様式 C -19

科学研究費補助金研究成果報告書

平成 22 年 6 月 16 日現在

研究種目：基盤研究（C）
研究期間：2007～2009
課題番号：19591434
研究課題名（和文）
光と RI の二重標識物質により癌関連遺伝子の移動と局在をイメージングする方法の開発
研究課題名（英文）
Optical and Nuclear Imaging of Tumor-related genes
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研究成果の概要（和文）：
多剤耐性遺伝子、*mdr1* に関係している細胞と *mdr1* に対してアンチセンスシークエンスを有する ASODN:mdr、乳癌遺伝子、*HER2* に関係している乳がん細胞と *HER2* に対してアンチセンスシークエンスを有する ASODN:her を用いた。

1. 安定な《Tc-99m- ASODN:mdr -ナノ粒子》と《蛍光- ASODN:mdr -ナノ粒子》を作成することに成功し、いずれもが *mdr1* を過剰発現する腫瘍へ *in vitro*、*in vivo* の双方において、特異的に集積することを確認した。
2. 上記のナノ粒子を更に工夫して、《Tc-99m-ASODN:her-抗体-ナノ粒子》を作成した。これを担がんマウスに静脈投与して、乳がんの遺伝子 *HER2* の鮮明なイメージングを世界で初めて成功させた。

研究成果の概要（英文）：
We have developed the simple preparation of Tc-99m-labeled or fluorophore-labeled nanoparticle which was reactive to *mRNA* in the tumor specifically. Nanoparticle consisted of Antisense-oligonucleotides and streptavidin or biotin. We have also developed second-generation nanoparticles which consisted of Antisense-oligonucleotides and streptavidin or biotin and monoclonal antibody which recognized the tumor specific receptor. HER2, which over expressed in the breast tumor implanted in the thigh of mouse, was viewed very clearly when Tc-99m-labeled nanoparticle was injected intravenously.

交付決定額

（金額単位：円）

	直接経費	間接経費	合　計
2007 年度	1,600,000	480,000	2,080,000
2008 年度	1,200,000	360,000	1,560,000
2009 年度	700,000	210,000	910,000
年度			
年度			
総　計	3,500,000	1,050,000	4,550,000

研究分野：医歯薬学
科研費の分科・細目：内科系臨床医学・放射線科学
キーワード：放射線；遺伝子；トランスレーショナルリサーチ；イメージング；光生命科学

１．研究開始当初の背景

遺伝子のin vivoイメージングに関する研究は国内・外にて精力的に行われていたが、その殆どが遺伝子の発現物質であるたんぱく質、レセプターを対象としており、ミクロの腫瘍細胞を検出しているとは言いがたかった。mRNAの局在を扱ったものは当初、熾烈な競争を展開しており、臨床で汎用されているSPECT核種を用いてイメージングを研究しているグループとしては、研究代表者が最も優位の位置にいた。一方、蛍光標識物質を用いたin vivoイメージングは、研究手段として国内・外にて活発に行われていたが、その殆どが蛍光標識した細胞の投与であり、生体内の腫瘍関連遺伝子mRNAを直接標的としたものは、多くはなかった。研究代表者は、蛍光標識したASODNの投与によって、生体内腫瘍の当該のmRNAのイメージングに成功していた。

ASODNのTc-99m標識に関しては、特殊な技法と知識と経験を有する。研究代表者は海外共同研究者、Donald J Hnatowich（University of Massachusetts Medical School教授）の指導を受け、信頼のできるTc-99m標識ASODNを作成し、世界初のジーンイメージングに成功し、本課題の発想の原点となった。また、キャリヤー物質を有効に複合（コンジュゲート）させる方法を見出し、本手法の成功も本申請課題の立案の基となった。

