厚生労働科学研究費補助金

がん臨床研究事業

オピオイド治療効果に対する実測可能な薬理学的効果予測システムORPSの開発

平成22年度～24年度　総合研究報告書

研究代表者　中　川　和　彦

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別添１

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オピオイド治療効果に対する実測可能な薬理学的効果予測ｼｽﾃﾑORPSの開発

平成２２年度～２４年度　総合研究報告書

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平成２５（２０１３）年　３月

別紙２

目　　次

別紙３

厚生労働科学研究費補助金（がん臨床研究事業）
総合研究報告書

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研究要旨　本研究の三年度目では、前向き臨床試験に対するモルヒネ薬物代謝関連SNPs解析において、*CYP3A4, UGT1A3, ABCC3, ABCB1, COMT*のSNPsがモルヒネ必要量及び有害事象と関連を有することを示した。COMT以外のSNPsは新規の候補SNPsであり有望である。また、モルヒネ必要量と一部の血中サイトカイン濃度（IL-8, G-CSF, IL-1α, IL-6, IL-10, IP-10）の相関を特定した。本研究の結果を臨床応用することによって、将来のがん性疼痛の個別化医療に貢献できると考える。

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A．研究目的

がん性疼痛へのオピオイド治療に対して、治療効果の指標およびモニタリングできる実測可能な薬理学的バイオマーカーの開発し、実測可能な薬理学的効果予測システムORPS（Opioid treatment Response Prediction System)の開発を通じて、がん性疼痛の定量化システムに相補的に寄与することを目的とする。　前向き臨床試験においてがん性疼痛へのオピオイド治療を受ける患者を対象に臨床的・定量的エンドポイントと各種薬理学的バイオマーカー候補分子との相関を統合的に検討し、実測可能な薬理学的バイオマーカーを得る。

B．研究方法

＜臨床試験の続行＞

前向き臨床試験において、速放性モルヒネ製剤によるタイトレーションを行った後、定期投与としてモルヒネ必要用量を投与する。オピオイド治療は通常の治療指針に従って行う。モルヒネ治療前、治療後1日目、治療後8日目に採血、NRS（Numeric Rating Scale)、心理テストおよびQOL評価尺度などを施行する。末梢血は血漿分離およびDNA・RNA用に専用採血管で保存する。

収集する臨床情報は、次の項目である（年齢、性別、PS、疼痛部位、癌種、TNM分類、投薬内容、NSAIDsの種類、量、モルヒネ投与量、オピオイドと相互作用を起こす薬剤の投与の有無、除痛に関連し得る鎮痛補助薬の種類、量、モルヒネの必要用量、治療抵抗性の有無）。

＜臨床検体の測定＞

薬理学的バイオマーカーとしては、①マイクロアレイを用いた薬力学的効果関連遺伝子の特定、②モルヒネ関連代謝酵素の遺伝子多型、③モルヒネ血中濃度、④血中サイトカイン濃度を実施する。モルヒネ関連遺伝子の遺伝子多型は、ゲノムDNAを試料として測定する。通常使用されるdirect sequence法を用い、PCR法で当該遺伝子の任意のDNA配列を増幅し、シークエンス機器で塩基配列を同定する。モルヒネ薬物代謝の代謝経路にフォーカスを絞ったSNPs解析については、Affymetrix社のDMETチップ（薬物代謝関連231遺伝子上の1936個のSNPsを対象）を用いてSNPs解析を行う。マイクロアレイは、末梢血白血球を対象にオピオイド治療前後で変動する遺伝子および、治療抵抗性に関連する遺伝子を特定する際に用いる。Affymetrix 社製のGeneChip HG-U133 Plus2.0 array を用いる。血中サイトカイン測定はBioplex systemを用いて測定する。モルヒネ血中濃度測定は、ボンドエルートC18カートリッジカラム、高速液体クロマトグラフィーを用いて血漿中のモルヒネ濃度を測定する。

［研究体制］

研究代表者は研究の統括・計画・測定・解析を実施する。研究分担者は近畿大学医学部腫瘍内科・近畿大学医学部堺病院の2施設において症例集積を行う。バイオマーカーの測定は近畿大学医学部ゲノム生物学教室が行なう。近畿大学総合社会学部心理学科は、心理テストについての評価を行う。大阪府立大学看護学部は、QOL調査票についての評価を行う。解析は九州がんセンター腫瘍統計学部門が行う。

（倫理面への配慮）

本研究による身体的な危険性は採血のみでありきわめて少ない。本研究に用いるゲノムDNA遺伝子多型の検出はモルヒネの代謝および薬理作用に関連した遺伝子に制限して解析を行う。本研究では、検体提供

者に登録前に同意説明文書・同意書に基づき、本研究の意義、目的、方法、予測される結果や不利益について説明し、文書により自由意思による検体提供者の同意を得る。
本研究のプロトコール「研究名：がん性疼痛へのモルヒネ治療に対する治療効果および薬力学的効果に関する探索的研究」は近畿大学医学部・近畿大学医学部堺病院の２施設の倫理委員会で承認を得ている（平成21年6月5日承認）。
個人情報は個人情報管理者により連結可能匿名化され、厳重に管理される。連結した遺伝子情報が第三者に渡ることはない。本研究では、３省合同「ヒトゲノム・遺伝子解析研究に関する倫理指針」を遵守する。各臨床試験の実施にあたっては「ヘルシンキ宣言」「臨床試験に関する倫理指針」「個人情報保護法」「ヒトゲノム・遺伝子解析研究に関する倫理指針」など関連の指針や法律・省令・告示等に従う。

