厚生労働科学研究費補助金

第3次対がん総合戦略　研究事業

平成23年度　総括研究報告書

肺癌糖鎖標的マーカーの実用化に向けた定量的糖鎖構造変動解析システムの構築

研究代表者　植田　幸嗣

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研究代表者　植田幸嗣　　独立行政法人理化学研究所　上級研究員

研究要旨：本研究は肺癌の早期診断を可能とする血清腫瘍マーカーを同定し、著しい増加を続ける肺癌死亡数を有意に減少させることを最終目標とする。同目標に対して、細胞の癌化や環境の変化に鋭敏に反応して構造が変化することが知られる糖鎖修飾に着目した「糖鎖標的バイオマーカー」候補は多数同定されるに至っているが、特定の糖鎖付加部位における糖鎖構造の変化を定量的に、かつ多検体を用いてバリデーションできる技術が存在しないため、糖鎖バイオマーカーの臨床、診断応用は長らく停滞していた。

この状況を打破するため、Multiple Reaction Monitoring (MRM) 法を応用して数百箇所の糖タンパク質上糖鎖付加部位について、個々に糖鎖構造変化を定量的にモニターできる Energy resolved oxonium ion monitoring technology (Erexim 法)を開発した。研究計画の一年目である平成 23 年度において、単一な糖鎖構造を持つ糖ペプチドライブラリ（ペプチド部分は一定）を独自に調製し、IgG1 分子が持つ糖鎖構造のモル比 99%以上を占める 29 種類の糖鎖が付加されたペプチドを単離、精製することに成功した。続いて、それら個々の糖ペプチド MRM 分析から得られた定量的質量分析データと糖鎖構造の関連付けを詳細に行い、各糖鎖構造に関して混合物中での含有率 0.1%以上、絶対量 10 amol（アトモル = 10^{-18} モル）以上、測定定量幅 10^4 以上の感度、定量性で糖鎖構造バリエーションの分析ができる質量分析条件を決定した。

A. 研究目的

本邦において肺癌は部位別がん死亡率で第一位を占めており、肺癌の罹患数、死亡者数を減少させることが急務となっている。そのためには喫煙対策などに加え、根治可能なより早い段階で肺癌、またはその前癌病変を発見できる診断技術の開発は重要な位置を占める。現状において肺癌の早期発見には胸部X線診断、CT診断など画像診断技術が主軸となっているが、これらよりも早期に、かつ検査技師の技量にかかわらず腫瘍を発見する技術は現時点では存在せず、本研究で開発する非侵襲的で安価に行える血清質量分析診断法にて初期肺癌の検出やリスク診断までもが可能になれば、それに伴って治療成績は劇的に改善されると期待できる。

研究代表者はこれまで、血清による肺癌の早期診断を目指して様々なグライコプロテオーム解析技術を用い、早期肺癌において糖鎖構造に変化が認められる多数の糖鎖標的マーカー候補の同定を行ってきた。そこで本研究ではそれら全てのマーカー候補糖タンパク質上に付加されている糖鎖の癌性変化を高感度に定量化、統合することによって肺癌を「治療可能な段階で早期に発見すること」、さらには「症状が出る前でのリスクの把握を可能にすること」を目標とする。本診断技術は従来のイムノアッセイとは異なり、約30分間の1アッセイで200項目までのバイオマーカーを同時に定量化し、非常に信頼性の高い、多岐にわたる早期癌関連情報を得ることができるようになる。

B. 研究方法

a. 糖ペプチド標準品の精製

市販品のヒトIgGタンパク質を8M Ureaを含む変性バッファーで溶解させ、還元アルキル化を行った。PD-Miditrap 脱塩カラム(GE Healthcare, Buckinghamshire, UK)を用いて重炭酸アンモニウムバッファーに置換した後、トリプシン GOLD (Promega, Madison, WI)で12時間消化した。消化後のペプチドサンプルを Oasis HLB カートリッジ(Waters, Milford, MA)で脱塩精製し、次の二次元HPLC分画に供した。

まず4.6 mm x 500 mm Cadenza CD-C18 column (Imtakt Corporation, Kyoto, Japan)を用いて、溶媒A [0.2% TFA]、溶媒B [75% acetonitrile, 0.1% TFA]、溶媒B% 5-20 90分グラジェント、流速0.6 ml/minの条件でIgG由来ペプチドの分画を行った。

分取した各フラクションを完全乾燥し、0.1% TFAに再溶解した後、二段階目の150 mm x 4.6 mm SunShell C18 column (Chromanik Corporation, Osaka, Japan)によるさらなる分画精製を行った。ここでは溶媒A [0.1% TFA]、溶媒B [12% acetonitrile, 0.1% TFA]、溶媒B% 10-90グラジェントの条件を用いた。分取した各フラクションは乾燥し、以降の実験に供した。

b. 蛍光HPLC分析

前項で単離精製した各種糖ペプチド表品の糖鎖構造を確定させるため、2-アミノピリジン蛍光標識法による HPLC 分析を行った。単離糖ペプチドを[0.5U N-glycosidase F (Roche Diagnostics, Basel, Switzerland), 0.01% ProteaseMax

(Promega, Madison, WI)]中で37℃、8時間消化を行い、糖鎖をペプチドから切断した。遊離糖鎖をセルロースカートリッジにて濃縮精製、乾燥後、2-アミノピリジンと混合して還元アミノ化反応によるラベル化を行った。

ラベル化糖鎖は MassPREP HILIC μElution plate (Waters, Milford, MA)を用いて脱塩精製を行い、Shimpack CLC-ODS column (0.6 x 15 cm)による逆相HPLC分析に供した。ここでは溶媒 A [10 mM リン酸ナトリウムバッファー(pH = 3.8)]、溶媒B [0.5% 1-ブタノールを含む溶媒A]を用いた。溶媒 B 20%で平衡化を行った後、%B 50 まで75分間のグラジェントを作成し、励起波長 320 nm、吸収波長 400 nm で標識糖鎖を測定した。各糖鎖溶出時間は、PA-glucose oligomer (Takara Bio Inc., Shiga, Japan)を利用したグルコースユニット (GU) で補正、構造データベースとの相対比較を行えるようにした。

c．抗体医薬品3種の分析前処理

80 μg のトラスツズマブ（ハーセプチン)、ベバシズマブ（アバスチン)、セツキシマブ（アービタックス）をそれぞれ 8M Urea を含む変性バッファーで溶解させ、還元アルキル化を行った後、4 μg endoproteinase Lys-C で37℃、2時間の消化を行った。この Lys-C 消化物をさらに Trypsin GOLD で37℃、4時間消化を行った。最終消化物を Oasis HLB カートリッジにて脱塩を行い、15% acetnitrile で溶出、糖ペプチド画分を回収した。分析に際してはこれを0.1%酢酸で5倍に希釈したものを用いた。

d．Multiple Reaction Monitoring (MRM)

