厚生労働科学研究費補助金

創薬バイオマーカー探索研究事業

重層的・定量的トキシコモディフィコーム解析を用いた安全性バイオマーカーの探索に関する研究

平成20－22年度　総合研究報告書

研究代表者　足立　淳

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重層的・定量的トキシコモディフィコーム解析を用いた安全性バイオマーカーの探索に関する研究

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研究要旨

毒性物質・薬剤の標的因子、作用メカニズムの解明のため、2 次元ゲルや、安定同位体標識技術を用いたショットガン式定量技術を用いたプロテオーム解析が幅広く取り入れられている。これまでのバイオマーカー探索では、蛋白質の“量”の変化が主に測定されてきたが、毒性の作用機序には、“質”の変化、例えばキナーゼ阻害によるリン酸化修飾変化のような、標的因子の“質”（翻訳後修飾）の変化が深く関わっている。そこで本研究では、直接“質”の変化を定量的に捉え、さらに複数の翻訳後修飾を同時に解析し、重層的にデータを統合・解析することで、バイオマーカー探索を行うという戦略を提唱し、そのための基盤整備から有用性の検証まで行うことを目指している。具体的には、研究期間の中で、バイオマーカー探索を行うために必要となる要素技術である、安定同位体アミノ酸標識法を用いた蛋白質の定量、nano LC-MS/MS の高感度化、修飾ペプチド・蛋白質の濃縮技術、バイオインフォマティクス解析についての基礎検討を行い、これらを組み合わせることで、高感度・大規模にマーカー探索を行い、有用なマーカー候補を効率的に絞り込む手法を確立した。本研究で確立された手法は普遍的であり、様々なバイオマーカー探索への貢献が期待される。

A. 研究目的

本研究は、安全性バイオマーカーの探索対象として、蛋白質の“質”の変化に注目し、システイン酸化・ユビキチン化・リン酸化の 3 種の蛋白質翻訳後修飾を解析し、解析データを一体化し、バイオインフォマティクス解析を行うことで、安全性バイオマーカーや毒性シグナルネットワークを推定することを研究目的とする。この目的設定の背景として、蛋白質翻訳後修飾は細胞内シグナル伝達等様々な機能を担い、毒性機序にも深く関わっているが、トランスクリプトーム解析では測定できないこと、近年の技術進歩により効率的な翻訳後修飾部位の濃縮が可能になりつつあり、毒性学研究への応用可能性が高まっていることが挙げられる。毒性学研究での大規模な翻訳後修飾解析例はほとんど例がなく、毒性物質の翻訳後修飾への影響を明らかにする点が、本研究の特色と言える。研究を構成する要素として、本研究で採用するプロテオーム定量法である、安定同位体標識アミノ酸による培養細胞内蛋白質標識法（SILAC 法）、システイン酸化・ユビキチン化・リン酸化の翻訳後修飾解析法、LC-MS/MS を用いた蛋白質の同定・

定量、バイオインフォマティクスが挙げられる。H20 年度は主に要素技術の基礎検討を行い、H21 年度は、要素技術の基礎検討と並行して、ダイオキシン受容体リガンドかつ CDK 阻害剤であるインディルビンを用いてリン酸化・システイン酸化プロテオーム解析を行った。H22 年度はトキシコモディフィコーム解析システムの有用性を検証することを目的として、リン酸化プロテオーム、ユビキチン化プロテオーム解析手法を用いて、ヒドロキシ尿素もしくはガンマ線照射による DNA 損傷応答を解析した。定量データを基に、複数のバイオインフォマティクス解析を行い、その結果を重層的に取り込むことによって、大規模データからマーカー候補を効率的に絞り込むことを試みた。

B. 研究方法

リン酸化プロテオーム解析（H20-21 年度）

[$^{12}C_6$]アルギニンと[$^{12}C_6$]リジンもしくは[$^{13}C_6$]-アルギニンと[$^{13}C_6$]-リジンで標識した SILAC-HepG2 細胞を用意した。一方にインディルビンを 1 μM、他方にコントロールとして DMSO をそれぞれ 10 分間曝露し、曝露後、等量混合した。変動したリン酸化ペプチド・蛋白質を検出するために、酸化チタンビーズ、チロシンリン酸化抗体による濃縮法を併用した。

○酸化チタンビーズを用いたリン酸化ペプチド濃縮

上述の SILAC-HepG2 細胞を等量混合し、細胞溶解し、細胞質画分を調製した。細胞質画分を定法に従いリシルエンドペプチダーゼ C とトリプシンで消化した後、ペプチドを C18 カートリッジを用いて固相抽出した。続いて酸化チタンビーズ（GL Science）にリン酸化ペプチドを選択的に結合させた。なお選択性を高めるエンハンサーとして 25%酪酸を用いた。その後、5%アンモニアと 0.5%ピペリジンでリン酸化ペプチドを溶出し、C18 Stagetip で脱塩濃縮して LC-MS/MS に供した。LC-MS/MS の設定、データ解析方法は以下の通りである。C18 Stagetip から溶出させたペプチドを 0.5%酢酸、5%アセトニトリルに溶解させて LC (Hitachi nano LC)-MS/MS (Waters QTof ultima) で分析した。カラムは ReproSil-Pur C_{18}-AQ 3 μm resin (Dr. Maisch GmbH)を長さ 10 cm 内径 50 μm のヒューズドシリカチューブにパックしたものを使用し、移動相は A: 2%アセトニトリル、0.5%酢酸、B: 98%アセトニトリル、0.5%酢酸によるリニアグラジエント（流速 200 nl/min、測定時間 85 分）であった。MS 及び MS/MS 測定は MS1 秒、第 1MS/MS～第 3MS/MS 各 2 秒の合計 7 秒の自動測定（Data dependent analysis）で行い、*m/Z* 300-750、750-900、900-1100、1100-1600 の 4 分割したレンジで MS 測定した。データ処理は解析ソフト Mascot Server と Mascot Distiller を用いて行い、デコイデータベースによる検索で偽陽性確率が 5%以内となるように同定基準を定めた。