C．研究結果
【前向き臨床試験の実施】
3年間で予定通り前期臨床試験50症例および後期臨床試験50症例の計100症例の登録と、100例x 3ポイント(治療前・Day 1・Day 8)の検体採取がすべて終了した。
【SNPs解析（DMETチップ)】
モルヒネ薬物代謝の代謝経路にフォーカスを絞ったSNPs解析を行うために、Affymetrix社のDMETチップ（薬物代謝関連231遺伝子上の1936個のSNPsを対象）を用いてSNPsとモルヒネ治療効果との相関解析を行った。Day 1のモルヒネ必要量に有意に（$p<0.05$）関連するSNPsを17個特定した。その中で疼痛の個人差に関連することが知られているSNP である*COMT-V108M*（rs4680, p=0.02）は、本研究で既にdirect sequence法により報告したSNPで、DMETチップでも再現性が確認された。Day 8のモルヒネ必要量に関連するSNPsは16個特定され、モルヒネをN脱メチル化する*CYP3A4*（rs2242480, p=0.03）、活性は弱いがモルヒネをグルクロン酸抱合する*UGT1A3*（rs7574296, p=0.04）のSNPsが選択されていた。Day 1の治療後の疼痛スコアにおいては、*COMT-V108M*（rs4680）が有意であった。一方、興味深いことにモルヒネの「眠気」の有害事象出現に関連する解析においては、モルヒネを基質とすることトランスポーターである*ABCC3/MRP3*（rs4148416, p=0.0002)、*ABCB1/MDR1*（rs2235013, rs2235033, rs1128503, rs10276036, p=0.02）などが同定された。まとめると、今回特定された*COMT, CYP3A4, UGT1A3, ABCC3, ABCB1*のモルヒネ薬物代謝関連SNPsは、COMT以外は新規のモルヒネ効果予測バイオマーカーの候補SNPsであることが示された。この結果は前期臨床試験50症例に基づくものであり、検証症例である独立した後期臨床試験50症例で結果を検証する予定である。
【サイトカイン解析】
前期臨床試験と同じ方法で26分子の血中サイトカイン測定研究の検証を行った。独立した後期臨床試験において、評価可能な49症例を対象にした。モルヒネ治療前後変化の検討では、3分子が治療後に有意に変動した。治療前血漿を試料とするモルヒネ必要量に対する検討では、6分子（IL-8, G-CSF, IL-1α, IL-6, IL-10, IP-10）の血中濃度が高容量のモルヒネ必要群で有意に低い結果が得られた。いくつかの分子は前回との結果に再現性が得られている。血中サイトカイン評価は、採血によるモルヒネ必要量の新しい予測方法として有望な結果と考えられる。

D．考察
モルヒネ薬物代謝関連SNPsについては、*CYP3A4, UGT1A3, ABCC3, ABCB1, COMT*のSNPsは、新しいモルヒネ効果予測バイオマーカーである可能性を示した。一部の血中サイトカイン濃度とモルヒネ必要量の関連に対して、独立サンプルで再現性が確認された。本研究の結果は、将来のがん性疼痛の個別化医療に貢献できる可能性があると考える。

E．結論
本研究では、採血によるモルヒネ治療効果予測の新規候補因子を多数特定した。がん性疼痛の定量化システムに相補的に寄与可能な分子の選定およびプロトタイプの「薬理学的バイオマーカーによるオピオイド治療効果予測システム」を構築が、近日中に可能と考えている。

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G．知的財産権の出願・登録状況

1. 特許取得

なし

2. 実用新案登録

なし

3.その他

なし

別紙４

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
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Amplification of GNAS may be an independent, qualitative, and reproducible biomarker to predict progression-free survival in epithelial ovarian cancer

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ABSTRACT

Objectives. The purpose of this study was to identify genes that predict progression-free survival (PFS) in advanced epithelial ovarian cancer (aEOC) receiving standard therapy.

Methods. We performed microarray analysis on laser microdissected aEOC cells. All cases received staging laparotomy and adjuvant chemotherapy (carboplatin + paclitaxel) as primary therapy.

Results. Microarray analysis identified 50 genes differentially expressed between tumors of patients with no evidence of disease (NED) or evidence of disease (ED) ($p<0.001$). Six genes (13%) were located at 8q24, and 9 genes (19.6%), at 20q11–13. The ratio of selected gene set/analyzed gene set in chromosomes 8 and 20 are significantly higher than that in other chromosome regions (6/606 vs. 32/13656, $p=0.01$) and (12/383 vs. 32/13656, $p=1.3\times10^{-16}$). We speculate that the abnormal chromosomal distribution is due to genomic alteration and that these genes may play an important role in aEOC and choose GNAS (GNAS complex locus, NM_000516) on 20q13 based on the p value and fold change. Genomic PCR of aEOC cells also showed that amplification of GNAS was significantly correlated with unfavorable PFS ($p=0.011$). Real-time quantitative RT-PCR analysis of independent samples revealed that high mRNA expression levels of the GNAS genes, located at chromosome 20q13, was significantly unfavorable indicators of progression-free survival (PFS). Finally, GNAS amplification was an independent prognostic factor for PFS.

Conclusions. Our results suggest that GNAS gene amplification may be an independent, qualitative, and reproducible biomarker of PFS in aEOC.