MRM 分析には 4000QTRAP トリプル四重極型質量分析計(AB Sciex, Foster City, CA) に Agilent 1200 nano-HPLC system (Agilent Technologies, Palo Alto, CA) を接続した LC/MS/MS システムを使用した。Nano-HPLC のカラムには 75 μm x 200 mm ESI sprayer tip packed with 3 μm C18 resin (Nikkyo Technos, Tokyo, Japan)を用い、溶媒 A [0.1% ギ酸]、溶媒 B [70% acetonitrile, 0.1% ギ酸]、流速 250 nl/min の条件で分離を行った。

4000QTRAP 質量分析計の設定は以下の通りである。2200 V ionization spray voltage; 12 psi curtain gas (N2); CAD = 4; 70 V declustering potential; 10 V entrance potential; Q1 resolution, HIGH; Q3 resolution, LOW; 2 ms pause in between。

e. データ解析

MRM で得られたデータは MultiQuant version 2.02 (AB Sciex, Foster City, CA) ソフトウェアを用いてプロセッシング、定量解析を行った。

C. 研究結果

糖鎖構造バリエーション定量化法を構築するにあたって、最初にペプチド部分のアミノ酸配列が同じで、かつ糖鎖構造が異なる糖ペプチドライブラリが必要であった。これは市販されていないので、ヒト IgG 分

子が持つ一本のN型糖鎖をモデルとして、同分子上に存在する全ての糖鎖構造バリエーションを単離精製することを試みた。図1に示すように、特殊な高分解能逆相カラムと至適化溶媒を用いることによって、糖ペプチドの状態であっても糖鎖構造にのみ依存する高度な分離が可能となった。ここで単離精製された糖ペプチドの糖鎖構造は、従来法である蛍光標識 HPLC 分析法によって全て確証実験を行った上でその後の質量分析に供している。

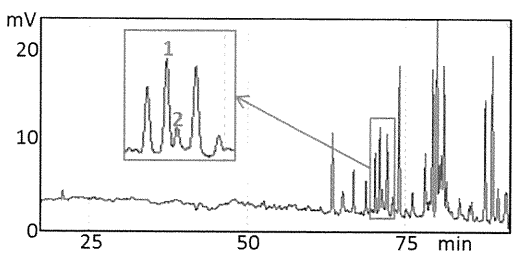

図1　単一糖鎖構造を持つ糖ペプチドライブラリの作成。IgG 分子の持つ一箇所のN型糖鎖付加部位を含むトリプシン消化ペプチド断片を単離し、さらにそれを高分解能逆相分析カラムで分画精製した際のHPLC クロマトグラム。図中赤字1、2で示したピークはそれぞれ図2の Peak 1、2に相当する。

糖ペプチドから PNGaseF を用いた酵素的消化により糖鎖部分を遊離させ、2-ピリジルアミン (2-PA)と反応させて蛍光標識を行った。これを高分解能逆相カラムを用いた蛍光 HPLC 分析に供したデータの一例が図 2 である。同時に分析したグルコースオリゴマーの溶出時間からグルコースユニットを求め、GALAXY など既知の糖鎖構造データベースに登録されている糖鎖構造と照合、詳細な糖鎖構造を確定した。ここでは合計29種類のN型糖鎖構造が単離精製できた。

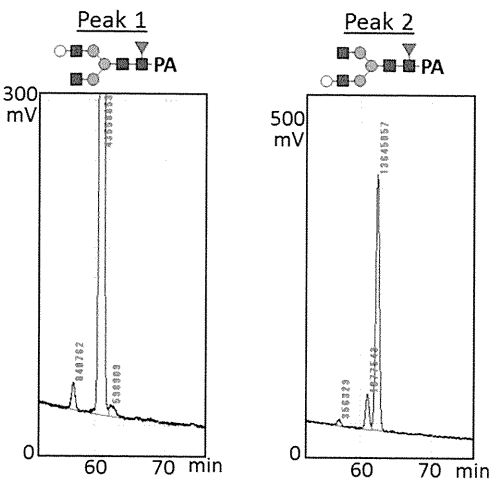

図2　図1中の糖ペプチド1、2から糖鎖を酵素的に切断し、蛍光標識後、HPLC による構造確認実験を行った結果。各ピークの溶出時間をグルコースユニット (GU) に変換し、糖鎖構造データベースと照合して求められた構造をクロマトグラム上部に示した。

前ページ記載の標準品測定データベースから作成した糖鎖構造バリエーション定量アルゴリズムを用いて、実用されている3つの抗体医薬品の品質評価を行い、本技術の実用性を検証した。図3は実際に国内で投薬された実績のあるハーセプチン(a)、アバスチン(b)の異なる4ロットを入手し、

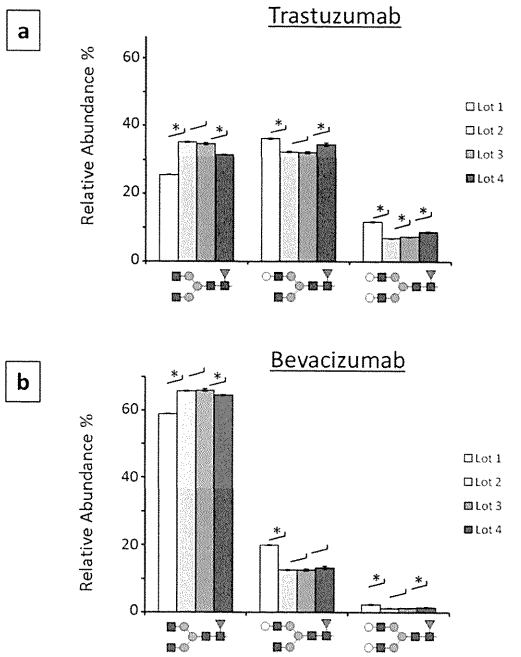

図3　Erexim 法を用いて二種抗体医薬品上の糖鎖構造に対するロット間比較を行った結果。(a)Trastuzumab、(b)Bevacizumab に存在する代表的な 3 種の糖鎖構造について、4 つの異なるロットを測定した結果。＊は Student T-test にて p < 0.05 の有意差が確認されたことを示す。エラーバーは triplicate 試験を 3 回独立に行った結果を集計して求めたものである。

