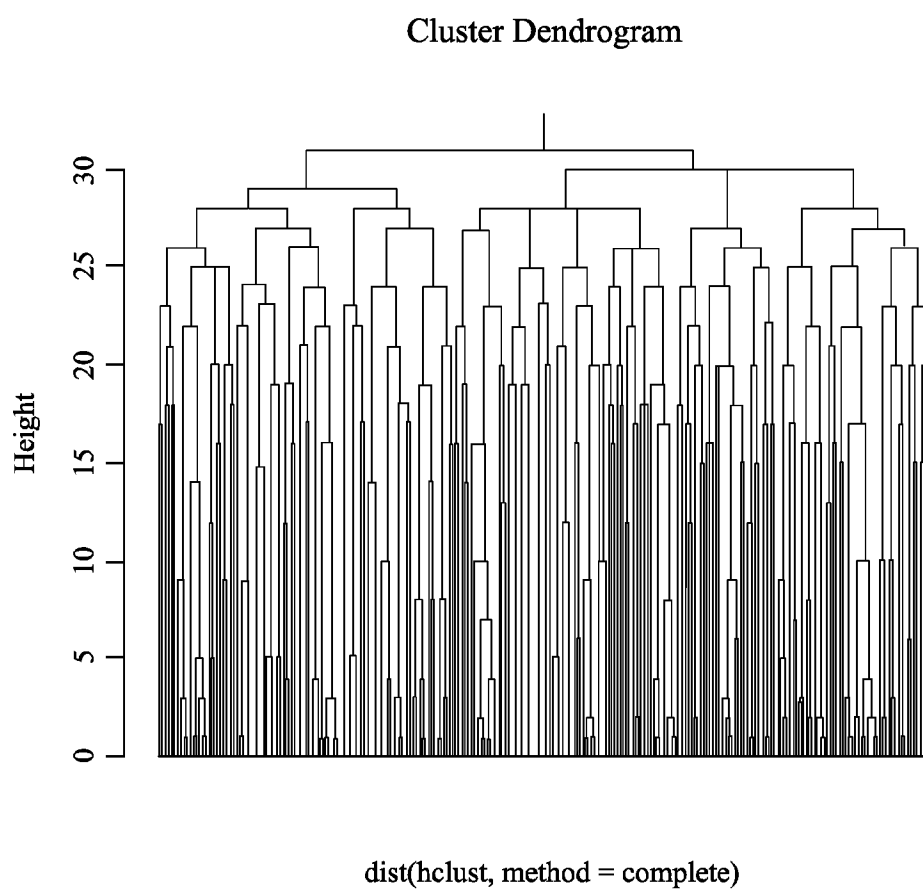


FIG. 3B

	Seq No.	Occurrence	Sequences
SEQ ID NO. 21	V199	4	ATTCGCGGCAAAAACCTCAGGCTCAGCGGCA
SEQ ID NO. 22	V86	105	GATCGCGGCAAAAGCTCAGGCTCAGCGGCA
SEQ ID NO. 4	V4	3846	GATCGCGGCAAAAACCTCAGGCTCAGCGGCA
SEQ ID NO. 23	V18	801	AGATCGCGGCAAAAACCTCAGGCTCAGCGGCA
SEQ ID NO. 24	V17	808	ATCGCGGCAAAAACCTCAGGCTCAGCGGCA

FIG. 3C

No.	Frequency of occurrence	Sequences
SEQ ID NO. 1	19338	TTTAACGATCCCAACATAGACCTGATTGTCA
SEQ ID NO. 2	13609	TATTTCCGGACACAGCTGGGTCTATGCAGG
SEQ ID NO. 25	3374	AGCCTGAGTTTTTGGCGGATCTCGACCTCC
SEQ ID NO. 26	2189	TCTCAAGAAGATCCCTGAAGTAACTTCCT
SEQ ID NO. 27	1836	ACACACGAGCGTGGTGGCGCGCATCGCC
SEQ ID NO. 28	1623	TTTAACGATCCCAACATAGACCTGATTGTCAT
SEQ ID NO. 29	1206	ATTGTCATTCTACACCAACGATACCATTC
SEQ ID NO. 30	1183	CGCGAAGCATCTTATTTCCGGACCAAGCTGG
SEQ ID NO. 31	1144	AAATAATTTTTGTATTCTCACTAATTTGCTT
SEQ ID NO. 32	1087	AAGCATCTGTTTAACGATCCCAACATAGAC
SEQ ID NO. 33	989	ACCTCCTTGCACTCGGCGATGCGCGGCCAC
SEQ ID NO. 34	936	TGGTGTTAAAACGCGGCTTTAATAATTTTA
SEQ ID NO. 35	791	CCTGAGTTTTTTGCCGCGATCTCGACCTCC
SEQ ID NO. 36	709	ACACACGAGCGTGGTGGCGCGCATCGC
SEQ ID NO. 37	633	CGAAAGCATCTTATTTTCCGGACCAAGCTGG
SEQ ID NO. 38	604	TTTAACGATCCCAACATAGACTGATTGTCAT
SEQ ID NO. 39	575	TTTAACGATCCCAACATAGACTGATTGTC
SEQ ID NO. 40	563	TCATTCCCTACACCAACGATACCCATTTC
SEQ ID NO. 41	552	CGCGCGCATCGCGGAGTGCAAGGAGGTGGA
SEQ ID NO. 42	515	ATCCCAACATAGACCTGATTGTCATTCTCTAC
		