Introduction

Epithelial ovarian cancer (EOC) remains the most common cause of cancer death in women and the leading cause of death from gynecologic cancer. Early diagnosis of EOC is extremely difficult because most patients with early-stage disease are asymptomatic, so that 80% of patients present with advanced disease. Standard therapy includes surgical procedures (bilateral adnexectomy + hysterectomy + greater omentectomy) with staging laparotomy, debulking surgery, and postoperative chemotherapy using a combination of platinum and taxane. In 70% of advanced EOC (aEOC) patients, complete clinical responses are achieved; however, tumor recurs in most patients within 1 to 2 years after diagnosis and death is due to the development of chemotherapy resistance. In contrast, small numbers of patients with aEOC are cured by standard therapy. Although several clinical features are associated with poor prognosis, including poor performance status, suboptimal debulking surgery, clear cell or mucinous histology, high histologic grade, old age, or slow decrease in serum CA125 during adjuvant chemotherapy, reliable predictive biomarkers for aEOC are still lacking. If such markers could be established, patients who are likely to relapse and die of disease might be identified. These patients would be appropriate candidates for experimental approaches using novel anticancer drugs or new combination chemotherapy.

Recently, molecular diagnostic methods have been developed and BAX or BRCA1 have been reported to be predictive biomarker for aEOC [1,2]. We also previously reported that abnormalities of cell cycle regulators are predictive prognostic indicators for EOC [3]. Similarly, gene expression profiles or array comparative genomic hybridization (aCGH) has been reported to offer predictive/prognostic information for aEOC [4–7]. However, in order to identify useful predictive biomarkers for EOC, it is important that the markers should be tested in the context of standard therapy. In addition, the histology of the

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tumors should be considered because clear cell and mucinous types are usually more chemoresistant than other histologic types [8,9].

In this study, we used oligonucleotide microarrays combined with RNA isolated from microdissected tumor tissue to identify new prognostic biomarkers for aEOC patients receiving standard therapy. We excluded clear cell or mucinous tumors from our analysis based on the chemosensitivity.

Materials and methods

Patients and samples

Subjects eligible for this study were patients with histologically confirmed stage IIc–IV EOC (excluding mucinous and clear cell types) receiving standard therapy. Histologic grade was determined using WHO grading system. Additional inclusion criteria included an Eastern Cooperative Oncology Group performance status of 0 to 2. Exclusion criteria included a history of prior chemotherapy or major surgery. All patients received standard surgery and chemotherapy using carboplatin and paclitaxel. Standard surgery was bilateral adnexectomy, hysterectomy, and greater omentectomy with staging laparotomy and debulking surgery. Thirty-three aEOC patients were enrolled for microarray analysis, and an additional 107 patients were for real-time PCR analysis. The progression-free survival (PFS) was defined from the date of primary surgery to the date of the first occurrence of any of the following events: appearance of any new lesions, tumor progression, elevation of the CA125 level to at least two times the upper limit of normal or a nadir CA125 level, or death from any cause. The patients were determined to be no evidence of disease (NED) or evidence of disease (ED) at the disease progression or final visit. The study was approved by the Institutional Review Board of the Osaka City General Hospital and School of Medicine, Keio University, and written informed consent was obtained from all patients. Tumor specimens were obtained at operation and were immediately stored at −80 °C.

Study design

One hundred and forty aEOC samples were evaluated. The samples were divided between microarray analysis ($n=33$) and a real-time PCR analysis ($n=107$). Microarray analysis was performed using 33 samples, and candidate genes showing significant correlation with disease progression were identified. The GNAS gene was evaluated in an independent set of 107 samples, and PFS was predicted using the results of real-time PCR analyses of both mRNA and DNA.

Microdissection

Microdissection was performed as described previously. In brief, frozen sections (6 μm) prepared from tumor tissue specimens were affixed to glass slides and stained by Histogene LCM Frozen Section Staining Kit (Arcturus Engineering, Mountain View, CA). Stained sections were microdissected using a PixCell IIe LCM system (Arcturus Engineering, Mountain View, CA). Tumor cells and adjacent non-tumor stromal cells were visualized under the microscope and tumor cells selectively released by activation of the laser. Approximately 15,000 tumor cells were dissected in each case.