２．研究の目的

本研究の目的は、担癌マウスをモデルとして、その原発巣からリンパ節への腫瘍関連遺伝子の移行経路を RI と光との双方の特性を利用して、in vivo にてイメージング（ジーンイメージング）することにある。当該の腫瘍に過剰発現する mRNA に対するアンチセンスシークエンスを有するオリゴヌクレオチド（ASODN）を放射性核種（RI）、Tc-99m、および蛍光にて二重標識する。これを担癌マウスの原発巣に局注して、癌からリンパ節への経路を Tc-99m にて追跡する。さらに、到達したリンパ節の局在を、蛍光にて同定する。即ち、癌から流れるリンパ管を通じてセンチネルリンパ節（SN）の存在を確認して、蛍光の集積の有無から、SN に腫瘍が転移しているかの判定を行う。最終的に、本方法を用いて、腫瘍に特有の遺伝子が存在するミクロの転移巣を非侵襲的に画像診断することで、当該の腫瘍の治療計画を精細に行うとする臨床診断へと繋げることを意図とした。

３．研究の方法

研究は；腫瘍特性と遺伝子情報の構築されている腫瘍細胞として多剤耐性遺伝子(*mdr1*)に関係している細胞（KB-31、KB-G2、TCO-1など）と、*mdr1* に対してアンチシークエンスを有する ASODN:mdr、乳癌遺伝子、*HER2* に関係している乳がん細胞（SK-BR-3 と MDA-MB-231 など）と *HER2* に対してアンチセンスシークエンスを有する ASODN:her を用いた。標識する核種は Tc-99m、In-111、I-125 を使用した。また、腫瘍細胞を植え付けたヌードマウスを *in vivo* のモデルとして使用した。

４．研究成果

研究成果はいずれも、英語論文として発表されており、その概要を以下に示す。

1. **Simplified preparation via streptavidin of antisense oligomers/carriers nanoparticles showing improved cellular delivery in culture**

METHODS: The model carriers were cholesterol, a 10 mer HIV-tat peptide, and a 10 mer polyarginine, each having been reported elsewhere to improve cellular delivery of oligomers. The model antisense oligomer was the 25 mer MORF targeting the survivin mRNA. The accumulations of the antisense MORF/carrier nanoparticle were compared to the sense MORF/carrier, to the carrier-free nanoparticles, and to the naked antisense MORF in the survivin-expressing MCF-7 cells. The MORFs and peptides were purchased biotinylated, while the cholesterol was biotinylated in-house. In all cases, the ^{99m}Tc radiolabel was placed on the oligomers. Cell studies were performed at low nM concentration as required for antisense imaging applications and at 37ºC primarily in 1% FBS. RESULTS: Each radiolabeled oligomer/streptavidin/carrier nanoparticle was successfully prepared by careful mixing at a 1:1 molar ratio. As evidence of carrier participation, the radiolabeled MORF showed increased accumulation in cells when incubated as the nanoparticle compared to the carrier-free nanoparticle and by as much as a factor of 11. Accumulation

of the antisense MORF/streptavidin/tat nanoparticle was significantly higher than the sense MORF/streptavidin/tat nanoparticle as evidence of specific antisense targeting. CONCLUSIONS: The preparation of oligomer/carrier nanoparticles was greatly simplified over covalent conjugations by using streptavidin as a linker. Furthermore, our results suggest that the addition of streptavidin did not interfere with the cellular delivery function of the tat, polyarginine, or cholesterol carriers nor with the specific antisense mRNA binding function of the MORF oligomer.