FIG. 4A

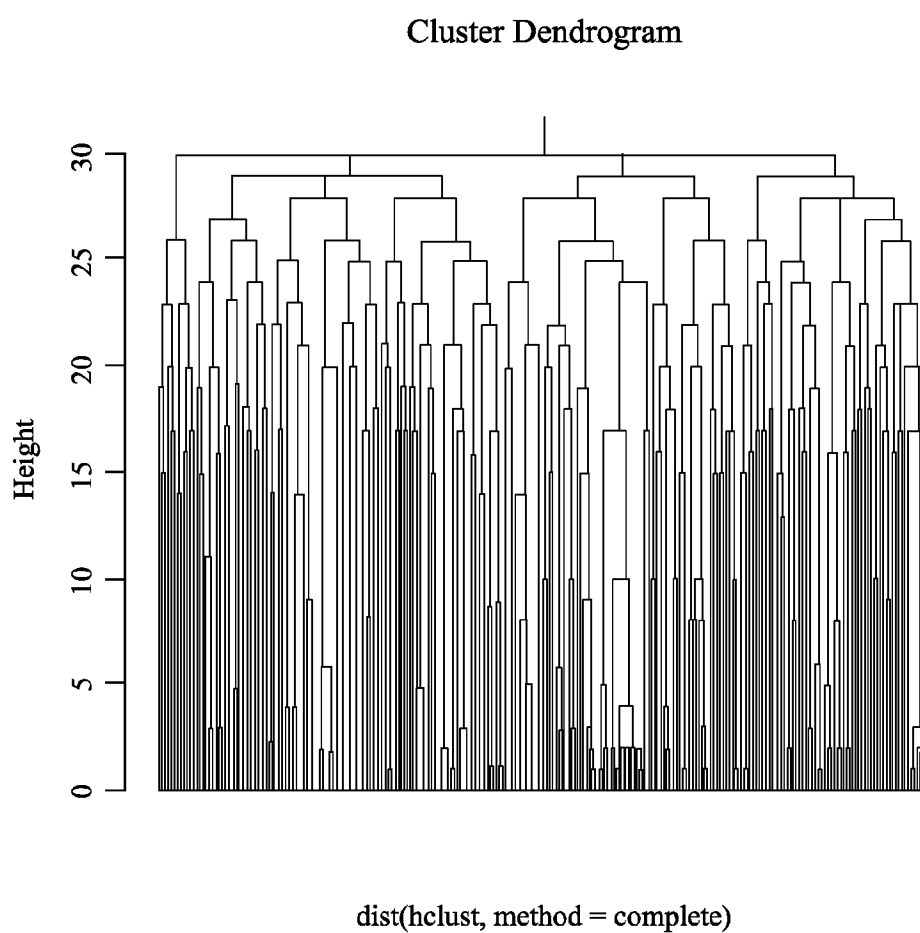


FIG. 4B

SEQ ID NO. 43	Seq_73	3 108	AGCCTGAGTTTTTGC GCGATCTCGACCTCC
SEQ ID NO. 44	Seq_13	3 791	CCTGAGT -TTTTTCCCGCATCTCGACCTCC
SEQ ID NO. 45	Seq_106	3 72	AGC-TGAGTTTTTCCCGCATCTCGACCTCC
SEQ ID NO. 25	Seq_3	3 3374	AGCCTGAGTTTTTCCCGCATCTCGACCTCC
SEQ ID NO. 46	Seq_195	3 33	AGCCTGAGTTTTTCCCGCATCTCAACCTCC