RNA and DNA extraction and amplification

Total RNA and DNA extractions were performed using the PicoPure RNA Isolation Kit and PicoPure DNA Extraction Kit according to the manufacturer's instructions (Arcturus Engineering, Mountain View, CA). RNA was amplified using a modified single-round T7 RNA amplification protocol. In brief, total RNA (600 ng) was first incubated with 1 μl of T7 primer(5′-GCATTAGCGGCCGCGAAATTAATACGACTCACTATAGGGAGATTTTTTTTTTTTTTTTTTVN-3′, 200 ng/μl) in a total volume of 50 μl for 3 min at 70 °C. First-strand cDNA synthesis was then performed by incubating 5 μl of primer-annealed sample and 5 μl of first strand master mix containing 2 μl of 5× first-strand buffer, 1 μl of 0.1 M DTT, 0.5 μl of DEPC water, 0.5 μl 10 mM dNTP mix, 0.5 μl RNase inhibitor, and 0.5 μl of MMLV reverse transcriptase (200 U/μl) for 1 h and 15 min at 37 °C. Subsequently, second-strand cDNA synthesis was performed by incubating the 10 μl first-strand reaction with 65 μl of second master mix, which contained 46 μl DEPC water, 15 μl 5×second-strand buffer, 1.5 μl of 10 mM dNTP mix, 0.5 μl of *Escherichia coli* DNA ligase (10 U/μl), 1.5 μl E. coli DNA polymerase I (10 U/μl), and 0.5 μl *E. coli* RNase H (2 U/μl), for 2 h at 16 °C, and then for 15 min at 70 °C. The entire 75 μl cDNA sample was loaded onto a ChromaSpin TE-200 spin column (BD Biosciences, San Diego, CA), which was centrifuged for 5 min at 2900 rpm (700×g) in an Eppendorf centrifuge. Purified cDNA was collected, lyophilized, dissolved in 8 μl of RNase-free water, and incubated at 70 °C for 10 min. In vitro transcription was subsequently performed by incubating the 8 μl post-lyophilization cDNA product with 12.2 μl of master mix containing 2 μl of 10× T7 reaction buffer, 6 μl of 25 mM rNTP Mix, 2 μl of 100 mM DTT, 0.2 μl of RNase inhibitor (40 U/ml), and 2 μl of T7 RNA polymerase for 3 h at 37 °C. The amplified RNA was purified on an RNeasy mini column (Qiagen, Valencia, CA) as per the manufacturer's protocol. The purified amplified RNA was quantified using RiboGreen RNA Quantitation Reagent (Molecular Probes, Eugene, OR).

Oligonucleotide microarray analysis

The microarray procedure was performed according to Affymetrix protocols (Santa Clara, CA). In brief, total RNA extracted from tumor samples was checked for quality using an Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany) and cRNA was synthesized using the GeneChip 3′-Amplification Reagents One-Cycle cDNA Synthesis Kit (Affymetrix). The labeled cRNAs were then purified and used for construction of probes. Hybridization was performed using the Affymetrix GeneChip HG-U133 Plus2.0 array for 16 h at 45 °C. Signal intensities were measured using a GeneChip Scanner3000 (Affymetrix) and converted to numerical data using the GeneChip Operating Software, Ver.1 (Affymetrix).

DNA copy number analysis

The method has been described previously [5,10]. From array data, we focused on 20q11–13 loci for further examination, because we thought that 20q11–13 loci are amplified in the ED group. We chose GNAS (GNAS complex locus, NM_000516) on 20q13 based on the *p* value and fold change. Results were normalized to the amount of RH78455 of chromosome 5q22.2 as genomic internal control locus. Regarding the internal DNA copy number control, we selected the genomic region of chromosome 5q, which is less frequently received the genomic alterations in ovarian cancers referred to previous report [11–13]. Next, we checked 10 primers (D5S818, D5S409, D5S349, D5S346, D5S519, D5S422, STSR33609, RH46186, RH78455, RH68508) of chromosome 5 region according to database of sequence tagged sites (STSs, http://www.ncbi.nlm.nih.gov/unists). Among them, RH78455 was most specific and reproducible primers, then we used it as internal DNA copy number control. The DNA was quantified using the Power SYBR Green PCR Master Mix (Applied Biosystems) and 7900HT Fast Real-time PCR system (Applied Biosystems) and reported relative to the control primer. The control DNA for standard DNA copy numbers was purchased from Invitrogen (Carlsbad, CA). The PCR conditions were as follows: one cycle of denaturation at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 60 s. If copy number was >1.5 relative to the chromosome control, we judged that there was amplification [5]. The primers used for estimating DNA copy numbers were as follows: GNAS: SHGC59923-FW: 5-GGG TGG GCT TTT GTT CTT TG-3, SHGC59923-RW: 5-AGG CAT AAA CGG GGG AGA TT-3, and