製造ロットの違いによって糖鎖構造は正確に維持されているかどうかを分析した結果である。図に示した含有量の多い代表的な糖鎖構造でも、統計学的有意差をもって糖鎖構造のロット間変動があることが分かった。ここに記した以外の、検出されうる全ての糖鎖構造を評価した結果、経時的に糖鎖長が短い構造の割合が有意に増加していることが明らかとなった。アバスチンに関しては糖鎖自体が付加されていない抗体が増えてくる傾向が観測された。

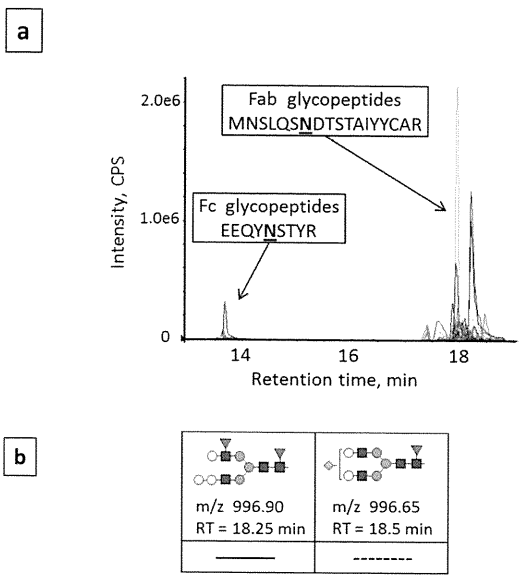

図4　Cetuximab の Fab 領域、Fc 領域に存在する糖鎖構造バリエーションの同時定量分析。(a)MRM クロマトグラムの全体図。上記 2 ヶ所の糖ペプチドが明瞭に分離された上で定量できていることが分かる。(b)本分析で証明されたヒト体内で抗原性を持ちうると考えられる非ヒト型糖鎖構造の一例。

次に、同じく投与実績のあるロットのアービタックスを入手し、FDA での認可当時から問題となっているアナフィラキシーショックの原因を Erexim 法で検討した。アービタックスは偶発的に可変領域にも N 型糖鎖が一本修飾された構造を持っており、従来技術ではこの Fab 糖鎖と Fc 糖鎖を分離し、どちらがヒト体内での抗原性を示して

いるのかが不明であった。また、どのような構造が上記抗原性の要因となっているかも網羅的に調べることができなかった。

それに対し本技術では、図4(a)に示すようにFab糖鎖とFc糖鎖を明確に質量、溶出時間で分離した上で、存在しうる糖鎖構造を同時定量化することが可能となった。さらにその分析結果からは、これまで知られていなかった非ヒト型糖鎖構造がFab領域からのみ検出され(b)、これが投薬時に高頻度で発症するアナフィラキシーショックの原因ではないかと推察された。(b)には、検出された代表的なシアリルルイスX型糖鎖と、N-グリコリルノイラミン酸含有糖鎖の構造を示している。

興味深い知見としては、図5に示すように、同一IgG分子（アービタックス）上の糖鎖であるにも関わらず、Fab領域に付加した糖鎖と、Fc領域に付加した糖鎖構造が全く独立した糖鎖構造構成を持っていた点である。同抗体医薬の副作用を考える上で、Fc領域にはヒト型糖鎖しか存在せず、Fab領域に結合した糖鎖の大多数は非ヒト型糖鎖であった点は極めて重要な情報と言える。

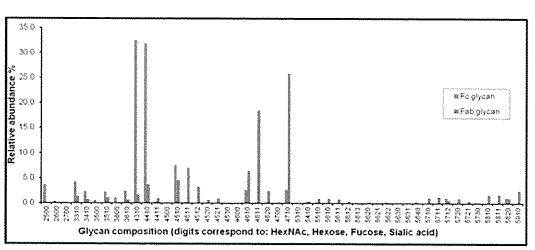

図5 Cetuximabの Fab領域（赤）、Fc領域（青）に付加された糖鎖構造の定量的プロファイル比較。

D. 考案

本Erexim テクノロジーの開発によって、従来技術では数週間から数ヶ月を要する特定糖鎖付加部位の糖鎖構造定量プロファイリングが1日に40～50サンプル測定できるようになった。さらに、解析可能な糖鎖構造も解離エネルギースキャンを導入したことにより、Immunoglobulin糖鎖のほぼ全てを網羅できるほどの種類を定量可能となった。複数ヶ所の糖鎖付加部位を持つタンパク質の解析において、それぞれの付加部位ごとのプロファイルを一分析で完了できるようになったのも革新的である。

本技術が実際に使用されている医薬品の評価に使用可能な水準の技術かどうかを確かめる目的で試行したハーセプチン、アバスチン、アービタックスの分析では、いずれも過去に知られていなかった重要な知見が多数得られた。前二者で観察された経時的な糖鎖構造の短縮、または消失はこれまで検証されていなかった明らかな品質変化と言えるが、その薬効への影響やヒト免疫系への抗原性もまた現時点では不明である。こうした製造ロット間で変化しやすい分子構造全てにおいて薬理学的意義を検証するのはほぼ不可能であるので、今後は本開発技術などを利用して、こうした微小変化が「起きない」工夫を講じてゆく必要があるだろう。

後者のアービタックスに関しては、約33%の投与患者においてアナフィラキシーショックが確認されたとういう報告があるが（N Engl J Med. 2008 Mar 13; 358(11): 1109-17)、その抗原性はアービタックスの持つFab領域糖鎖のほとんどを占める非ヒ

ト型糖鎖構造である可能性が強く示唆された。ここでも、多数検出された非ヒト型糖鎖構造のうちどれが実際にアナフィラキシーショックの原因になっているか、なぜ同一抗体分子上にもかかわらずFab領域とFc領域で糖鎖プロファイルが完全に異なるのか、など分子生物学的に解明すべき課題は多い。少なくとも、Ereximテクノロジーを使用すればどの部位にどのような糖鎖構造が何%付加されているかが即座に判明するため、非ヒト型糖鎖構造を付加させないような製造工程を開発する、またその事実をロットごとに検証することは今後の抗体医薬開発において重要であると考えられる。