FIG. 4C

TV2

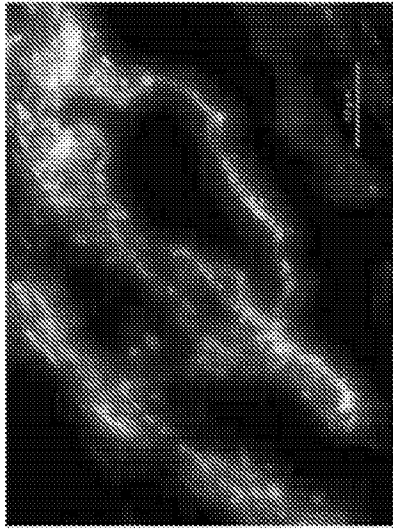


FIG. 5B

TV1

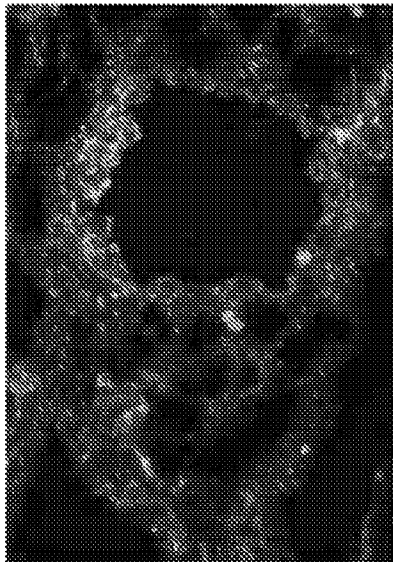


FIG. 5A