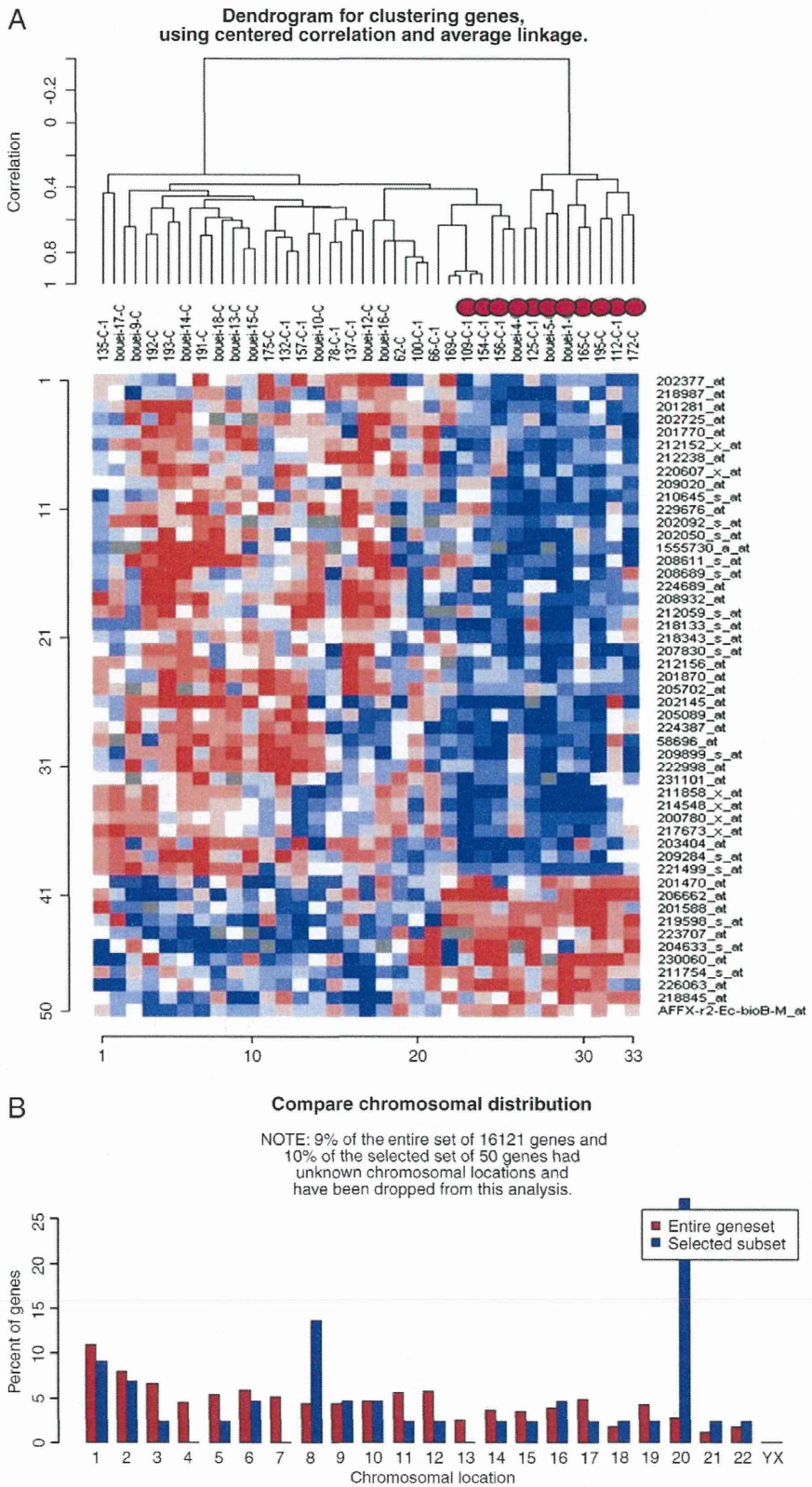

Fig. 1. (A) Fifty genes differentially expressed between tumors in ED and NED patients. Dendrogram for clustering genes using centered correlation and average linkage. Red circles indicate NED cases. (B) The chromosome distribution of the entire gene set and the selected subset of 50 genes. Among genes, which are located on chromosome 8 and 20, 50 genes are frequently selected in aEOC.

Table 1
Identification of 50 candidate PFS-related genes from microarray analysis.

No.	Name	Symbol	Cytoband	Fold Difference	Probe Set
1	Zinc finger, MYM-type 4	ZMYM4	Chr:1p32-p34	1.6	202050_s_at
2	Putative homeodomain transcription factor 1	PHTF1	Chr:1p13	1.5	205702_at
3	Protein phosphatase 1, regulatory (inhibitor) subunit 8	PPP1R8	Chr:1p35	1.8	207830_s_at
4	AT-rich interactive domain 1A (SWI-like)	ARID1A	Chr:1p35.3	1.8	212152_x_at
5	NIF3 NGG1 interacting factor 3-like 1 (S. pombe)	NIF3L1	Chr:2q33	1.6	218133_s_at
6	General transcription factor IIIC, polypeptide3, 102 kDa	GTF3C3	Chr:2q33.1	1.5	218343_s_at
7	Cell division cycle associated 7	CDCA7	Chr:2q31	0.4	230060_at
8	Chromosome 3 open reading frame 63	C3orf63	Chr:3p14.3	1.9	209284_s_at
9	Glutaredoxin (thioltransferase)	GLRX	Chr:5q14	0.3	206662_at
10	Dual specificity phosphatase 22	DUSP22	Chr:6p25.3	0.5	218845_at
11	RWD domain containing 1	RWDD1	Chr:6q13-q22.33	0.5	219598_s_at
12	Lymphocyte antigen 6 complex, locus E	LY6E	Chr:8q24.3	3	202145_at
13	Zinc finger protein 7	ZNF7	Chr:8q24	1.6	205089_at
14	Fuse-binding protein-interacting repressor	SIAHBP1	Chr:8q24.2-qter	1.8	209899_s_at
15	MAF1 homolog (S. cerevisiae)	MAF1	Chr:8q24.3	1.8	222998_at
16	COMM domain containing 5///COMM domain containing 5	COMMD5	Chr:8q24-qter	1.8	224387_at
17	Exosome component 4	EXOSC4	Chr:8q24.3	1.7	58696_at
18	Spectrin, alpha, non-erythrocytic 1 (alpha-fodrin)	SPTAN1	Chr:9q33-q34	2	208611_s_at
19	vav 2 oncogene	VAV2	Chr:9q34.1	0.6	226063_at
20	Glutathione S-transferase omega 1	GSTO1	Chr:10q25.1	0.6	201470_at
21	PAP-associated domain containing 1	PAPD1	Chr:10p11.23	1.8	229676_at
22	Cofilin 1 (non-muscle)	CFL1	Chr:11q13	2.4	1555730_a_at
23	Activating transcription factor 7 interacting protein	ATF7IP	Chr:12p13.1	1.6	218987_at
24	Ribosomal protein S6 kinase, 90 kDa, polypeptide 5	RPS6KA5	Chr:14q31-q32.1	0.5	204633_s_at
25	Vacuolar protein sorting 39 (yeast)	VPS39	Chr:15q15.1	1.6	212156_at
26	ADP-ribosylation factor-like 2 binding protein	ARL2BP	Chr:16q13	1.5	202092_s_at
27	Protein phosphatase 4 (formerly X), catalytic subunit	PPP4C	Chr:16p12-16p11	1.9	208932_at
28	Polymerase (RNA) II (DNA-directed) polypeptide A, 220 kDa	POLR2A	Chr:17p13.1	1.8	202725_at
29	Thioredoxin-like 1	TXNL1	Chr:18q21.31	0.6	201588_at
30	Small nuclear ribonucleoprotein polypeptide A	SNRPA	Chr:19q13.1	1.7	201770_at
31	GNAS complex locus	GNAS	Chr:20q13.3	1.4	200780_x_at
32	Adhesion regulating molecule 1	ADRM1	Chr:20q13.33	1.6	201281_at
33	Ribophorin II	RPN2	Chr:20q12-q13.1	1.9	208689_s_at
34	Chromosome 20 open reading frame 111	C20orf111	Chr:20q13.11	1.5	209020_at
35	GNAS complex locus	GNAS	Chr:20q13.3	1.9	211858_x_at
36	Transient receptor potential cation channel, subfamily C, member 4-associated protein	TRPC4AP	Chr:20q11.22	1.7	212059_s_at
37	Additional sex combs like 1 (Drosophila)	ASXL1	Chr:20q11.1	1.7	212238_at
38	GNAS complex locus	GNAS	Chr:20q13.3	1.8	214548_x_at
39	GNAS complex locus	GNAS	Chr:20q13.3	1.6	217673_x_at
40	TH1-like (Drosophila)	TH1L	Chr:20q13	1.7	220607_x_at
41	Ayntaxin 16	STX16	Chr:20q13.32	1.7	221499_s_at
42	Mannosidase, beta A, lysosomal-like	MANBAL	Chr:20q11.23-q12	1.5	224689_at
43	Tetratricopeptide repeat domain 3	TTC3	Chr:21q22.2	1.5	210645_s_at
44	Solute carrier family 25 (mitochondrial carrier; peroxisomal membrane protein, 34 kDa), member 17	SLC25A17	Chr:22q13.2	0.6	211754_s_at
45	Translocase of outer mitochondrial membrane 34	TOMM34		1.6	201870_at
46				1.7	202377_at
47	Hypothetical protein MGC10850	MGC10850		0.5	223707_at
48	Transcribed locus			1.6	231101_at
49	Armadillo repeat containing, X-linked 2	ARMCX2	Chr:Xq21.33-q22.2	2.8	203404_at
50				0.7	AFFX-r2-Ec-bioB-M_at