2. Optical antisense imaging of tumor with fluorescent DNA duplexes

Antisense targeting of tumor with fluorescent conjugated DNA oligomers has the potential of improving tumor/normal tissue ratios over that achievable by nuclear antisense imaging. When administered as a linear duplex of two fluorophore-conjugated oligomers arranged in a manner that inhibits fluorescence as the duplex and designed to dissociate only in the presence of the target mRNA, the fluorescence signal should in principle be inhibited everywhere except in the target cell. Optical imaging by fluorescence quenching using linear fluorophore-conjugated oligomers has not been extensively investigated and may not have been previously considered for antisense targeting. We evaluated in cell culture and in KB-G2 tumor bearing nude mice a 25-mer phosphorothioate (PS) anti-mdr1 antisense DNA conjugated with the Cy5.5 emitter on its 3' equivalent end and hybridized as a linear duplex with a shorter 18-mer phosphodiester (PO) complementary DNA (cDNA) with the Black Hole inhibitor BHQ3 on its 5' end. In serum environments, 90% of the DNA25-Cy5.5 fluorescence was inhibited immediately following addition of the cDNA18-BHQ3 and showed only slight loss of inhibition over 24 h at 37ºC. As evidence of antisense specific binding, when incubated with the DNA25-Cy5.5/cDNA18-BHQ3 duplex, the fluorescence was lower in KB-31 (Pgp+/-) cells compared to KB-G2 (Pgp++) cells, but when incubated with the control cDNA18-Cy5.5/-DNA25-BHQ3 duplex in which the fluorophores were reversed, the fluorescence of both cell types was low. As further evidence of specific binding, the fluorescent intensity of total RNA from KB-G2 cells incubated with the study duplex showed evidence of dissociation and hybridization with the target mRNA. Furthermore, the fluorescence microscopy images of KB-G2 cells incubated with DNA25-Cy5.5 as the singlet or study duplex show that migration in both cases is to the nucleus. The animal studies were performed in mice bearing KB-G2 tumor in one thigh and receiving iv the study or control duplexes. The tumor/normal thigh fluorescence ratio was clearly positive as early as 30 min post injection in the study mice and reached a maximum at 5 h. By contrast, much lower fluorescence was observed in mice receiving the control duplex at the same dosage. Fluorescence microscope imaging showed that the Cy5.5 fluorescence was much higher in tumor sections from the animal that had received the study rather than control duplex. Thus combining a fluorophore conjugated antisense DNA with an inhibitor conjugated shorter complementary cDNA inhibited fluorescence both in cell culture and in tumored animals except in the presence of the target mRNA. This proof of concept investigation of optical antisense targeting therefore suggests that further studies including optimization of this approach are appropriate.

3. Cell culture and xenograft-bearing animal studies of radiolabeled antisense DNA carrier nanoparticles with streptavidin as a linker

METHODS: The 3 carriers were cholesterol, a 10-mer Tat peptide, and a 10-mer polyarginine peptide. A 20-mer DNA targeting the mdr1 messenger RNA coding for Pgp expression was used as the phosphodiester (PO) DNA as well as the phosphorothioate (PS) DNA. In all cases, the ^{99m}Tc radiolabel was on the DNA. The 8 nanoparticles were first tested in mdr1(++) KB-G2 and TCO-1 cells and in mdr1(+/-) KB-31 cells in culture for evidence of improved accumulation and antisense targeting. Thereafter, the PSDNA-streptavidin-Tat, PODNA-streptavidin--Tat, and PS DNA-streptavidin-cholesterol nanoparticles were administered intravenously to KB-G2 xenograft-bearing mice, and tissue distributions were measured. RESULTS: In culture, the PO nanoparticles showed increased accumulation compared with the corresponding nanopar-

ticles without the carrier in all 3 cell types; in contrast, with the PS nanoparticles, any similar carrier-mediated increase may have been obscured by the much higher protein-binding affinity of PS DNA. As evidence of antisense targeting, the Tat and cholesterol PS nanoparticles showed statistically significant accumulation at 23 h in cells in the descending order TCO-1, KB-G2, and KB-31, although there were no significant differences among the PO nanoparticles. In xenograft-bearing mice, the tissue accumulation of both forms of the PS nanoparticles greatly exceeded that of the PO nanoparticles and, including in the tumor, were similar to that obtained previously for naked PS DNA. CONCLUSION: The presence of the streptavidin linker had no obvious detrimental effect on the functions of the carriers and antisense DNAs. The higher protein-binding affinity of the PS nanoparticles than the PO nanoparticles was still apparent both in vitro and in vivo, the pharmacokinetics of the PS nanoparticles were similar to that of naked PS DNA, and the carriers improved cellular accumulation, at least for the PO nanoparticles. These observations, taken together with the higher accumulation of both forms of the antisense PS nanoparticles in mdr1(++) KB-G2 and TCO-1 cells than in mdr1(+/-) KB-31 cells, suggest that further effort is justified to confirm that the antisense properties of the DNAs were not compromised by the presence of streptavidin.