Ovarian tumor vessel

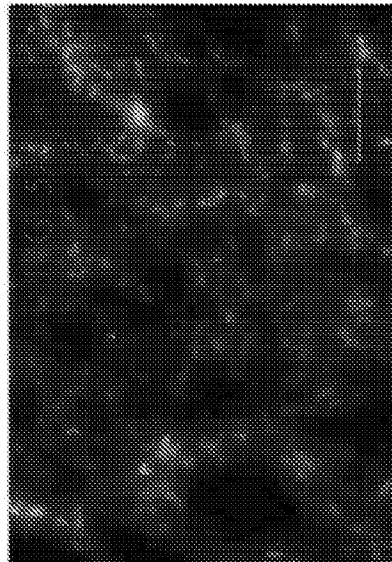


FIG. 5C

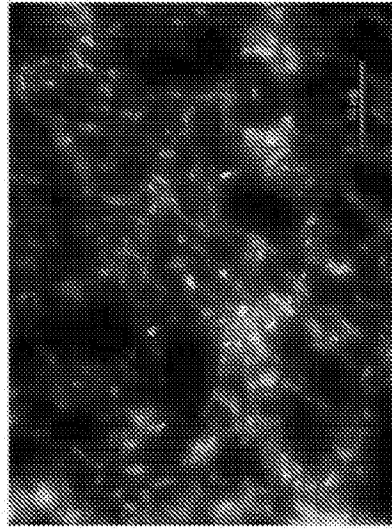