○チロシンリン酸化抗体による濃縮

上述の SILAC-HepG2 細胞を等量混合し、チロシンリン酸化抗体ビーズ（4G10、ミリポア）を用いて、チロシンリン酸化蛋白質を濃縮した。濃縮した蛋白質を 1 次元電気泳動で分離し、ゲル内消化法を用いてペプチドに断片化し、C18 Stagetip で脱塩濃縮して LC-MS/MS に供した。LC-MS/MS の設定、データ解析方法は上述の手法に以下の変更点を加えて行った。MS 及び MS/MS 測定は MS1 秒、第 1MS/MS～第 5MS/MS 各 1 秒の合計 1 秒の自動測定（Data Dependent Analysis）で行い、*m/Z* 300-1600 のレンジで MS 測定した。

リン酸化プロテオーム解析（H22 年度）

[$^{12}C_6$]アルギニンと[$^{12}C_6$]リジン(light)、[$^{13}C_6$]-アルギニンと[$^{2}D_4$]-リジン(Medium)、[$^{13}C_6$, $^{15}N_4$]- アルギニンと [$^{13}C_6$, $^{15}N_2$]リジン(Heavy)で標識した 3 種類の SILAC-Hela-S3 細胞を用意した。6 Gy のガンマ線を照射し、1 分、5 分、20 分、60 分後に細胞を回収し、等量混合した。照射後の培養時間と SILAC 標識の種類の組み合わせを変えることで、合計 3 回の繰り返し実験を行った。なお予備検討として、ヒドロキシ尿素を 2 mM、1 時間曝露した。

変動したリン酸化ペプチド・蛋白質を検出するために、Fe-IMAC 法を用いた。

○Fe-IMAC 法を用いたリン酸化ペプチド濃縮

上述の SILAC-Hela-S3 細胞から total lysate を 6 mg 分等量混合し、定法に従いリシルエンドペプチダーゼ C とトリプシンで消化した後、ペプチドを C18 カートリッジを用いて固相抽出した。続いて Nickel-Chelating Resin（invtrogen）のニッケルを鉄に置換させたレジンを用いて、リン酸化ペプチドを選択的に結合させ、1%リン酸でリン酸化ペプチドを溶出し、C18 カートリッジを用いて脱塩濃縮した。リン酸化ペプチドは、陽イオン交換カラム (ZORBAX 300SCX, agilent)で 20 分画した後に C18 Stagetip を用いて脱塩濃縮し、LC-MS/MS に供した。LC-MS/MS の設定、データ解析方法は以下の通りである。

C18 Stagetip から溶出させたペプチドを 0.5%酢酸、4%アセトニトリルに溶解させて LC (AMR, Paradigm)-MS/MS (Thermo, LTQ-Orbitrap Velos) で分析した。カラムは L-column ODS 3 μm resin (化学物質評価研究機構)を長さ 20 cm 内径 100 μm のヒューズドシリカチューブに充填したカラムを使用し、移動相は A: 2%アセトニトリル、0.1%蟻酸、B: 90%アセトニトリル、0.1%蟻酸によるグラジエント（流速 500 nl/min、測定時間 120 分）であった。MS は *m/Z* 350-1500 を orbitrap で測定し、MS/MS 測定は Data dependent analysis (top 20 peak/scan)で CID 法でフラグメント化後、LTQ で測定した。データ処理は解析ソフト Mascot Server(version 2.3) と MaxQuant(version 1.0.0.13)を用いて行い、ペプチド・蛋白質の偽陽性同定率が 1%以下となるように解析した。またリン酸化部位については MaxQuant によって判定されたクラス 1 レベル（同定確率 75%以上）のリン酸化部位のみを対象に解析した。

システィン酸化プロテオーム解析

上述の SILAC-HepG2 細胞の一方にインディルビンを 1 μM、他方にコントロールとして DMSO を 8 時間曝露し、曝露後、等量混合し、細胞質画分を調製した。ヨード酢酸で分画中の蛋白質の還元型システイン(-SH)をブロックし、次に還元剤（DTT）で酸化型システインを還元し、Biotin-HPDP タグで標識した。その後、トリプシン消化、ストレプトアビジンビーズによる標識ペプチドの精製を行い、タグの付いたペプチドを脱塩濃縮してナノ LC-MS/MS でペプチドの同定・定量を行った。LC-MS/MS の設定、データ解析方法はチロシンリン酸化抗体による濃縮時と同じ手法を用いた。

ユビキチン化プロテオーム解析

上述のヒドロキシ尿素を 2 mM、1 時間曝露した SILAC- Hela-S3 細胞を等量混合し、K48 結合型ユビキチン抗体、K63 結合型ユビキチン抗体を用いて、ユビキチン化蛋白質を濃縮した。濃縮した蛋白質を 1 次元電気泳動で分離し、ゲル内消化法を用いてペプチドに断片化し、C18 Stagetip で脱塩濃縮して LC-MS/MS に供した。LC-MS/MS の設定、データ解析方法はリン酸化プロテオーム解析と同じ手法を用いた。