以上のように、ペプチド部分のアミノ酸配列に関わらず、糖鎖構造の定量的バリエーションプロファイルがハイスループットに可能となる新技術の開発に成功した。本技術を適用するモデルとして上記抗体医薬品を用いて新たな知見が多くもたらされたが、Erexim法は原理的にその解析対象糖タンパク質を選ばない。すなわち、本研究の目的である、同定済みの肺癌糖鎖標的腫瘍マーカー候補分子に対する糖鎖付加部位ごとの大規模検証試験にも応用が可能である。現在はより幅広い血清糖タンパク質に対して同時分析が可能な前処理プロトコルを至適化し、順次糖鎖標的バイオマーカー候補タンパク質の測定に進めてゆく段階である。

E. 結論

平成23年度の研究において、極めて稀な構造を含む29種類のN型糖鎖構造を1回のMRM分析で構造決定、含有率の定量を行うことが可能となった（Ereximテクノロジー）。本技法を実際に使用し、ハーセプチンやアバスチンといった抗体医薬上の糖鎖構造バリエーションをモデル系として解析し終わったので、これらの測定データを元にして「糖鎖バリエーション高速定量化アルゴリズム」を平成24年度前半までに確立する。次に、これまでに同定した肺癌糖鎖標的マーカー候補について、500症例規模の血清サンプルと上記開発技術を用いた検証実験を行い、肺癌の早期診断に有用なバイオマーカー群の確定、及び診断システムの確立を行う（平成24年度後半～25年度）。

G. 研究発表

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H. 知的財産権の出願・登録状況（予定を含む）

・「発明の名称：肺癌マーカー補体 C3dg 分子及び肺癌マーカーの分析方法」
出願番号：特願 2011-201380
出願日：2011/09/15

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
Piao, L. H. Nakagawa **K. Ueda** S. Chung K. Kashiwaya H. Eguchi H. Ohigashi O. Ishikawa Y. Daigo K. Matsuda Y. Nakamura	C12orf48, termed PARP-1 binding protein, enhances poly(ADP-ribose) polymerase-1 (PARP-1) activity and protects pancreatic cancer cells from DNA damage.	Genes Chromosomes Cancer	50	13-24	2011
C. Tanikawa M. Espinosa A. Suzuki K. Masuda K. Yamamoto E. Tsuchiya **K. Ueda** Y. Daigo Y. Nakamura K. Matsuda	Regulation of histone modification and chromatin structure by the p53-PADI4 pathway.	Nature communications	3	676	2012
A. Toyama H. Nakagawa K. Matsuda N. Ishikawa N. Kohno Y. Daigo T. A. Sato Y. Nakamura **K. Ueda.**	Deglycosylation and label-free quantitative LC-MALDI MS applied to efficient serum biomarker discovery of lung cancer.	Proteome Science	9	18	2011

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C12orf48, Termed PARP-1 Binding Protein, Enhances Poly(ADP-Ribose) Polymerase-1 (PARP-1) Activity and Protects Pancreatic Cancer Cells from DNA Damage

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To identify novel therapeutic targets for aggressive and therapy-resistant pancreatic cancer, we had previously performed expression profile analysis of pancreatic cancers using microarrays and found dozens of genes trans-activated in pancreatic ductal adenocarcinoma (PDAC) cells. Among them, this study focused on the characterization of a novel gene *C12orf48* whose overexpression in PDAC cells was validated by Northern blot and immunohistochemical analysis. Its overexpression was observed in other aggressive and therapy-resistant malignancies as well. Knockdown of C12orf48 by siRNA in PDAC cells significantly suppressed their growth. Importantly, we demonstrated that C12orf48 protein could directly interact with Poly(ADP-ribose) Polymerase-1 (PARP-1), one of the essential proteins in the repair of DNA damage, and positively regulate the poly(ADP-ribosyl)ation activity of PARP-1. Depletion of C12orf48 sensitized PDAC cells to agents causing DNA damage and also enhanced DNA damage-induced G2/M arrest through reduction of PARP-1 enzymatic activities. Hence, our findings implicate C12orf48, termed PARP-1 binding protein (PARPBP), or its interaction with PARP-1 to be a potential molecular target for development of selective therapy for pancreatic cancer.

INTRODUCTION

Pancreatic cancer is the fourth leading cause of cancer death in the western world and shows the worst mortality among common malignancies with a 5-year survival rate of lower than 5% (DiMagno et al., 1999; Wray et al., 2005). It is estimated that a total of 42,470 new cases were diagnosed to have pancreatic cancer and 35,240 deaths were caused by it in the United States in 2009 (Jemal et al., 2009). At present, only surgical resection can offer a chance for cure or long-term survival to the patients suffering from pancreatic cancer. However, only 10–20% of patients with pancreatic cancer are able to have radical surgery because most of the patients are already at an advanced stage at the time of diagnosis (DiMagno et al., 1999; Wray et al., 2005). Gemcitabine or 5-fluorouracil chemotherapy coupled with radiotherapy could improve the quality of life of the patients (DiMagno et al., 1999; Wray et al., 2005), but its survival benefit is very limited. Hence, there has been no substantial improvement in relative 5-year survival rate for pancreatic cancer in the past 3 decades (Jemal et al., 2009). To overcome this dismal situation, development of novel molecular therapies targeting a molecule specifically functioning in pancreatic cancer is eagerly awaited. We had previously performed extensive genome-wide expression profile analysis of pancreatic cancer cells in combination with microdissection to enrich cancer cell population (Nakamura et al., 2004), and demonstrated some genes trans-activated in PDAC cells to be possible molecular targets for development of new therapeutic modalities to treat pancreatic cancers (Taniuchi et al., 2005a,b; Iiizumi et al., 2006; Takehara et al., 2006, 2007; Hosokawa et al., 2007, 2008; Kashiwaya et al., 2009).

Poly(ADP-ribose) polymerase-1 (PARP-1), a nuclear enzyme, catalyzes the transfer of the ADP-ribose unit from its substrate, NAD^+, to some protein acceptors such as histones, p53, and PARP-1 itself. The addition of negatively charged polymers profoundly alters the properties

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and functions of the target proteins. Through its physical association with partner proteins or by the poly(ADP-ribosyl)ation of them, PARP-1 is involved in multiple cellular processes including DNA repair, transcriptional regulation, chromatin modification, cell cycle progression, or genomic stability (Ogata et al., 1981; Kameshita et al., 1984). PARP-1 is a molecular nick-sensor of DNA breaks and has a critical role in the spatial and temporal organization of the DNA repairs (de Murcia et al., 1994). The activation of PARP-1 after DNA damage provides rapid signals to halt transcription and recruits enzymes required for DNA repair to the site of DNA damage, including XRCC1, DNA ligase III, and DNA polymerase β. PARP-1 is essential in the repair of both DNA single-strand breaks (SSB) as well as double-strand breaks (DSB) (Durkacz et al., 1980; D'Silva et al., 1999; Dantzer et al., 2000; Audebert et al., 2004, 2006; Wang et al., 2006). The involvement of PARP-1 in the DNA repair system prompted us to investigate the effect of PARP-1 inhibition on DNA-damaging anticancer therapies (Daniel et al., 2009; Horton et al., 2009). Inhibition of PARP-1 enhanced the cytotoxicity of DNA-damaging agents and seemed to overcome one of the causes of resistance in cancer cells to anticancer treatment (Hoeijmakers et al., 2001; Longley and Johnston, 2005). Currently, several PARP-1 inhibitors have already been taken into the clinical trials as chemopotentiating or radio-potentiating agents, and have shown promising results (Miknyoczki et al., 2007; Plummer et al., 2008; Rottenberg et al., 2008; Horton et al., 2009; Jones et al., 2009; O'Shaughnessy et al., 2009).