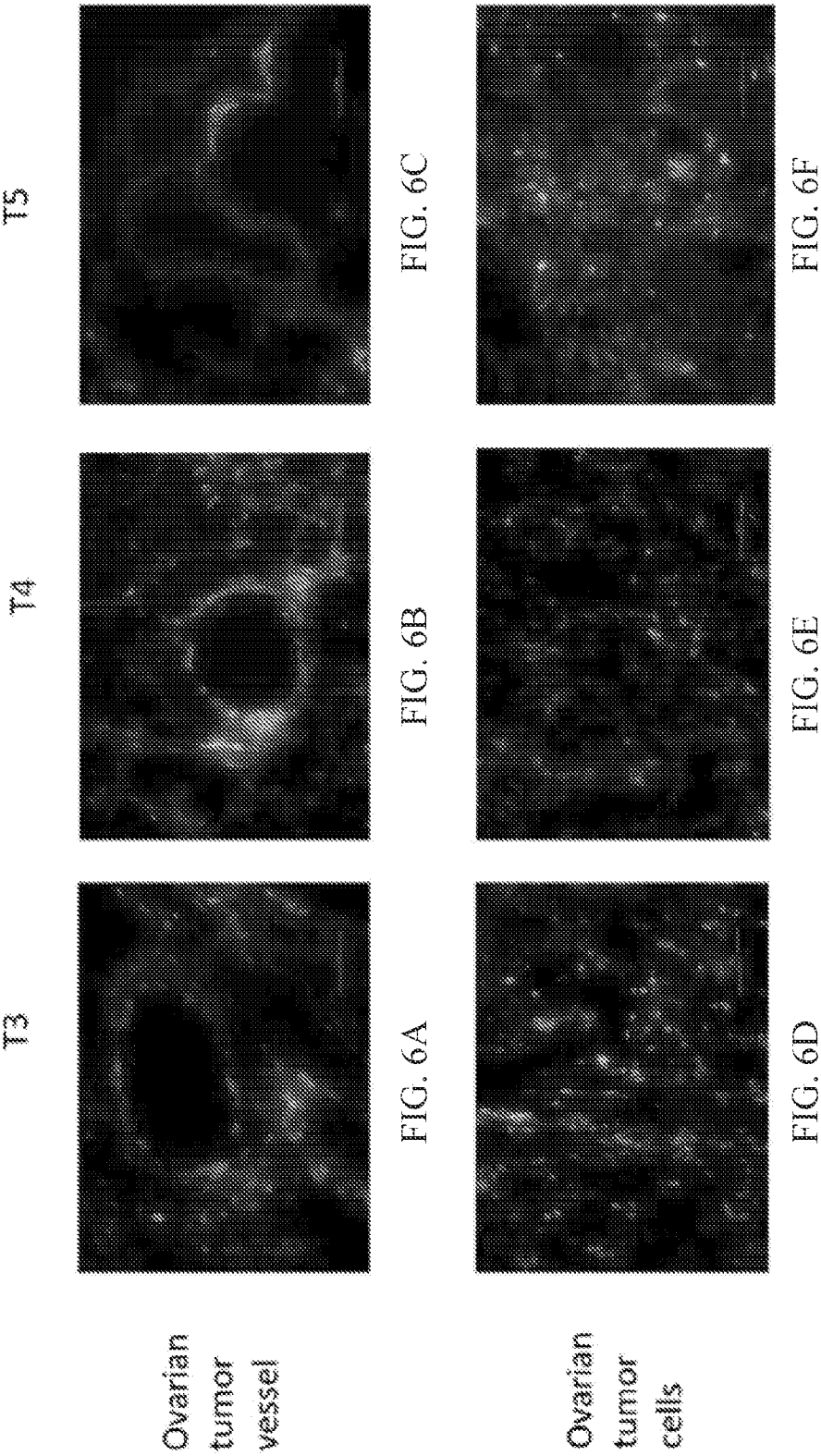
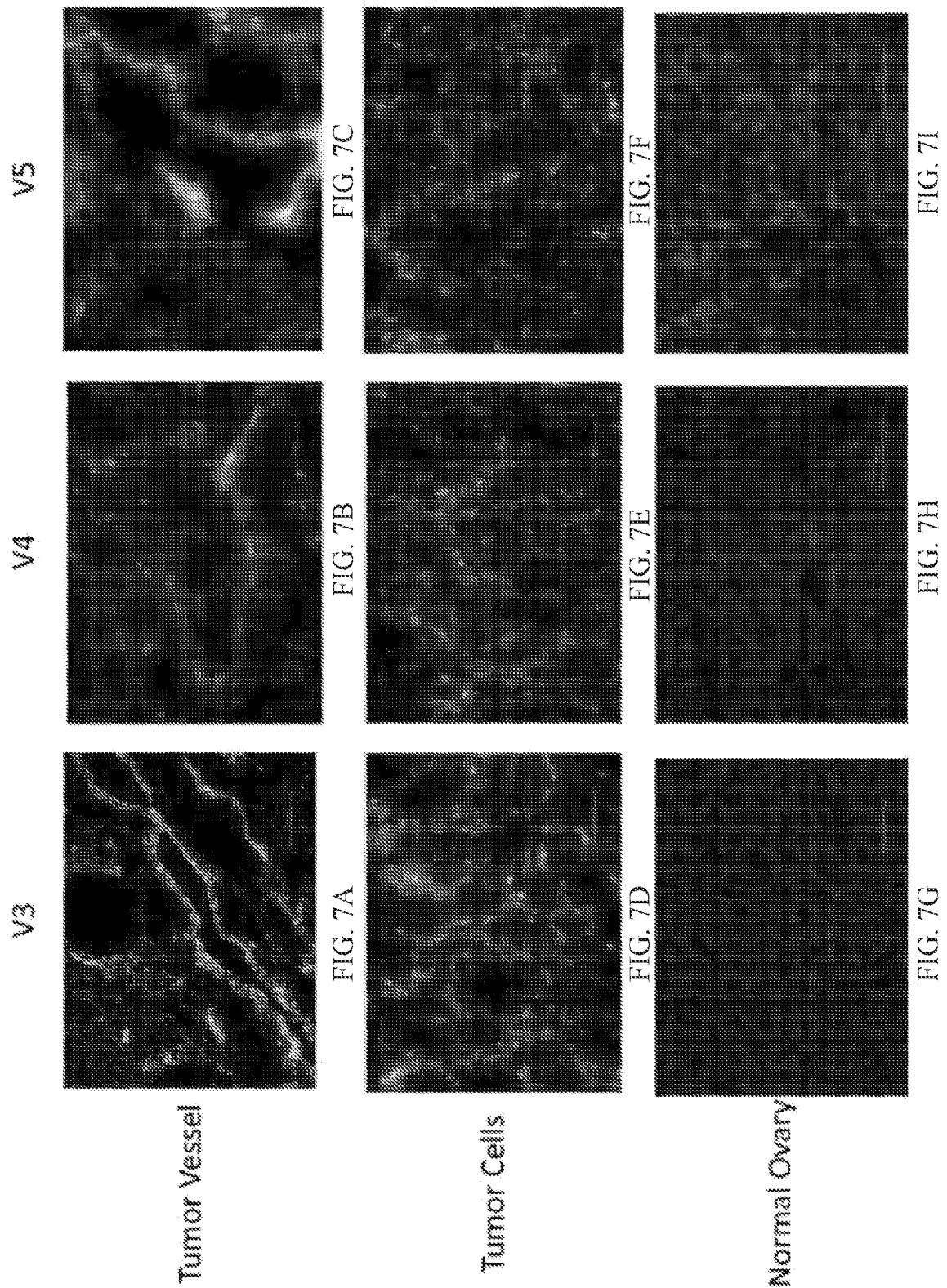
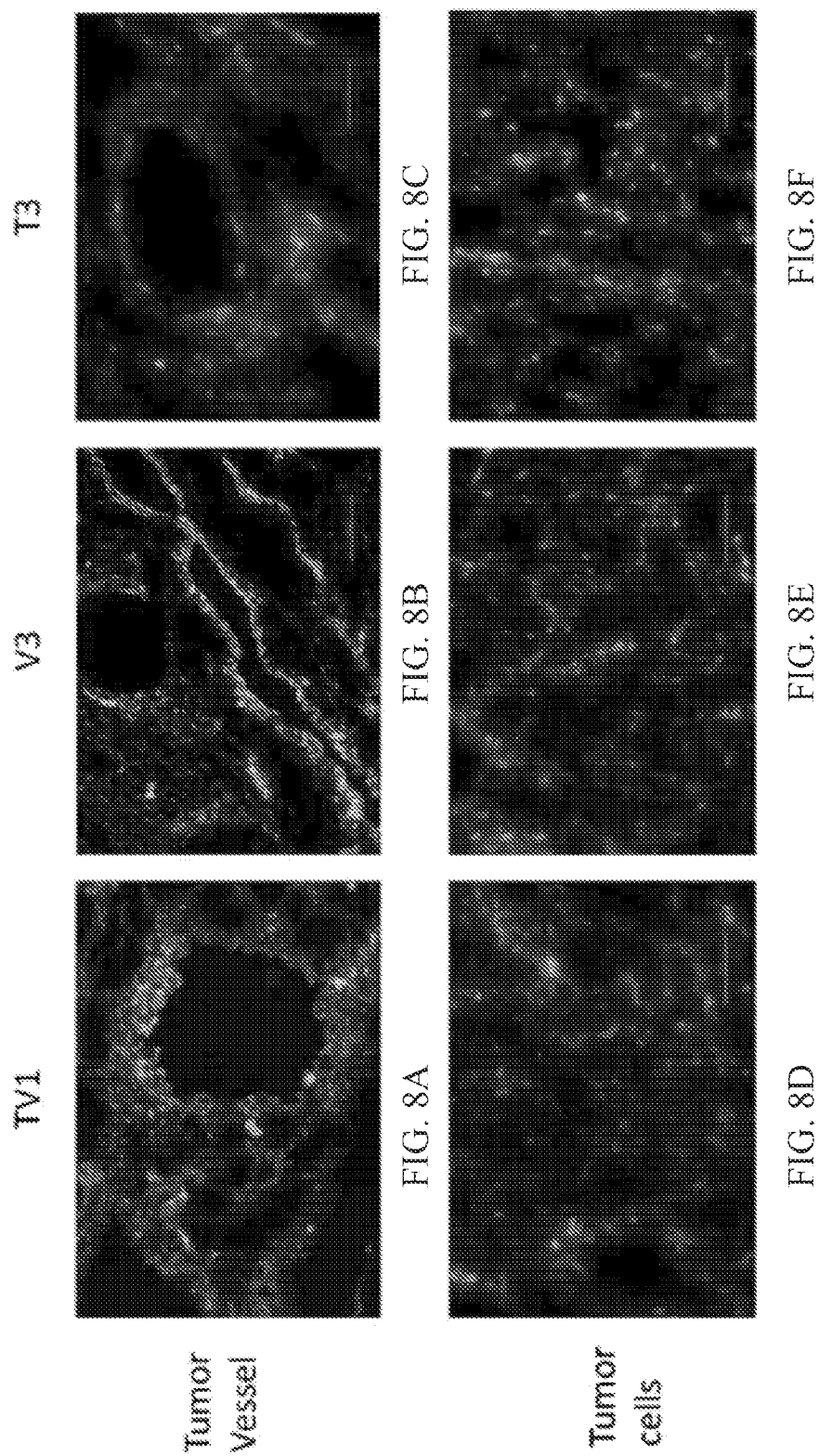