（倫理面への配慮）

本研究ではサンプルとして培養細胞を用いるため、人権・動物愛護に関する問題には抵触しません。

C. 研究結果

SILAC法の定量精度について

SILAC 法は実験の初期段階で曝露群と非曝露群を混合し、その後の操作過程による影響を受けないため、極めて優れた定量精度を持つ。H20 年度は 1.5 倍以上の差異を検出可能であることを確認したが、H21 年度は、曝露群と対照群の細胞を入れ替えるスワップ実験をすることによって、1.33 倍以上の差異を検出可能であることを確かめた。蛋白質の翻訳後修飾サイトの同定は、通常の非修飾ペプチド配列の同定と比較して、擬陽性確率が

高まるが、SILAC 法を用いた場合、同一配列の「軽い」ペプチドと「重い」ペプチドが偶然同一配列のペプチドに誤同定される確率は極めて低く、また MS/MS スペクトルを比較することで、y イオン、b イオンシリーズの判別がつくため (y イオンシリーズのみがリジンもしくはアルジニンを含むため、質量差を有する)、擬陽性確率を減らすことができる。この SILAC 法のメリットは特にリン酸化プロテオーム解析にて、ペプチド同定が正しいかどうか、MS/MS スペクトルをマニュアルでチェックする際に大きく貢献した。この利点は iTRAQ 法では得られないものであり、SILAC 法の大きなメリットである。

リン酸化プロテオーム解析

H21 年度はインディルビンを曝露して 10 分後という短期間の non genomic な影響を、リン酸化ペプチドを濃縮する手法とチロシンリン酸化蛋白質を濃縮する方法を併用して調査した。その結果 702 個の蛋白質、794 個のリン酸化ペプチド、961 個のリン酸化サイトを同定した。そのうち 1.33 倍以上に増減が見られた蛋白質は ADP-ribosylation factor GTPase-activating protein 2 (0.65 倍)、Tight junction protein ZO-2 (1.36 倍)、FAM134C(1.42 倍)の 3 個であった。

チロシンリン酸化抗体による濃縮法では、236 個の蛋白質を同定した。そのうち 1.33 倍以上に増減が見られた蛋白質は Non-receptor tyrosine-protein kinase TYK2 (1.67 倍)、GTF21(1.50 倍)、40S ribosomal protein S10 (1.40 倍)、β actin(0.53 倍) の 4 個であった。上記蛋白質のリン酸化はこれまでダイオキシン受容体シグナルの対象としては知られていないものであった。

リン酸化ペプチドを濃縮する手法とチロシンリン酸化蛋白質を濃縮する方法で同定された蛋白質は大部分が重複していないため、リン酸化蛋白の同定数を増やすために有効であった。リン酸化ペプチドを濃縮する手法では、リン酸化サイトを直接同定・定量することが出来る反面、そのペプチドがうまくフラグメント化しなかったり、イオン化効率が低いなどの理由で同定できなかった場合も多かった。一方で蛋白質を濃縮する方法では、1 次元のゲルによって、細かく分離することができ、分子量情報も得られるが、リン酸化サイトまで同定できることはまれであった。

H22 年度は Fe-IMAC 法を用いたリン酸化プロテオーム解析を行った。これまでの研究で培ってきた高感度化技術を導入し、さらに質量分析計を LTQ-Orbitrap velos を用いることでさらなる高感度化を図った結果、ガンマ線照射サンプルにおいて 5041 個の蛋白質、27422 個のリン酸化部位を同定した。これは、過去に報告された DNA 損傷応答におけるリン酸化プロテオーム解析データで最も大規模な研究(Sci.Signal., 3, 151, re3, 2010)で同定された 1099 蛋白質、2871 部位を大幅に上回る規模である。さらに定量性についても、SILAC 法の定量性については、triplicate 実験を 2 回繰り返して、定量値の再現性で評価すると、SD 値が 0.31~0.33 の範囲であった（図 1）。リン酸化修飾の定量はペプチドレベルで行わなければならず、蛋白レベルの定量に比べて、定量精度が落ちるが、本研究では、照射後の培養時間と SILAC 標識の種類の組み合わせを変えて、合計 3 回の繰り返し実験を行うことで、上述の定量精度を確保することができた。

2 倍以上に増減が見られた蛋白質は 2473 個、リン酸化部位は 6555 個と極めて多くのリン酸化部位がガンマ線照射によって変動していることが明らかになった。これは今までの予想を遙かに超える数であり、DNA 損傷応答が極めて多くの蛋白質に影響を及ぼしていることを示唆している。またこれまでに知られている DNA 損傷応答蛋白質のリン酸化についても、既知部位だけでなく、未知部位も数多く同定された（図 2）。これらの変動リン酸化蛋白質は、DNA 損傷のマーカーもしくは機能因子候補であるが、これだけ多数の候補を検

証することは困難なので、さらにバイオインフォマティクス解析を駆使して、有力な候補を絞り込むことを試みた。

システイン酸化プロテオーム解析

DTT によって可逆的に酸化されるシステインを含むペプチドを 115 個、蛋白質を 107 個同定した。表 3 に示すようにこのなかで 1.33 倍以上に増減するペプチド、蛋白質は検出されなかった。

ユビキチン化プロテオーム解析

H21 年度は、解析手法の確立を目的として、プロテアソーム阻害剤 MG132 の有無による、蛋白質の定量を行った。具体的には K48 結合型ユビキチン抗体を用いた免疫沈降法で濃縮したユビキチン化蛋白質を 1 次元電気泳動で分離し、ゲル内消化法を用いてペプチドに断片化し LC-MS/MS で同定、定量を行った結果、699 個の蛋白質が同定された。そのうち 0.75 倍以下に減少する蛋白質を 185 個、1.33 倍以上に増加する蛋白質は 66 個同定した。この中には、HSP90 のように、同定された分画によって、ratio が異なる蛋白質も見られた。