4. Cationic transfectors increase accumulation in cultured tumor cells of radiolabeled antisense DNAs without entrapment

While cellular accumulations in culture of oligomers, such as interfering RNA and antisense DNA, are reported to benefit from the addition of transmembrane transfectors (TFs), the extent to which individual TFs improve cellular delivery is usually inferred and rarely measured. The goal of this investigation was to use radioactivity to measure in cells in culture the degree to which accumulations of DNA increased when complexed with TFs and without DNA entrapment in vesicles. The antisense (AS) DNA targeting mdr1 mRNA coding for P-glycoprotein (Pgp) and its sense (S) complement DNA were radiolabeled with ^{99m}Tc and mixed with jetPEI, Chariot, or Neophectin over a range of TF/DNA ratios. Thereafter, the radiolabeled DNAs with and without TFs were incubated with KB-G2 (mdr1(+/+)) and KB-31 (mdr(+/-))cells at 37ºC in serum or serum-free media for 20-24 hours at a fixed DNA concentration of 13 nM. Cellular accumulations were increased under most incubation conditions and by as much as threefold with jetPEI and eightfold with Neophectin. As evidence against entrapment, the accumulations of ASDNAs were higher than SDNAs in virtually all measurements and higher in virtually all accumulations in the mdr1(+/+) cells, compared to the mdr1(+/-) cells. In conclusion, by using radiolabeled DNAs, definitive evidence was obtained showing that the addition of Neophectin and jet PEI increased cellular accumulations of both AS and S DNA without evidence of vesicle entrapment.

5. Cell studies of a three-component antisense MORF/tat/Herceptin nanoparticle designed for improved tumor delivery

The three-component nanoparticle of this investigation consisted of an anti-type I regulatory subunit alpha of the cyclic AMP-dependent protein kinase A (RIα) antisense phosphorodiamidate morpholino (MORF) oligomer, a tat peptide and the anti-HER2 Herceptin antibody each biotinylated and each linked via streptavidin and tested in SUM190 (HER2+), SUM149 (HER2-) and SK-BR-3 (HER2+) cells in culture, using both radioactivity and fluorescent labels on the antisense and control sense MORF. Within the nanoparticle, the antibody provides specific binding to the target cells, the tat improves cellular delivery and the MORF provides the specific retention of the radioactivity in the target cell nucleus. The results show that within the nanoparticle, the Herceptin was still able to bind to its determinant; that the MORF escaped entrapment with its mRNA-binding ability preserved and that the tat maintained its carrier function. Fluorescence microscopy showed evidence of antisense MORF internalization, separation from Herceptin and migration to the nucleus. In conclusion, streptavidin appears to provide an easy means of mixing and matching components to improve the tumor-specific targeting, cell membrane transport, pharmacokinetics and other properties of antisense and other oligomers. Combining the three

components of this investigation with streptavidin apparently did not interfere with the properties of each component in cell culture and significantly improved delivery.

6. **Auger radiation-induced, antisense mediated cytotoxicity of tumor cells using a 3 component streptavidin delivery nanoparticle with ^{111}In**

METHODS: Our nanoparticle consists of streptavidin that links 3 biotinylated components: the antiHer2 antibody trastuzumab (to improve pharmacokinetics), the tat peptide (to improve cell membrane transport), and the ^{111}In-labeled antiRIα messenger RNA antisense morpholino (MORF) oligomer. RESULTS: As evidence of unimpaired function, tumor cell and nuclear accumulations were orders of magnitude higher after incubation with ^{99m}Tc-MORF/tat/trastuzumab than after incubation with free ^{99m}Tc-MORF and significantly higher with the antisense than with the sense MORF. In mice, tumor and normal-tissue accumulations of the ^{99m}Tc-MORF/-tat/trastuzumab nanoparticle were comparable to those of free ^{99m}Tc-trastuzumab, confirming the improved pharmacokinetics due to the trastuzumab component. Although kidneys, liver, and other normal tissues also accumulated the nanoparticle, immunohistochemical evaluation of tissue sections in mice receiving the Cy3-MORF/-tat/trastuzumab nanoparticle showed evidence of nuclear accumulation only in tumor tissue. In a dose escalation study, as measured by the surviving fraction, the nanoparticle significantly increased the kill of SK-BR-3 breast cancer Her2+/RIalpha+ cells, compared with all controls. CONCLUSION: Significant radiation induced antisense mediated cytotoxicity of tumor cells in vitro was achieved using an Auger electron emitting antisense MORF oligomer administered as a member of a 3 component streptavidin delivery nanoparticle.