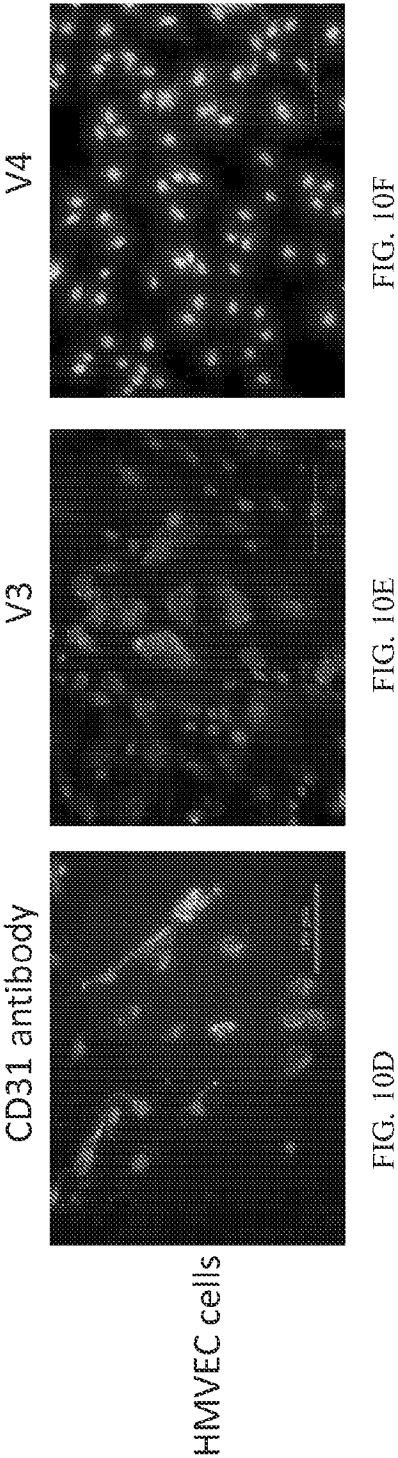
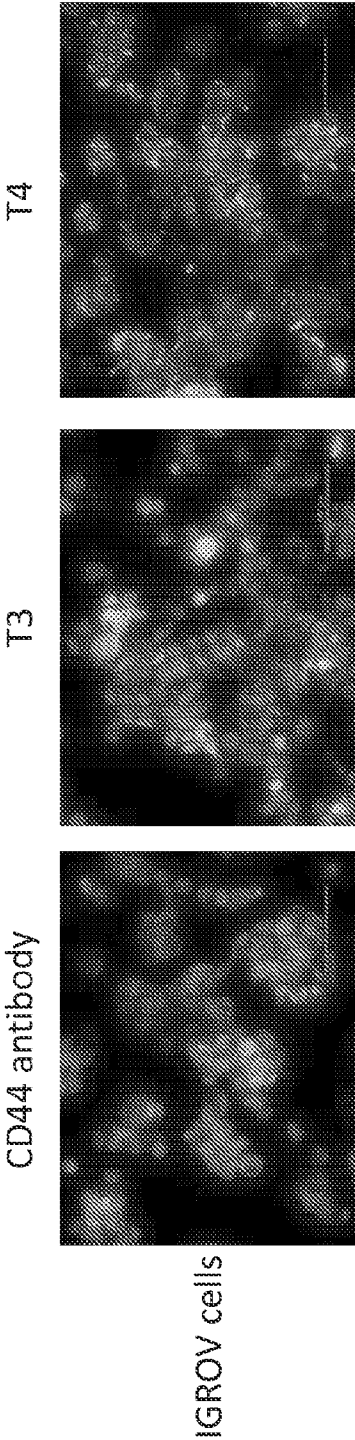
FIG. 5D