We here focus on the characterization of a novel gene *C12orf48* (*Chromosome 12 open reading frame 48*). We demonstrate that C12orf48 protein can interact with PARP-1 directly and be involved in the repair of DNA breaks through enhancing PARP-1 activity. Thus, we termed this molecule PARP-1 binding protein (PARPBP). These findings indicate that C12orf48, or its interaction with PARP-1 could be a promising molecular target for the development of novel treatment for pancreatic cancer.

MATERIALS AND METHODS

Cell Lines

PDAC cell lines, KLM-1, SUIT-2, KP-1N, PK-1, PK-45P, and PK-59, were provided from Cell Resource Center for Biomedical Research, Tohoku University (Sendai, Japan). MIAPaCa-2, Panc-1 and COS7 cell lines were purchased from the American Type Culture Collection (ATCC, Rockville, Maryland). KLM-1, SUIT-2, PK-1, PK-45P, PK-59 and Panc-1, were grown in RPMI 1640 (Sigma-Aldrich, St. Louis, Missouri), and COS7, MIAPaCa-2 in DMEM (Sigma-Aldrich), with 10% fetal bovine serum and 1% antibiotic/antimycotic solution (Sigma-Aldrich).

Semi-Quantitative Reverse Transcription-PCR

Microdissection of PDAC cells and normal pancreatic ductal cells were described previously (Nakamura et al., 2004). RNAs from these cells were subjected to two rounds of RNA amplification using T7-based in vitro transcription (Epicenter Technologies, Madison, Wisconsin). Total RNAs from human PDAC cell lines were extracted using Trizol reagent (Invitrogen) according to the manufacturer's instructions. Extracted RNAs were treated with DNase I (Roche, Mannheim, Germany) and reversely transcribed to single-stranded cDNAs using oligo $(dT)_{12-18}$ primer with Superscript II reverse transcriptase (Invitrogen). The primer sequences were 5′-TTGGCTTGACTCAGGATTTA-3′ and reverse 5′-ATGCTATCACCTCCCCTGTG-3′ for β-*actin* (*ACTB*), and 5′-CTCAGCTGGG AAAGCTACAGAT-3′ and 5′-CATGCCAGGT AGTTCTTCCATC-3′ for *C12orf48* (GenBank Accession no. NM_017915). Each PCR regime involved initial denaturation at 94°C for 2 min followed by 23 cycles (for *ACTB*), 28 cycles (for *C12orf48*) at 94°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min.

Northern Blot Analysis

One μg each of polyA RNA extracted from eight PDAC cell lines (KLM-1, PK-59, PK-45P, MIAPaCa-2, KP-1N, Panc-1, PK-1, and SUIT-2) and seven adult normal tissues (heart, lung, liver, kidney, brain, testis, and pancreas, from BD Bioscience, Palo Alto, CA) was blotted onto a nylon membrane. The 305-bp probe specific to *C12orf48* was prepared by PCR using the primer set described above. The cancer membrane and human Multiple Tissue Northern blot membrane (Clontech, Mountain View, CA) were hybridized with the cDNA probe labeled with α^{32}P-dCTP using Mega Label kit (GE Healthcare, Piscataway, New Jersey). Prehybridization, hybridization, and

washing were performed according to the manufacturer's instruction. The blots were autoradiographed at −80°C for 10 days.

Generation of Antibodies to C12orf48 and Immunocytochemistry/Immunohistochemistry

Plasmids expressing two fragments of C12orf48 (codons 1–150 and 328–498) in pET21a(+) vector (Novagen, Madison, Wisconsin) were constructed to produce recombinant proteins in *E. coli*. The recombinant C12orf48 proteins were purified using Ni-NTA resin agarose (Qiagen, Valencia, CA) and used to immunize rabbits. The sera from the immunized rabbits were purified by antigen-Affi-Gel 10 (Bio-Rad Laboratories, Hercules, CA) affinity column chromatography. For immunocytochemical analysis, KLM-1 cells fixed by 4% paraformaldehyde were incubated with rabbit anti-C12orf48 polyclonal antibody for 1 hr. After washing with PBS, the cells were stained by Alexa 488-conjugated anti-rabbit IgG secondary antibodies (Molecular Probes, Eugene, Oregon) for 1 hr. Stained preparations were mounted with VECTASHIELD mounting medium with DAPI (Vector Laboratories, Burlingame, CA). For immunohistochemistry, tissue sections of PDACs were obtained from the Osaka Medical Center for Cancer and Cardiovascular Diseases under the written informed consent. Human PDAC tissue microarrays were purchased from ISU-ABXIS (Accurate Chemical Corp., Westbury, New York). The sections were deparaffinized and autoclaved at 108°C in Dako Cytomation Target Retrieval Solution High pH (Dako Cytomation, Carpinteria, CA) for 15 min. After blocking, the sections were incubated with rabbit anti-C12orf48 antibody (dilution 1:2,500) at room temperature for 1 hr, washed three times in PBS, and incubated with peroxidase labeled anti-rabbit immunoglobulin (Envision kit; Dako Cytomation). Finally, the reactants were developed with 3,3′-diaminobenzidine. Counterstaining was performed using hematoxylin.