7. **A convenient thiazole orange fluorescence assay for the evaluation of DNA duplex hybridization stability**

When study duplexes shown to be stable in serum were incubated with their target cells, the assay successfully detected evidence of dissociation, most likely by an antisense mechanism. Thus, a TO fluorescence assay has been developed that is capable of detecting the dissociation of DNA duplexes.

8. **Optical antisense tumor targeting in vivo with an improved fluorescent DNA duplex probe**

Fluorescent conjugated DNA oligonucleotides for antisense targeting of mRNA has the potential of improving tumor/normal tissue ratios over that achievable by nuclear antisense imaging. By conjugating the Cy5.5 emitter to the 3' equivalent end of a 25 mer phosphorothioate (PS) antisense major DNA and hybridizing with a shorter 18 mer phosphodiester (PO) complementary minor DNA (cDNA) with the Black Hole inhibitor BHQ3 on its 5' end, we previously achieved antisense optical imaging in mice as a proof of this concept. In a process of optimization, we have now evaluated the stability of a small series of duplexes with variable-length minor strands. From these results, a new study anti-mdr1 antisense duplex was selected with a 10 mer minor strand. The new study duplex shows stability in serum environments at 37ºC and provides a dramatically enhanced fluorescence in KB-G2 (Pgp++) cells when compared with KB-31 (Pgp+/-) as evidence of antisense dissociation at its mdr1 mRNA target. The duplex was also administered to KB-G2 tumor bearing mice, and when compared to the duplex used previously, the fluorescence from the tumor thigh was more obvious and the tumor-to-background fluorescence ratio was improved. In conclusion, by a process designed to optimize the duplex for optical antisense tumor targeting, the fluorescence signal was improved both in cells and in tumored mice.

9. **In vivo delivery of antisense MORF oligomer by MORF/carrier streptavidin nanoparticles**

Tumor targeting by oligomers is largely limited by the pharmacokinetics and cell-membrane transport obstacles. In this article, we describe the use of a delivery nanoparticle, in which streptavidin served as a convenient bridge between a biotinylated oligomer and a biotinylated cell-membrane-penetrating peptide, to improve the delivery of an antisense phosphorodiamidate morpholino (MORF) oligomer in vivo. A biotinylated ^{99m}Tc-radiolabeled MORF oligomer with

a base sequence antisense to the RIα mRNA and its sense control were incorporated separately into nanoparticles, along with biotinylated tat or polyarginine carrier. The streptavidin nanoparticles were administrated intravenously to both normal and nude mice bearing SUM149 breast tumor xenografts. The biodistributions showed much higher normal tissue levels for the radiolabeled MORFs, independent of antisense or sense or tat or polyarginine, when administered as the nanoparticles, compared to naked. A statistically significant higher accumulation of both antisense nanoparticles, compared to the respective sense control nanoparticles, was observed, along with much higher tumor accumulations, compared to historical naked controls. This study has provided evidence that the in vivo function of an antisense oligomer within the streptavidin nanoparticle is not impeded, and, as such, the MORF/streptavidin/carrier nanoparticles may be suitable for in vivo tumor delivery of antisense MORF and other oligomers.

５．主な発表論文等

（研究代表者、研究分担者及び連携研究者には下線）

〔雑誌論文〕（計 12 件）全て査読有り

以下代表論文

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4. Liu X, Wang Y, Nakamura K, et al; Auger radiation-induced, antisense- -mediated cytotoxicity of tumor cells using a 3-component streptavidin delivery nanoparticle with ^{111}In. J Nucl Med. 2009; 50(4):582-590.
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〔学会発表〕（計 9 件）：以下招待講演のみ

1. **中村佳代子；シンポジウム『悪性リンパ腫の診断と治療－核医学の役割－』Y-90抗CD20抗体の基礎。第48回日本核医学会学術総会；2008年10月24日～26日；幕張**
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3. D. Hnatowich and K. Nakamura; The Evolution of Molecular Imaging Radiopharmaceuticals. **第47回日本核医学会学術総会；2007年11月4日～6日；仙台**

〔図書〕（計 0 件）

〔産業財産権〕

○出願状況（計 0 件）

○取得状況（計０件）

６．研究組織

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