H22 年度は、DNA 損傷を引き起こすヒドロキシ尿素を曝露したサンプル中のユビキチン化修飾蛋白質を、K48 結合型、K63 結合型ユビキチン抗体を用いた免疫沈降法で濃縮し、1 次元電気泳動で分離し、ゲル内消化法を用いてペプチドに断片化し LC-MS/MS で同定を行った。その結果、K48 結合型ユビキチン抗体を用いた場合 740 個、K63 結合型ユビキチン抗体を用いた場 723 個の蛋白質が同定された（図 3)。同時にリン酸化プロテオーム解析も行い、K48-Ubi プロテオーム、K63-Ubi プロテオーム、リン酸化プロテオームの重なりを比較すると、合計 2022 個同定された中で、それぞれのプロテオーム解析で同定された蛋白質は、296 個、265 個、941 個であった。また K48-Ubi プロテオームと K63-Ubi プロテオームの違いを Gene Ontology 解析で調べると、K48-Ubi プロテオームではプロテアソーム複合体、リボソーム、蛋白質分解が濃縮されており、K63-Ubi プロテオームでは、RNA プロセッシング、シグナル伝達が濃縮されており (図 4)、それぞれの抗体を用いた濃縮法の有効性が示唆された。

データ解析

Mascot Server システムを導入し、LC-MS/MS による蛋白質同定・定量解析処理作業の自動化を進め、処理効率を大幅に向上させた。さらに、パスウェイ解析、ジーンオントロジー解析、学習型蛋白質機能推定解析を行い、試験物質が作用する蛋白質、シグナル経路、細胞内部位、細胞内機能を推定するための情報処理基盤を整備した。

バイオインフォマティクス解析

基質からのリン酸化酵素予測には、NetWorkin2.0 を用いて、同定されたすべての基質についてそのリン酸化酵素を予測した。ガンマ線未照射サンプルと比較して、リン酸化レベルが上がっている群（2 倍以上)、下がっている群（0.5 倍以下)、その他の群に分けて、各群中の予測リン酸化酵素のカウントが有意に上昇しているか減少しているかをフィッシャーの正確検定を用いて、p 値を算出した。その結果、DNA 損傷に応答することがしられている ATM/ATR の活性化が予測された他、リン酸化酵素（kinaseX）の活性化が予測された。Networkin によって予測された kinaseX の基質群を用いてネットワーク解析を行うと、ATR や Chk1 など DNA 損傷に関わる蛋白質が蛋白質ネットワークを形成していた。さらにゲノムワイドな siRNA ノックダウン法を用いたガンマ線感受性遺伝子の探索結果からも kinaseX は感受性であると報告されており（Genes & Dev., 24, 1939-1950, 2010)、kinaseX が DNA 損傷応答に関与する有力な候補蛋白質と思われる。DNA 損傷応答における kinaseX の機能解析は今後の課題である。また上述のように、大規模かつ正確な翻訳後修飾定量データと様々なバイオインフォマティクス解析を組み

合わせることで、目的の事象を象徴するバイオマーカーや、機能因子を絞り込んで、検証段階に橋渡しできる可能性を示すことができた。

D. 考察

C.研究結果の欄に記載

E. 結論

バイオマーカー探索を行うために必要となる要素技術である、安定同位体アミノ酸標識法を用いた蛋白質の定量、nano LC-MS/MS の高感度化、修飾ペプチド・蛋白質の濃縮技術、バイオインフォマティクス解析についての基礎検討を行い、これらを組み合わせたトキシコモディフィコーム解析システムを構築した。本システムを用いて高感度・大規模にマーカー探索を行い、有用なマーカー候補を効率的に絞り込む手法を確立した。

F. 健康危険情報

なし

G. 研究発表

① 論文発表

1 .Sasaki K, Nishida Y, Adachi J, Okawa K, Nakayama A, Yoneda M, and Morisawa S. Proteomic Analysis for the Purpose of Understanding the Mechanisms of Benzene and X-ray Induced Leukemia Using Human Bone Marrow Cells. Journal of Proteomics & Bioinformatics, 2010; 3(3): 66-73.