chromosome 5q22.2: RH78455-FW: 5-TCC TGC AAA CAT TTA AAC TCC A-3, RH78455-RW: 5-AAC AGC AAC TGT TTT TTC CCC-3. Finally, for PCR, 1.5-fold was used as the cutoff for amplification, respectively [5].

Real-time quantitative RT-PCR for mRNA expression

In addition, mRNA expression levels were validated for GNAS (GNAS complex locus, NM_000516) on chromosome 20q13. All results were normalized to the amount of glyceraldehyde 3 phosphate dehydrogenase (GAPD, NM_002046). RNA was converted to cDNA using a GeneAmp RNA PCR Core kit (Applied Biosystems, Foster City, CA). The cDNAs were quantified using the Power SYBR Green PCR Master Mix (Applied Biosystems) and 7900HT Fast Real-time PCR system (Applied Biosystems) and reported relative to the GAPD expression levels. The PCR conditions were as follows: one cycle of denaturation at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s, and 60 °C for 60 s. To amplify the target genes, all of the primers used for real-time RT-PCR were purchased from Takara (Yotsukaichi, Japan), which is the major company of molecular biology in Japan. We are frequently using their primers [14–16], and we consider that the primers are reliable one. GNAS-FW: 5-TGT ACA AGC AGT TAA TCA CCC ACC A-3, RW: 5-TCT GTA GGC CGC CTT AAG CTT TC-3, GAPD-FW: 5-GCA CCG TCA AGG CTG AGA AC-3, RW: 5-ATG GTG GTG AAG ACG CCA GT-3. Finally, we determined the case as overexpression when the relative mRNA expression is larger than median relative mRNA expression in all cases.

Statistical analysis

The microarray analysis was performed using the BRB Array Tools software ver. 3.3.0 (http://www.linus.nci.nih.gov/BRB-ArrayTools.html) developed by Dr. Richard Simon and Dr. Amy Peng. In brief, a log base 2 transformation was applied to the raw microarray data, and global normalization was used to calculate the median over the entire array. Genes were excluded if the percentage of data missing or filtered out exceeded 20% or if less than 20 % of expression data had at least a 1.5-fold change in either direction from the median value.

The 16,121 genes that passed the filtering criteria were then considered for further analysis. The *t*-test ($p<0.001$) was used to identify the genes differentially expressed between NED and ED. Clustering for 50 identified genes used centered correlation and average linkage.

Both mRNA and DNA copy numbers were validated using real-time PCR in the 107 independent cases of EOC. PFS was calculated by the Kaplan-Meier method. Univariate and multivariate Cox's proportional hazard test was applied to identify variables associated with PFS. A p value of <0.05 was considered to be significant (SAS software ver. 9.1.3; SAS Institute Inc., Cary, NC).