Ovarian tumor cells









INTERACTING PROTEIN	FUNCTION
TSGA10	Influences the function of antigen presenting cells (APC)
AptA (A. phagocytophilum toxin A)	Activates mammalian Erk1/2 mitogen-activated protein kinase
Scrib	Cell migration and aggregation
Rab9	Intracellular lipid transport
Caveolin-1	Anterior polarization of caveolin-1 in transmigrating cells
Plectin	Integration of cytoplasm
IFAP-300	Lens cell differentiation
hsc70	Regulation of heat-shock genes

INTERACTING PROTEIN	FUNCTION
Filamin	Formation of cell extensions
Alpha-crystallin	Enhanced vimentin aggregation
cGMP kinase	Vimentin phosphorylation
Yes kinase	Molecular support for Yes kinase
Desmoplakin	Links IFs with desmosomes
Periplakin	Mediates cellular localization
Actin-containing structures	Vimentin filament organization
hnRNP	Viral replication
Uridine phosphorylase	Function unknown
Formiminotransferase cyclodeaminase	Integrates Golgi complex with IF cytoskeleton

FIG. 11

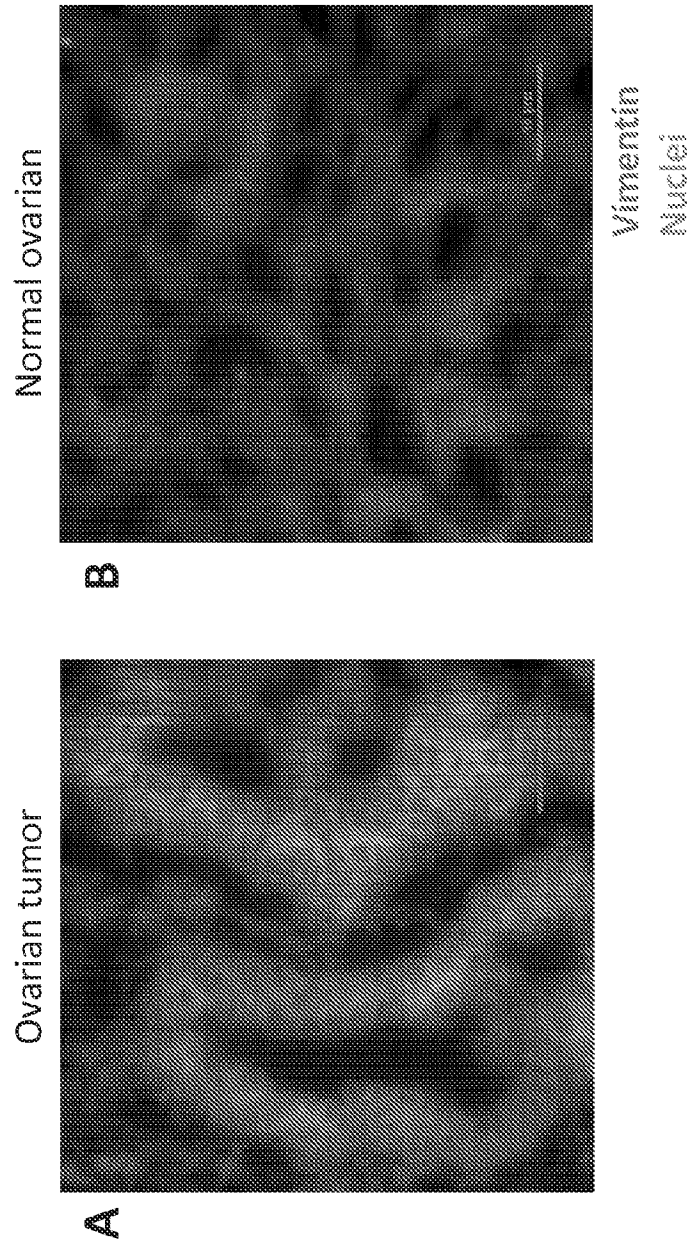


FIG. 12

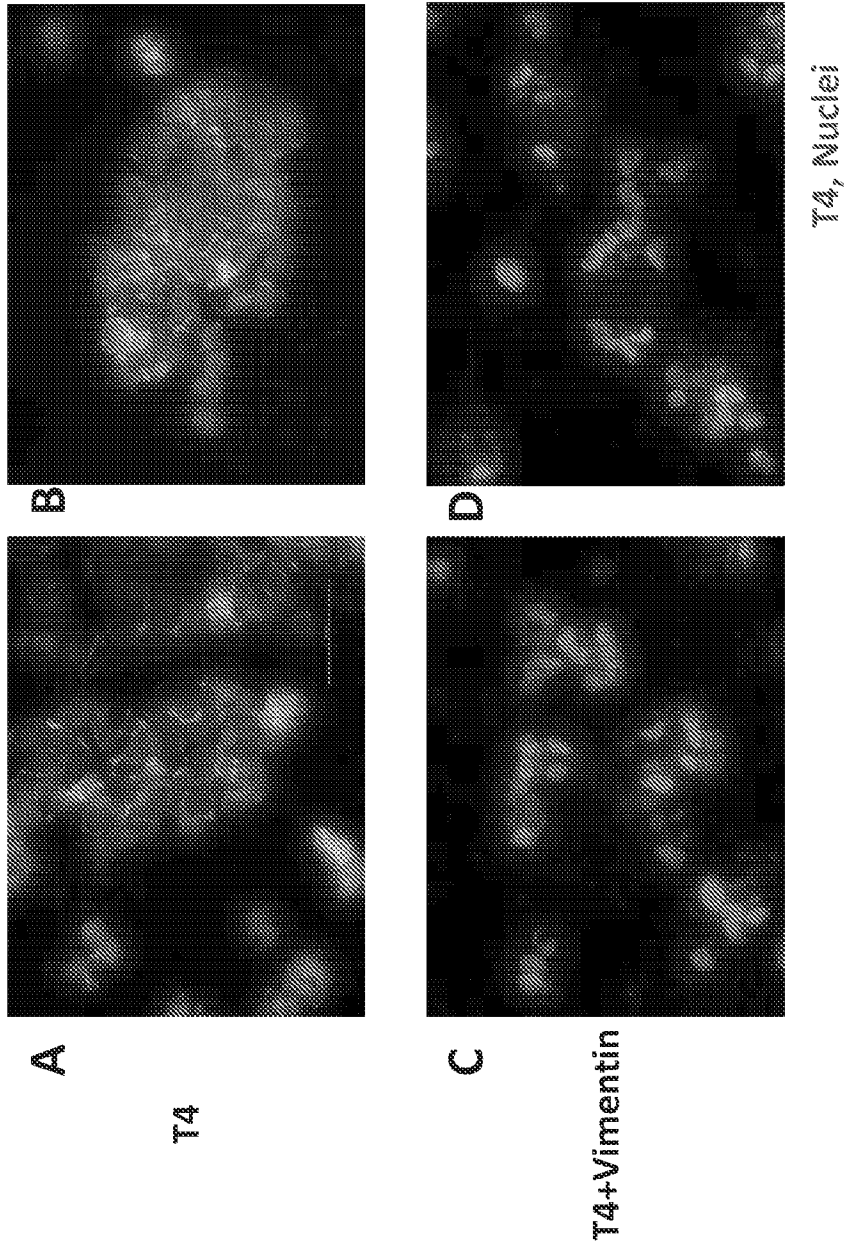


FIG. 13

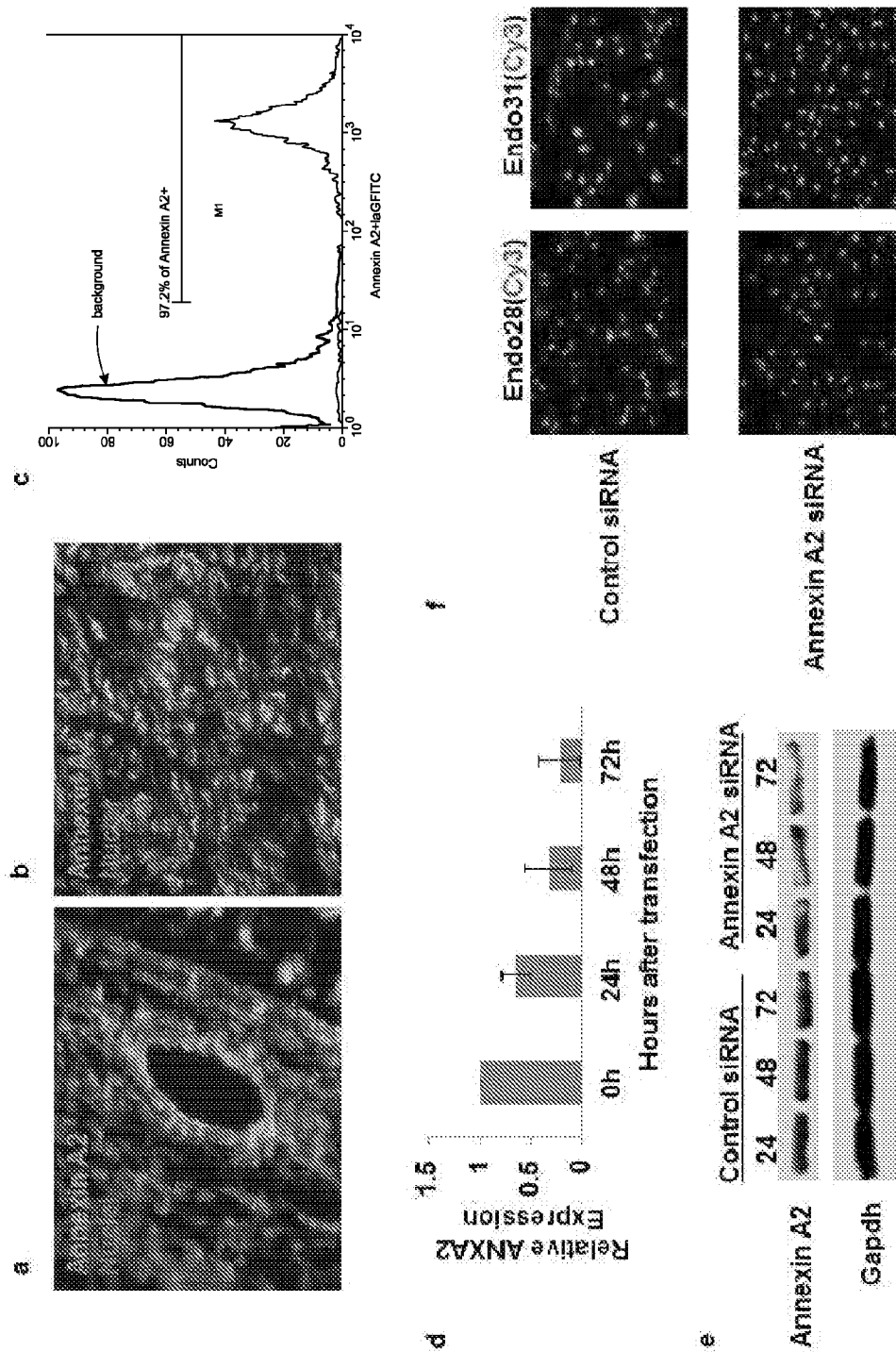


FIG. 14A-F

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2015/042515**A. CLASSIFICATION OF SUBJECT MATTER****C12Q 1/68(2006.01)i, C12N 15/115(2010.01)i, A61K 47/48(2006.01)i, A61P 35/00(2006.01)i**

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12Q 1/68; C12N 15/09; C07K 14/00; C40B 30/04; G01N 33/574; C12N 15/115; A61K 47/48; A61P 35/00

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Korean utility models and applications for utility models

Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

eKOMPASS(KIPO internal) & Keywords: aptamer selection, laser microdissection, cancer, sequencing, biomarker

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HANCOCK et al., (L-AU-0002) Abstracts of Current Investigations, In: The Robert A. Welch Foundation 57th Conference on Chemical Research, "Large problems in life chemistry", 28 October 2013, pp.114-115 See page 115.	1-6, 11, 22-24, 29, 40-49
Y		7, 15-21
A		14, 36, 39