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H. 知的財産権の出願・登録状況

特になし

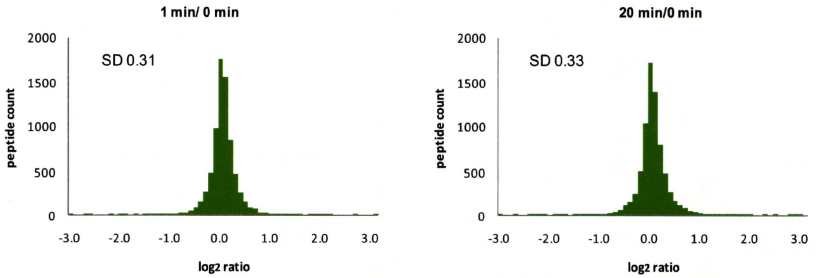

図 1　SILAC 法によるリン酸化プロテオーム解析の定量精度

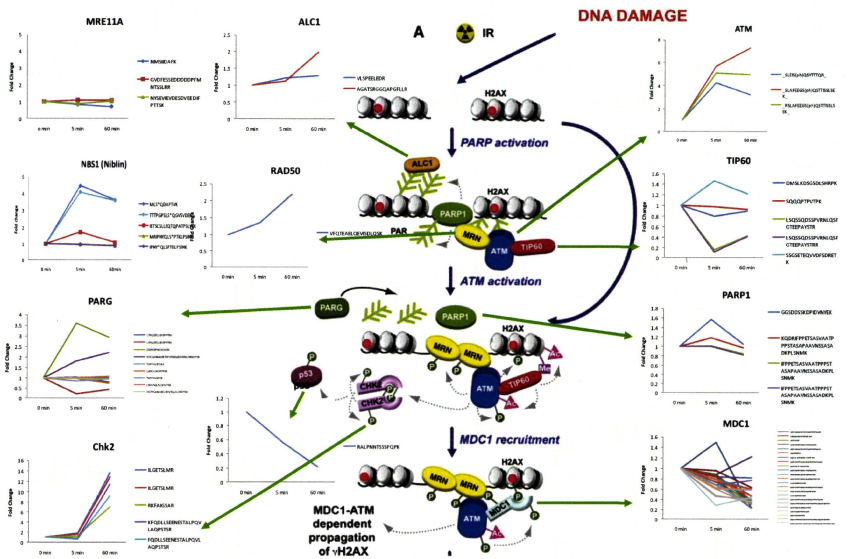

図 2　DNA 損傷初期応答因子のリン酸化ダイナミクス

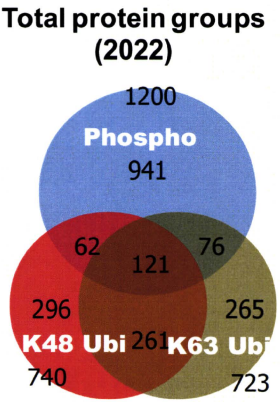

図 3 ヒドロキシ尿素曝露時のリン酸化・ユビキチン化プロテオーム解析結果

K48,K63結合型ユビキチンプロテオームで濃縮されているGO term

K48-link

Enriched GO term	K48 (%)	K63 (%)	p value
proteasome complex	11.71	0.99	5.04E-10
ribosome	22.55	8.23	9.13E-07
proteolysis	14.73	5.38	6.08E-05

K63-link

Enriched GO term	K48 (%)	K63 (%)	p value
RNA processing	5.36	16.56	1.98E-05
response to unfolded protein	0	3.43	2.41E-03
signal transduction	7.69	14.98	6.16E-03

図 4 K48-Ubi プロテオーム、K63-Ubi プロテオームで濃縮されている GO term

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
Sasaki K, Nishida Y, Adachi J, Okawa K, Nakayama A, Yoneda M, and Morisawa S.	Proteomic Analysis for the Purpose of Understanding the Mechanisms of Benzene and X-ray Induced Leukemia Using Human Bone Marrow Cells.	Journal of Proteomics & Bioinformatics	3(3)	66-73	2010

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Proteomic Analysis for the Purpose of Understanding the Mechanisms of Benzene and X-ray Induced Leukemia Using Human Bone Marrow Cells

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Abstract

Benzene and the ionizing radiation are well known as leukemogens. There have been many studies on leukemia accumulated, but the mechanisms underlying the leukemogenicity are not fully understood. Since there are differences and similarities in leukemogenesis by benzene and radiation, comparative analysis could offer insight toward understanding basic leukemogenesis. In this study, we extracted proteins from CD34+ cells from human bone marrow, the target organ of leukemia, exposed to benzene metabolites (catechol and hydroquinone) or/and X-rays, and performed two dimensional gel image analysis. As a result, we identified 8 proteins specific to benzene metabolites exposure, and 14 to X-ray irradiation. Notably, we found 2 proteins, protein SET and cofilin-1, which showed changes in expression levels common to both benzene metabolites and X-ray exposure. These results suggest that the SET-PP2A-JNK pathway might play a key role in the mechanisms of the leukemia.

Keywords: Benzene; Human bone marrow cells; Protein SET; Proteomics; Radiation; Two-dimensional gel electrophoresis

Abbreviations: ALL: Acute Lymphocytic Leukemia; AML: Acute Myeloid Leukemia; BEIR: Committee on the Biological Effects of Ionizing Radiation; CAT: Catechol; CERI: Chemicals Evaluation and Research Institute; CC100: Cytokine Cocktail 100; CLL: Chronic Lymphocytic Leukemia; CML: Chronic Myeloid Leukemia; DDA: Data Dependent Analysis; HQ: Hydroquinone; IEF: Isoelectric Focusing; IPCS: International Programme on Chemical Safety; IPG: Immobilized PH Gradient; JNK: Jun N-terminal Kinase; LC: Liquid Chromatography; LET: Linear Energy Transfer; MALDI-TOF: Matrix-Assisted Laser Desorption/Ionization Time-Of Flight; MS: Mass Spectrometry; PAGE: Polyl-Acrylamide Gel Electrophoresis; PP2A: Protein Phosphatase 2A; ROSs: Reactive Oxygen Species; SDS: Sodium Dodecyl Sulfate; SFEM: Serum-Free Expansion Medium; 2DE: Two Dimensional gel Electrophoresis