Short-Hairpin RNA-Expressing Constructs

The psiU6BX3.0 vector for expression of short-hairpin RNA (shRNA) was constructed to knock down the expression of the target genes, as described previously (Taniuchi et al., 2005b). The target sequences for *C12orf48* were 5′-CACAGTATCTCCTAGTCAA-3′ (si1), 5′-GTT GCTCAGGATTTGGATT-3′ (si2), 5′-GCAGC TAATGCTCCTACCA-3′ (si3), and 5′-GAAG CAGCACGACTTCTTC-3′ (siEGFP) as a negative control. PDAC cell lines, KLM-1 and SUIT-2, were transfected with each of these shRNA-expression vectors using FuGENE6 (Roche), and selected with Geneticin (GIBCO, 0.5 mg/mL for KLM-1 cells and 0.9 mg/mL for SUIT-2 cells, respectively). Cell viability was measured using cell-counting kit-8 (DOJINDO, Kumamoto, Japan) 6 days after the transfection. Absorbance was measured at 490 nm, and at 630 nm as reference, with a Microplate Reader 550 (Bio-Rad). After 2 weeks of the selection, cancer cells were fixed with 100% methanol and stained with 0.1% of crystal violet-H_2O.

Immunoprecipitation and Mass-Spectrometric Analysis

The pCAGGS Flag-C12orf48-HA vector was constructed by PCR cloning, and was transfected to HEK293 cells. The transfected cells were lysed in lysis buffer (50 mmol/L Tris-HCl [pH 8.0], 0.4% NP-40, 150 mmol/L NaCl, Protease Inhibitor Cocktail Set III [Calbiochem, San Diego, CA]). Cell extracts were precleared by incubation with CL-4B sepharose (Sigma-Aldrich) at 4°C for 1 hr, and incubated with anti-FLAG M_2-agarose (Sigma-Aldrich) for 1 hr. The proteins were separated in 5–20% gradient SDS-PAGE gels (Bio-Rad) and stained with a silver-staining kit (Invitrogen). Protein bands that specifically observed in the cell extracts transfected with pCAGGS Flag-C12orf48-HA were excised and analyzed by liquid chromatography-mass spectrometry (LC-MS/MS). The excised proteins were reduced in 10-mM tris(2-carboxyethyl)phosphine (Sigma-Aldrich) with 50-mM ammonium bicarbonate (Sigma-Aldrich) for 30 min at 37°C and alkylated in 50-mM iodoacetamide (Sigma-Aldrich) with 50-mM ammonium bicarbonate for 45 min in the dark at 25°C. Porcine trypsin (Promega, San Luis Obispo, CA) was added for a final enzyme to protein ratio of 1:20. The digestion was conducted at 37°C for 16 hr. The resulting peptide mixture was separated on a 100 μm × 150 mm HiQ-Sil C18W-3 column (KYA Technologies, Tokyo, Japan) using 30 min linear gradient from 5.4 to 29.2% acetonitrile in 0.1% trifluoroacetic acid (TFA) with total flow of 300 nL/min. The eluting peptides were automatically mixed with matrix solution (4 mg/mL α-cyano-4-hydroxy-cinnamic acid (Sigma-Aldrich), 0.08 mg/mL ammonium citrate in 70% acetonitrile, 0.1% TFA) and spotted

onto MALDI target plates (KYA Technologies). Mass spectrometric analysis was performed on 4800 Plus MALDI/TOF/TOF Analyzer (AB SCIEX Foster City, CA). MS/MS peak list was generated by the Protein Pilot version 2.0.1 software (AB SCIEX) and exported to a local MASCOT search engine version 2.2.03 (Matrix Science) for protein database search.

Flow Cytometry and Synchronization

KLM-1 cells were transfected with *C12orf48*-specific siRNA duplex (5′-CUAGUCAACUA CUGGAUUU-3′), *PARP-1*-specific siRNA duplex (5′-GAUAGAGCGUGAAGGCGAA-3′), and siEG FP duplex (5′-GAAGCAGCACGACUUCUUC-3′) as a negative control, respectively, by using Lipofectamine RNAiMAX (invitrogen) according to the manufacturer's recommendations. 96 hr after the transfection, the cells were fixed with 70% ethanol in PBS at 4°C, and incubated with 500 μL of PBS containing 0.5 mg of boiled RNase at 37°C for 30 min. Finally, 2×10^4 cells stained with 50 μg/mL propidium iodide were analyzed by means of Cell Lab Quanta^{TM} SC MPL Flow Cytometer (Beckman Coulter, USA). A complete block at G1/S-phase was achieved by treatment with 2 μg/mL aphidicolin for 24 hr. Then, cells were released from the cell-cycle arrest, harvested, and prepared for flow cytometry analysis (FACS).

In Vitro PARP-1 Auto-Poly(ADP-Ribosyl)ation Assays

In vitro PARP-1 automodification assays were performed as described previously (Di Palma et al., 2008). Briefly, 200 ng of the purified C12orf48 recombinant protein and 25 ng of the recombinant human PARP-1 (Alexis, San Diego, CA) were incubated in binding buffer (10 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 1 mM DTT) plus 10 μg/mL of sonicated DNA at 37°C for 10 min. The reactions were started by adding ^{32}P-labeled NAD^+, and incubated at 37°C for 10 min. After terminating the reactions with SDS sample buffer, the proteins were fractionated by 8% SDS-PAGE gel. Incorporation of ^{32}P-labeled NAD^+ to poly(ADP-ribosyl)ated proteins was visualized by autoradiography.

PARP-1 Activity in Cell Extracts

KLM-1 and SUIT-2 cells were transfected with *C12orf48*-siRNA, *PARP-1*-siRNA, or siEGFP (as a control), and collected 72 hr after the transfection. The knockdown effects were confirmed with anti-C12orf48 antibody and anti-PARP-1 antibody (Santa Cruz Biotechnology), respectively. PARP-1 activities in cell extracts were assayed using the universal colorimetric PARP assay kit (Trevigen, Gaithersburg, Maryland) based on the incorporation of biotinylated ADP-ribose onto histone H1 proteins. Briefly, cell extracts were loaded into a 96-well plate coated with histone H1, and incubated with biotinylated poly(ADP-ribose) and nicked DNA (Trevigen), size of which are 200–500 base pairs that are considered to be optimal for the PARP activation, for 1 hr. After wash with PBS containing 0.1% (v/v) Triton X-100, streptavidin-HRP (horseradish peroxidase) was added and incubated additionally for 20 min. TACS-Sapphire^{TM} was added subsequently to develop colors and the reaction was stopped by addition of 5% phosphoric acid. Finally, the absorbance was measured at 450 nm in a spectrometrophotometer. PARP-1 enzymatic activities were also evaluated by the use of mouse anti-poly(ADP-ribose) (PAR) monoclonal antibody (Trevigen). 25 μg of the cell extracts obtained from the KLM-1 cells that were transfected with *C12orf48*-siRNA, *PARP-1*-siRNA, or siEGFP (as a control), or 5 ng of recombinant human PARP-1 (Trevigen) were incubated for 20 min at 37°C in binding buffer (10 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 1 mM DTT) plus 10 μg/mL of sonicated DNA, and 200 μM NAD^+ (Sigma-Aldrich).