Results

Clinical backgrounds of 140 EOC

The clinical backgrounds of the 33 aEOC samples used for array analysis are as follows: median age (54 years: range 33–80), stage (II:4, III:19, IV:10), histologic types (endometrioid: 4, serous: 22, undifferentiated: 7), histologic grade (1+2:9, 3:24), and operation status (optimal:14, suboptimal:19). The median follow-up period is 907 days (range: 292–2136 days), and 11 patients are alive without relapse.

The clinical backgrounds of 107 aEOC samples assayed for real-time PCR are as follows: median age (54 years: range 29–85), stage (II:12, III:74, IV:21), histologic types (endometrioid: 27, serous: 61, undifferentiated: 19), histologic grade (1+2:46, 3:61), operation status (optimal: 61, suboptimal: 46). The median follow-up period is 699 days (range: 92–2885 days) and 49 patients are alive without relapse.

Identification of 50 candidate disease progression-related genes by microarray analysis

To identify candidate disease progression-related genes from 54,675 transcripts, microarray analysis was performed on a training set of 33 samples. A total of 16,121 genes passed the filtering criteria and were further analyzed. Fifty genes were significantly correlated with disease progression, with a p value of <0.001 (Fig. 1A, Table 1). The chromosome distribution of the gene set analyzed and a selected subset are shown in Fig. 1B. Fifty selected genes on chromosome 8 and 20 show a higher than expected chromosomal distribution (Fig. 1B). Of these 50 genes, 6 are located at chromosome 8q24 and 9 (12 probes) at 20q11–13. The ratio of selected gene set/analyzed gene set in chromosome 20 is significantly higher than that in other chromosome regions (12/383 vs. 32/13656, $p=1.3\times10^{-16}$) and the selected gene set/analyzed gene set in chromosome 8 is significantly higher than that in other chromosome regions (6/606 vs. 32/13656, $p=0.01$). We speculate that the abnormal chromosomal distribution is due to genomic alteration and that these genes may play an important role in aEOC.

The results suggested that 8q24 and 20q11–13 loci are amplified in the ED group and that this might be related to treatment of aEOC.

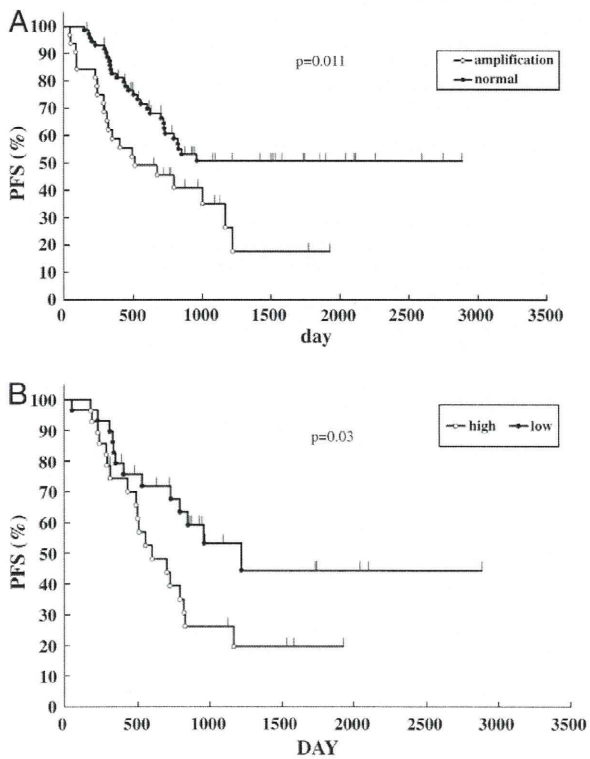

Fig. 2. The relationship between amplification and expression status of GNAS and PFS. (A) The relationship between copy number change of GNAS and PFS. (B) The relationship between mRNA expression of GNAS and PFS.

Table 2
Relationship between gene amplification and clinical factors.

Clinical Factor	GNAS	
	Frequency	*p* Value
Age		
Young	30%(16/53)	0.999
Old	30%(16/53)	
Grade		
1+2	33%(15/45)	0.689
3	28%(17/61)	
Operation		
Optimal	26%(16/61)	0.392
Suboptimal	36%(16/45)	

Relationship between GNAS gene amplification and PFS

DNA marker is thought to be more reliable and qualitative than RNA, because RNA expression changes according to the condition and environment of cancer cells. Therefore, we focused on detecting genomic alterations of chromosome 20q11–13 loci using real-time quantitative PCR on the 107 independent validation samples. The amplification of GNAS is significantly related to poor PFS ($p=0.011$) (Fig. 2A). GNAS shows gain of gene copy number rates of 30% (32/106), respectively. Correlations between amplification of GNAS and clinical factors are shown in Table 2. There are no significant associations between gene amplification at this loci and clinical factors such as age, histological grade, histologic type, and operation status. In addition, we performed multivariate Cox's proportional hazard test to identify variables including age, histologic grade, debulking status, histologic type, and GNAS copy number change associated with PFS. Finally, GNAS amplification was independent prognostic factor in aEOC (Table 3).

Table 3
Univariate and multivariate analysis of the effect of various prognostic factors on PFS.