Introduction

Benzene (C_6H_6) is an important industrial chemical. It is commonly used as an industrial solvent and synthetic material, and the main sources of exposure are cigarette smoke and exhaust gas of gasoline (Chemicals Evaluation and Research Institute (CERI), 1997). Benzene is known to cause adverse health effects on humans such as acute myelogenous leukemia. Epidemiological studies of benzene-exposed workers have demonstrated a causal relationship between benzene exposure and the induction of myelogenous leukemia (International Programme on Chemical Safety (IPCS), 1993). Among them, Pliofilm cohort study from 1970 is a sufficiently large well-studied cohort with enough benzene exposure to lead benzene risk estimation statistically. When benzene is taken into the body, it is metabolized to various metabolites by the action of metabolic enzymes such as cytochrome P-450 and myeloperoxidase (Parke, 1996). Benzene itself is a stable substance, but some of these metabolites have cytotoxicity and mutagenicity, and they are thought to contribute to the development of benzene-induced leukemia (Sammett et al., 1979). Among various metabolites, catechol (CAT) and hydroquinone (HQ) are reported to have an especially close connection with the leukemogenesis of benzene (Robertson et al., 1991). There have been many studies on benzene-induced leukemia accumulated, but the mechanisms underlying benzene-induced leukemogenicity are still not fully understood. The analysis of the effects of CAT and HQ on human bone marrow, which is the target organ of leukemia, is beneficial for getting better understanding on the mechanisms of benzene-induced leukemogenicity. In such analysis, the synergistic effects of CAT and HQ should be considered because CAT and HQ are simultaneously in bone marrow of humans and because it has reported that the combination exposure of CAT and HQ showed stronger toxicity than the individual exposure of CAT of HQ (Roberston et al., 1991; Igarashi, 2004, Kyoto University, Japan, unpublished observation; Levay and Bodell, 1991; Stillman et al., 1999).

As well as benzene, the radiation exposure causes various health effects including leukemia. It has been reported that the exposure to 1Gy of radiation leads to an increasing incidence of cancers by 0.05-0.16% (Bertell, 1984). 0.1Gy of low Linear En-

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ergy Transfer (LET) radiation has also been estimated to increase the carcinogenic rate by 0.5-1.4% (Committee on the Biological Effects of Ionizing Radiation (BEIR), 1980). In addition, it was shown that gene mutations could be induced artificially by X-ray exposure (Muller, 1927). X-ray is a form of electromagnetic radiation with a wavelength in the range of 0.01 to 10 nanometers. It is primarily used for medical diagnostics with the help of its high penetration. Today the exposure from medical X-rays has the highest percentage of exposure from man-made radiation. X-ray has been frequently used for biological study because the X-ray machines are easier to handle than other radiation sources.

Both benzene and radiation are similarly known to induce leukemia. They induce in common some DNA damages to human bone marrow cells, resulting in the induction of leukemia. For example, it was reported that benzene and radiation induced same kinds of chromosome aberrations such as t(8;21) and the losses of long arms or whole chromosome 5 and 7, which were thought to have some connections with leukemogenesis (McHale et al., 2008; Deininger et al., 1998; Domracheva et al., 2002). These results suggested that the inductions of leukemia by benzene and radiation exposure might have some similar mechanisms of action. However, there are also some differences. At first, there is a difference on the subtypes of leukemia induced by benzene and radiation. The relationship of benzene with acute myeloid leukemia (AML) is already proven, but there was no persuasive evidence the link of benzene to other subtypes including acute lymphocytic leukemia (ALL), chronic myeloid leukemia (CML), and chronic lymphocytic leukemia (CLL) (Schnatter et al., 2005). On the other hand, there was strong evidence of radiation-induced risks for all subtypes of leukemia (Preston et al., 1994). In addition, there have been another differences in the mechanisms of leukemia by them. In radiation-induced leukemia, DNA damages of bone marrow cells, which are the primary steps of leukemogenesis, is inducted mainly by two kinds of actions: one is the direct action in which the exposed radiation ionizes or excites the atoms constituting DNA and DNA damages are directly generated, and the other is the indirect action in which water molecules are ionized or excited by radiation and free radicals are produced, then these radicals induce DNA damages (Iida, 2009). On the other hand, in benzene-induced leukemia, DNA damages are mainly induced by binding of benzene metabolites such as CAT and HQ to intracellular molecules or by reactive oxygen species (ROSs) generated by the oxidation of benzene metabolites (Irons, 1985). It has also reported that benzene metabolites inhibit enzymes involved in DNA replication and maintenance such as topoisomerases, which are likely to contribute to benzene-induced leukemias (Eastmond et al., 2005; Lindsey et al., 2005).

Since there are differences and similarities in leukemogenesis by benzene and radiation described above, comparative analysis could give the insight of understanding basic leukemogenesis. As this comparative analysis, we conducted a comprehensive proteomic analysis by two-dimensional gel electrophoresis (2DE) using $CD34^-$ cells from human bone marrow, which are hematopoietic stem cells and thought to be the target of leukemia.

Proteins are involved with various cancers including leukemia. Mutations on a gene level are very important as the first steps in carcinogenesis. However, the change in protein expression alters more directly biological process than gene expressions and the essence of carcinogenesis is that mutant genes produce abnormal proteins and these proteins can not fulfill their original functions (Hirai, 1994). A translocation t(8;21) is one of the high frequency chromosome aberrations observed in acute myeloid leukemia. In this aberration, the AML1 gene on chromosome 21 and the MTG8 gene on chromosome 8 fuse together, and formed the chimera gene AML1/MTG8. The fusion protein produced by this chimera gene is regarded as a protein involved in leukemogenesis by disturbing the differentiation of myeloid cells to mature granulocytes. Additionally, one group recently reported that the primary target of radiation-induced leukemia might be not DNA itself but rather proteins (Kumagai et al., 2003; Suzuki et al., 2005; Urushibara et al., 2004). Therefore, monitoring the change of proteins may be useful as a means of getting better understanding the mechanisms underlying leukemia. Combined with the progress of analysis technology, recent researchers have actively analyzed the protein expression profile in cancers, and tumor markers are often used for the diagnosis and cure (Honda et al., 2005).

2DE is a form of gel electrophoresis commonly used for protein analysis. Using this method, we can analyze the changes of so many kinds of proteins at a time comprehensively. So, 2DE must be helpful for shedding light on the similarities and differences in leukemogenesis by benzene and radiation. Hence, we applied 2DE in this study to find proteins specific to benzene metabolites (CAT and HQ) or/and X-ray irradiation in human bone marrow cells.