Sensitivity to DNA Damage

KLM-1 cells were transfected with oligo *C12orf48*-siRNA or siEGFP (as a control), and incubated for 48 hr to knockdown C12orf48 expression as described above. These transfected KLM-1 cells were trypsinized, and the number of living cells was counted. 5×10^5 cells were re-seeded into the 6-well plates, and incubated with indicated concentrations of Adriamycin for 24 hr, H_2O_2 for 6 hr, or exposed to indicated intensity of UV radiation, and then incubated for 24 hr. Cell viability was measured using Cell-counting kit-8 as described above.

RESULTS

Overexpression of C12orf48 in PDAC Cells

Among the transactivated genes that were identified through our genome-wide microarray analysis of pancreatic cancer cells (Nakamura

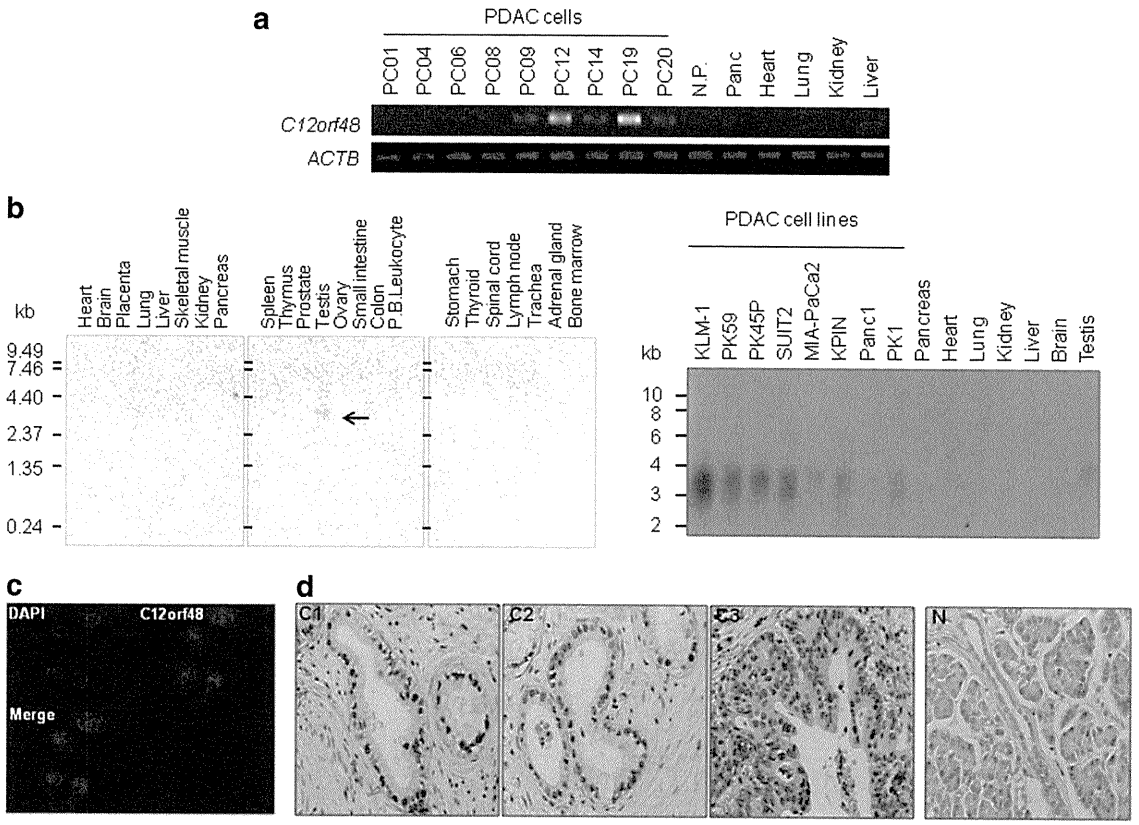

Figure 1. Overexpression of C12orf48 in PDAC cells. (a) Semi-quantitative RT-PCR validated that C12orf48 expression was upregulated in the microdissected PDAC cells (Lanes 5–9), compared with microdissected normal pancreatic ductal cells (NP), whole normal pancreatic tissue (Panc), and vital organs (heart, lung, kidney, and liver). Expression of *ACTB* served as the quantitative control. (b) Left panel; multiple tissue Northern blot analysis showed the limited expression of *C12orf48* in the testis, among the human adult organs. Right panel; Northern blot analysis for *C12orf48* expression showed that several PDAC cell lines (KLM-1, PK-59, PK-45P, and SUIT-2) strongly expressed *C12orf48*, while other normal adult organs did not. (c) Immunocytochemical analysis with anti-C12orf48 polyclonal antibody showed that C12orf48 protein (green) was localized in the nuclei of KLM-1 cells. (d) Immunohistochemical study on PDAC tissues with anti-C12orf48 antibody. C12orf48 was strongly stained in the nuclei of PDAC cells (C1 × 200, C2 × 200, C3 × 200), while it was not stained in acinar cells and ductal epithelium cells of normal pancreatic tissues (N, ×200). In total, 21 of 31 (67.7%) PDAC tissues showed positive staining for C12orf48.

et al., 2004), we here focused on a novel gene *C12orf48* for this study. Semi-quantitative reverse transcription (RT)-PCR confirmed *C12orf48* overexpression in five of the nine pancreatic cancer cases examined (Fig. 1a). Northern blot analysis using the *C12orf48* cDNA fragment as a probe confirmed abundant expression of a 4-kb transcript in most of the eight PDAC cell lines we examined, but its expression was hardly detectable in any normal organs except the testis (Fig. 1b). The predicted C12orf48 protein does not contain any reported motifs or conserved domains in the database, but PSORTII program indicated C12orf48 likely to be a nuclear protein, which was confirmed by following immunocytochemical analysis using anti-C12orf48 polyclonal antibody we generated (Fig. 1c). Moreover, immunohistochemical analysis using anti-C12orf48 antibody showed positive signals in the nuclei of 21 of 31 PDAC tissues (Fig. 1d, panels C1–C3), whereas no staining was observed in any of normal pancreatic tissues (panel *n* in Fig. 1d).