X	US 2011-0124015 A1 (TAN et al.) 26 May 2011 See abstract; claims 1-6; and paragraphs [0018]-[0019].	14
Y		7, 15-21
A	JP 2009-195187 A (OLYMPUS CORP) 03 September 2009 See abstract; and claims 1-11.	1-7, 11, 14-24, 29, 36, 39-49
A	VAN SIMAEYS et al., 'Study of the molecular recognition of aptamers selected through ovarian cancer cell-SELEX' PLoS One, Vol.5, No.11, Article No.e13770 (internal pages 1-7) (2010) See abstract; pages 3-5; and Table 2.	1-7, 11, 14-24, 29, 36, 39-49
A	WO 2011-060557 A1 (GOVERNORS OF THE UNIVERSITY OF ALBERTA) 26 May 2011 See the whole document.	1-7, 11, 14-24, 29, 36, 39-49

☐ Further documents are listed in the continuation of Box C.☒ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

06 October 2015 (06.10.2015)

Date of mailing of the international search report

06 October 2015 (06.10.2015)

Name and mailing address of the ISA/KR

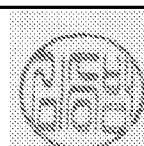
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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2015/042515

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 30-35, 50-52
because they relate to subject matter not required to be searched by this Authority, namely:
Claims 30-35 and 50-52 pertain to methods for treatment of the human body by therapy, and thus relate to a subject matter which this International Searching Authority is not required, under PCT Article 17(2)(a)(i) and PCT Rule 39.1(iv), to search.
2. ☒ Claims Nos.: 8-10, 38
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
Claim 8 refers to itself, and claims 9-10 depend on the claim 8. Therefore, claims 8-10 do not comply with PCT Article 6.
Claim 38 is worded in reference to the reporter agent of claim 16. However, said reporter agent has not been worded in claim 16. Therefore, claim 38 is not clear and concise contrary to PCT Article 6 (Claim 38 seems to refer to claim 37).
3. ☒ Claims Nos.: 12-13, 25-28, 37
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of any additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

Information on patent family members

PCT/US2015/042515

None