Materials and Methods

Reagents and cells

Reagents were obtained from WAKO (Osaka, Japan) unless otherwise stated.

$CD34^-$ cells used here were purchased from Lonza. (Hispanic, female, twenty six years old).

$CD34^-$ cells were cultured at 1.3 to 2.0×105 cells/mL in StemSpan® Serum-Free Expansion Medium (SFEM) (StemCell Technolgies, Inc.) supplemented with 1% StemSpan® Cytokine Cocktail 100 (CC100) (StemCell Technologies, Inc.). The cultures were incubated in a humidified atmosphere of 5% CO_2 in air at 37 degrees C for 6 days until cultures reach early- or mid-log phase.

Chemical treatment and X-ray irradiation

For chemical treatment, the half amount of medium was exchanged for new medium after 6 days of culture, and cells were seeded at a density of 5.0×10^4 cells/mL in a 100-mm dish and treated for 30 hr with CAT or HQ dissolved in dH_2O at the concentrations of 6 μM, or 1% of dH_2O. Cells were also treated with the mixture of CAT and HQ at the concentration of 6 μM (CAT 2 μM + HQ 4 μM). This ratio was determined according to literature, which reported the median values of CAT and HQ in the urine of exposed workers (1 to 25 ppm, n=20) were 7.2 and 16.4, 25 percentile values were 5.2 and 9.8, and 75 percentile values were 14.6 and 31.9, respectively (Rothman et al., 1998).

For X-ray irradiation, cells were re-seeded at a density of

1.0×10^5 cells/mL in a 50-mm dish. An X-ray exposure machine, Radioflex 350 (Rigaku, Tokyo, Japan) was used for the X-ray irradiation (5 Gy/min, 250 kV, 15 mA, Al 2 mm filter). The irradiated doses were 0, 0.5, 1 and 1.5 Gy. After irradiation, cells were cultured in the same medium for 30 hr.

Cytotoxicity analysis

Harvested cells of each treatment condition or X-ray irradiation were counted in a hematocytometer by using a phase-contrast microscope (Axiovert 25 ,Carl Zeiss, Germany). The survival rate was defined as the cell number ratio between treated cells and non-treated (control) cells. The Student's t-test was applied to test the difference of the survival rates between each dose and control.

Protein extraction

Proteins were extracted from $CD34^+$ cells by ultrasonication using SONIFIER150 (BRANSON, Japan) directly in 100 mL of cellular lysis buffer containing 8 M urea, 4% CHAPS (GE healthcare bioscience, UK), and 40 mM Tris. Ultrasonication was conducted at 4 W for 20 sec, and after that, solutions were set on ice for 30 sec. This process was repeated five times. The suspension was centrifuged at 15000 rpm for 10 min at 4 °C. For 2-DE, interfering components were removed using the 2-D Clean-Up Kit (GE healthcare bioscience), and proteins were diluted in cellular lysis buffer described above. The protein concentration was determined using the 2-D Quant Kit (GE healthcare bioscience). The coefficients of variation of these extraction processes were less than 15% (n = 5 per exposure condition).

2DE

For 2DE, we used 50 μg of proteins per gel. 2DE was performed in the following method.

Proteins were resuspended in 450 μL of buffer containing 8 M urea, 4% CHAPS, 40 mM Tris, 0.28% DTT (GE healthcare bioscience), and 0.5% immobilized pH gradient (IPG) buffer (GE healthcare bioscience). IPG gel strips (24 cm, pH 4-7, GE healthcare bioscience) were rehydrated with samples for 2DE using Immobiline DryStrip Reswelling Tray (GE healthcare bioscience) for 16 hr at room temperature. In order to avoid drying during the rehydration, DryStrip cover fluid (GE healthcare bioscience) was piled up on the strips. After the rehydration, isoelectric focusing (IEF) was performed with an Ettan IPGphor II electrophoresis unit (GE healthcare bioscience) for a total of 46.9 kVh at room temperature. The detailed conditions for IEF is 1 hr at 100 V, 1 hr at 500 V, gradually increased to 1000 V over 7 hr, then gradually increased to 8000 V over 3 hr, and finally run at 8000 V for 3.45 hr.

Before the second dimensional separation, each focused IPG strip was equilibrated, firstly in a buffer (50mM Tris-HCl, 6M urea, 30% glycerol, 2% SDS, 0.002% bromophenol blue (MP biomedicals), pH8.8) containing 1% DTT for 15 min, and then in the same equilibration buffer containing 2.5% iodoacetamide for another 15 min. Both incubations were carried out at room temperature with gentle shaking using MULTI SHAKER MMS (EYELA, Japan). In this equilibration process, DTT cut the disulfide bindings in proteins, and iodoacetamide protected the exposed cysteine residues to hold primary structures of proteins. The second dimension, sodium dodecyl sulfate - poly-acrylamide gel electrophoresis (SDS-PAGE), was carried out using an Ettan Dalt six (GE healthcare bioscience). The upper buffer chamber of Ettan Dalt six was filled with a SDS buffer I containing 50 mM Tris, 384 mM Glycine (MP Biomedicals), and 0.2% SDS. The lower buffer chamber was filled with a SDS buffer II containing 25 mM Tris, 192 mM Glycine, and 0.1% SDS. The equilibrated IPG strips were loaded on 12.5% gels (255 mm × 205 mm × 1 mm) at 10 °C, which contained 25% 40(w/v)%-Acrylamide/Bis Mixed Solution (37.5:1) (nacalai tesque, Kyoto, Japan), 375 mM Tris-HCl, 0.1% SDS, 0.05% ammonium persulphate (nacalai tesque), and 0.33 μL/mL TEMED (nacalai tesque). The agarose solution (0.5% agarose and 0.002% bromophenol blue in a SDS buffer II described above) was applied to seal the IPG strips, then SDS-PAGE was run at 2.5 W/gel for 30 min, followed by 25 W/gel until the bromophenol blue reached the bottom of the gel. A constant-temperature unit, NCB-2500 (EYELA) was used in order to keep the gels and buffers at 10 °C. After SDS-PAGE, analytic gels were stained by silver staining using 2-D silver staining kit II (Daiichi, Japan).