Variable[a]	Univariate			Multivariate		
	Hazards Ratio[b]	95% CI	*p*	Hazards Ratio[b]	95% CI	*p*
GNAS	2.035	1.164–3.558	0.013	1.906	1.081–3.36	0.026
Age	0.975	0.563–1.688	0.927	0.765	0.434–1.348	0.354
Histologic grade	1.376	0.774–2.447	0.277	1.248	0.697–2.233	0.22
Debulking status	0.26	0.143–0.471	<0.001	0.256	0.139–0.493	<0.001

[a] GNAS: amplification vs. non-amplification, AGE: >median vs.<median, histologic grade: G3 vs. G1 + 2, debulking status: optimal vs. suboptimal.

[b] Hazard ratio refers to risk of survival, with values<1.0 indicating reduced risk. CI, confidence interval.

Relationship between GNAS gene expression and PFS

Of the 107 independent samples, RNA from 62 was available for real-time RT-PCR analysis. There are no significant associations between GNAS expression and clinical factors such as age, histological grade, and operation status. GNAS expression correlates significantly with PFS of aEOC ($p = 0.03$) (Fig. 2B).

Discussion

The platinum/taxane regimen has improved the prognosis of EOC; however, this remains poor. For this reason, it is very important to find new prognostic markers for EOC receiving standard therapy, so that future clinical trials can be focused on patients with a poor prognosis. In this study, we used oligonucleotide microarrays to identify new prognostic biomarkers for aEOC excluding clear cell or mucinous types receiving standard therapy. In the past decade, several studies have analyzed chromosomal imbalances using comparative genomic hybridization (CGH) in EOC [10,17–20]. Arnold et al. investigated 47 malignant ovarian tumors and 2 ovarian tumors of low malignant potential using CGH and demonstrated that common genetic changes include DNA gains of chromosome arms 8q24 (51%) and 20q13.2-qter (40%) [10]. Iwabuchi et al. presented CGH data from 31 ovarian carcinomas and reported that increased copy numbers were most commonly observed in their cases at 3q26 (42%), 8q24 (35%), and 12q11.1–12 (25%) [17], while Sonoda et al. demonstrated that the most frequent sites of copy number increase were 8q24.1 (56%) and 20q13.2-qter (48%) in tumor DNA from 25 malignant ovarian carcinomas and 2 tumors of low malignant potential [18]. Tanner et al. focused on 20q12–q13 amplification in 24 sporadic, 3 familial and 4 hereditary ovarian carcinomas, and 8 ovarian cancer cell lines [19]. They demonstrated high-level amplification of at least one of the five non-syntenic regions at 20q12–q13.2 in 13 sporadic (54%) and in all four hereditary tumors [19]. Hu et al. focused on ovarian serous carcinomas and demonstrated DNA copy number gain at 8q22q24 and 20q12q13 in 60% and 45% of samples, respectively [20] In our study, amplification rates of GNAS at 20q13 is 30%, respectively.

Furthermore, Tanner et al. showed a tendency toward correlation between amplification and poor survival (not significant) and Hu et al. reported that 20q12q13 amplification may indicate a high risk for recurrence of serous ovarian cancer [19,20]. These reports included various histologic types and stages and even cell lines as well as primary and recurrent cases. Furthermore, they provide no information on therapy or operation status, include small number of patients, and do not perform validation assays. As it is well established that patients with ovarian carcinoma of different histologic types vary in their response to chemotherapy [8,9], it is important to take this into account in testing new biomarkers for their utility in clinical practice. In this study therefore, we focused on patients receiving standard therapy, excluding those with mucinous and clear cell tumors, and performed microarray analysis in 33 patients and validation assays in 107 patients.

Recent attempts to develop accurate predictors of clinical outcome in ovarian cancer have focused on techniques that are capable of assessing global gene status such as expression profiling and array CGH [4–7]. Birrer et al. performed oligonucleotide array CGH on 42 microdissected high-grade serous ovarian tumors and reported that amplification at 5q31–5q35.3 exhibited the strongest correlation with overall survival, identifying FGF-1 on 5q31 as a prognostic marker in 81 independent samples [7]. These data are not in agreement with our own. However, Birrer et al.'s report provides no information about the chemotherapy used, so that we speculate that prognostic biomarkers may be dependent on the chemotherapeutic regimen. Spentzos et al. also reported expression profiles for EOC and established a Chemotherapy Response Profile (CRP) and Ovarian Cancer Prognostic Profile (OCPP) [4,6].

We selected GNAS gene based on the p value and fold change in array data and examined the amplification status of GNAS as prognostic marker of aEOC. GNAS gene amplification was an independent prognostic factor. The GNAS locus encodes the G (alpha) protein, which stimulates the formation of cyclic AMP (cAMP). The cAMP pathway mediates pleiotropic effects including regulation of apoptosis and proliferation [21–23] and different genotypes of the single nucleotide polymorphism (ANP) T393C in the GNAS gene predict the clinical outcome of urothelial carcinoma, sporadic colorectal cancer, renal cell carcinoma, and chronic lymphocytic leukemia [24–27]. However, the role of GNAS in EOC remains unclear.

In conclusion, we identified amplification of GNAS on 20q13 as markers of prognosis in patients with aEOC treated with standard therapy. Our finding identifies qualitative and reproducible biomarker to predict the PFS of aEOC.

Conflict of interest statement

All of the authors are aware of and agree to the content of the article and have no conflict interest.

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Molecular Cancer Therapeutics

Enhanced Anticancer Effect of the Combination of BIBW2992 and Thymidylate Synthase –Targeted Agents in Non–Small Cell Lung Cancer with the T790M Mutation of Epidermal Growth Factor Receptor

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