Attenuation of PDAC Cell Viability by C12orf48 Knockdown

To investigate the biological significance of C12orf48 in PDAC cells, we constructed shRNA-expression vectors specific to *C12orf48* (si1, si2, si3) as well as that to siEGFP as a negative control, and transfected each of them into KLM-1 and SUIT-2 cells. Semi-quantitative RT-PCR showed significant knockdown effects on *C12orf48* expression in the cells transfected with si1 and si3, compared with the control (Fig. 2a). MTT assay and colony formation assay revealed that depletion of C12orf48 in KLM-1 and SUIT-2 cells (Figs. 2b and 2c) caused dramatic

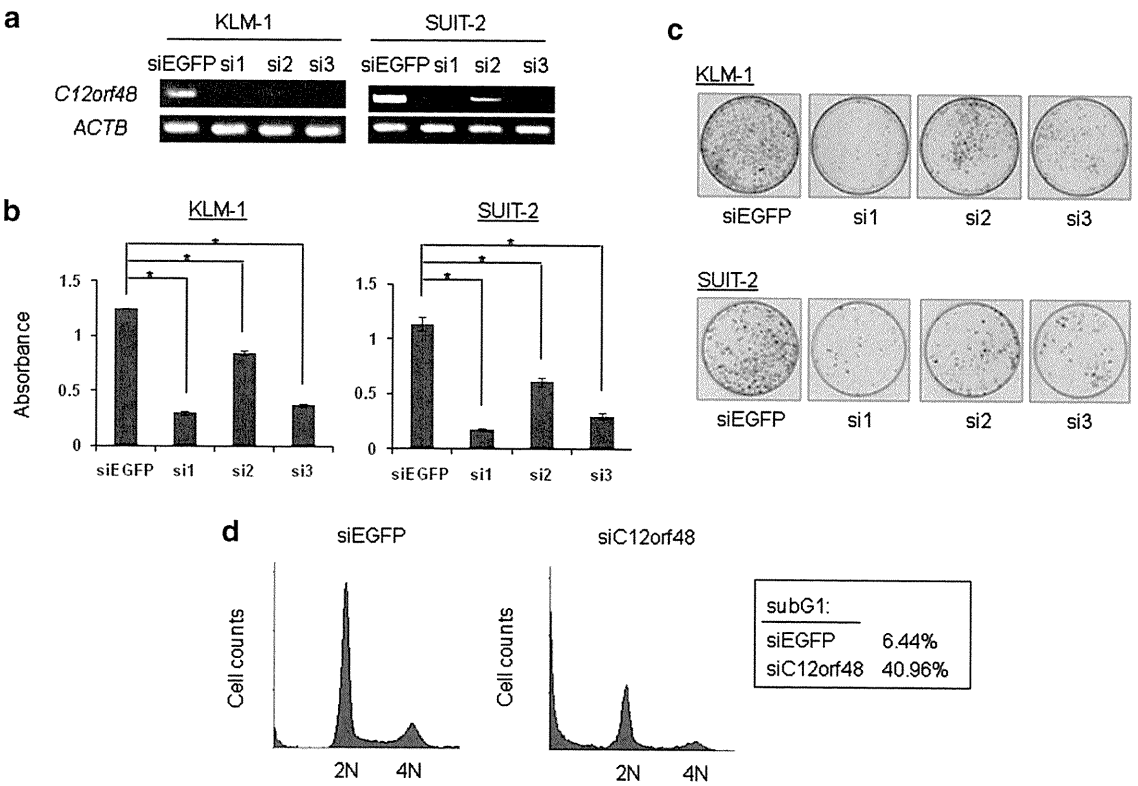

Figure 2. Effect of C12orf48-shRNA on growth of PDAC cells. (a) Semi-quantitative RT-PCR examined the knockdown effect on *C12orf48* expression in PDAC cells (KLM-1 and SUIT-2 cells) transfected with shRNA-expressing vectors specific to *C12orf48* (si1, si2, si3) or control shRNA (siEGFP). (b) PDAC cells transfected with C12orf48 si1, or si3 shRNA vectors showed a drastic reduction in their viabilities. Each average is plotted with error bars indicating the standard deviation (SD) after 6-day incubation with Geneticin. Y-axis means absorbance at 490 nm, and at 630 nm as reference, measured with a microplate reader. These experiments were carried out in triplicate (*$P < 0.005$, Student's *t* test). (c) Colony formation assays in PDAC cells after *C12orf48* knockdown. Cells were stained with 0.1% crystal violet after 14-day incubation with geneticin. (d) FACS analysis was performed 96 hr after transfection with the indicated siRNA. The percentage of cells in subG1 phase was calculated. Treatment of KLM-1 with siRNA specific to *C12orf48* caused a drastic increase in sub-G1 population (40.96%).

reduction in the number of viable cells. Furthermore, we performed FACS analysis after depletion of C12orf48 by siRNA oligonucleotide in KLM-1 cells and found a drastic increase of cells at sub-G1 population (40.96%, Fig. 2d). We also observed similar effects of siRNA oligonucleotide for C12orf48 in SUIT-2 cells (data not shown). These findings indicated that C12orf48 could play critical roles in the growth of PDAC cells.

Interaction of C12orf48 with PARP-1

Since the biological functions of C12orf48 remain totally unknown, we attempted to isolate a protein(s) that could physically interact with C12orf48 protein. Protein complexes were immunoprecipitated by anti-Flag M_2 agarose from the lysates of the HEK293 cells in which Flag-tagged C12orf48 was exogenously introduced. The immunoprecipitated complexes were separated on SDS-PAGE and silver-stained. We found three bands (110, 90, and 63 kDa) in the immunoprecipitated complexes from the lysates of C12orf48-overexprerssing cells, but not in those from the mock cells (Fig. 3a). Among the three bands, the 63 kDa-band was considered to be Flag-tagged C12orf48 itself. We excised 110- and 90-kDa bands, and analyzed them by LC-MS/MS as described in Materials and Methods. As a result, the 110-kDa protein coimmunoprecipitated with C12orf48 protein was identified to be PARP-1 and the 90-kDa protein to be HSP90α (Fig. 3a). Immunoblotting by anti-PARP-1 antibody confirmed that PARP-1 was coimmunoprecipitated with Flag-tagged C12orf48 protein (Fig. 3b). Moreover, C12orf48 was also confirmed to be coimmunoprecipitated with PARP-1 protein (Fig. 3c).

Positive Regulation of PARP-1 Activity by C12orf48

To investigate the functional significance of the interaction between PARP-1 and C12orf48, PARP-1 automodification was investigated by incorporation of [^{32}P]NAD$^+$ in the absence or presence of purified