Identification of specific proteins

For image analysis, all the silver-stained gels were scanned by image scanner GT-X 8000 (SEIKO EPSON Corporation, Japan). Electronic gel images were exported as tagged image format (TIF) in 8-bit black-and-white color with 160 μm of pixels. Images were analyzed using the PDQuest software (Bio-Rad Laboratories, Inc., USA). Twenty-four TIF images obtained from experiments (2 images from 2 treatment conditions as 0.5 Gy and 1.0 Gy, and 3 images from other 6 treatment conditions, i.e., control, CAT, HQ, CAT+HQ, 0Gy, 1.5 Gy) were loaded into the program and grouped. Spot detections were carried out automatically, followed by the manual editing of each image to remove artifacts such as streaks and splotches. All the spots on the gels were matched either automatically or manually. After each matching, the background subtraction and spot volume normalization were performed. We used the local regression model for the normalization because it is not easily affected by abnormal values. In this normalization method the variance of each spot volume was minimized. Using normalized volume as a parameter, the spots showing at least twofold changes in expression levels were identified.

For the spots specific to benzene metabolites exposure, mass spectrometric identification of proteins was performed as previously described (Jensen et al., 1996). Briefly, after SDS-PAGE, proteins were visualized by silver staining and excised separately from gels, followed by the in-gel digestions with trypsin (Promega Corporation) in a buffer containing 50mM ammonium bicarconate (pH 8.0) and 2% acetonitrile overnight at 37 degrees C. Molecular mass analyses of triptic peptides were performed by matrix-assisted laser desorption/ionization time-of flight mass spectrometry (MALDI-TOF/MS) using an ultraflex TOF/TOF (Bruker Daltonics). Proteins were identified by comparison between the molecular weights determined by MALDI-TOF/MS and theoretical peptide masses from the proteins registered in NCBInr.

For the spots specific to X-ray irradiation, mass spectrometric identification of proteins was performed as follows. The gels were subjected to in-gel tryptic digestion essentially as described (Wilm et al., 1996). Briefly, the gel pieces were destained and

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washed, and, after dithiothreitol reduction and iodoacetamide alkylation, the proteins were digested with porcine trypsin (mass spec grade) overnight at 37 degrees C. The resulting tryptic peptides were extracted from the gel pieces with 30% acetonitrile, 0.3% trifluoroacetic acid and 100% acetonitrile. The extracts were evaporated in a vacuum centrifuge to remove organic solvent, then desalted and concentrated on reversed-phase C18 StageTips as previously described (Rappsilber et al., 2003). Then, Nanoflow-Liquid Chromatography (LC)-MS and MS/MS experiments were performed on HITACHI Nano LC (HITACHI, Tokyo, Japan) and Q-Tof Ultima API (Waters, Milford, USA). Chromatographic separation of the peptides took place in a 10 cm fused silica column (50 μm inner diameter) in-house packed with reversed-phase ReproSil-Pur C_{18}-AQ 3 μm resin (Dr. Maisch GmbH, Ammerbuch-Entringen, Germany). Peptide mixtures were injected onto the column with a flow of 200 nl/min and subsequently eluted with a flow of 200 nl/min from 3.8% to 11.6% acetonitrile in 0.5% acetic acid, in a 5 min gradient, from 11.6 % to 26 %, in a 15 min gradient, and from 26% to 69.2%, in a 10 min gradient. Data were acquired in MS mode and data-dependent analysis (DDA) mode using MassLynx software (Waters, Milfold, USA). Proteins were identified via automated database searching (Mascot; Matrix Science, London, United Kingdom) of all tandem mass spectra against an MSIPI database (versions 3.53; European Bioinformatics Institute, www.ebi.ac.uk/IPI/msipi.html). Carbamidomethyl cysteine was set as fixed modification, and oxidized methionine and deamidation of asparagine and glutamine were searched as variable modifications. Initial mass tolerances for protein identification on MS peaks were 100 ppm and on MS/MS peaks were 0.3 Da. Two "missed cleavages" were allowed. The instrument setting for the Mascot search was specified as "ESI-QUAD-TOF". Peptides and proteins were identified using criteria as follows. Peptides which MS2 scores were above the 95th percentile of significant (Mascot score > XX). Only fully tryptic peptides with 6 amino acids or longer were accepted for identification. Proteins were considered positively identified when they were identified with at least two fully tryptic peptides.

Results

Cytotoxicity analysis

Figure 1 shows the survival rates of cells in each treatment condition. The experiments on benzene metabolites were performed thrice, and those on X-ray were performed five times. In Figure 1, error bars were set based on the standard deviations. For chemical exposure, CAT treatment lowered the survival rate to 83.9%, HQ to 84.9%, and CAT+HQ to 69.2%, respectively. CAT and CAT+HQ showed significant decrease in the survival rate of cells ($P < 0.05$), but HQ did not show significant decrease ($P = 0.106$). The X-ray irradiation induced dose-response decrease in the survival rate significantly ($P < 0.01$). 0.5 Gy X-ray lowered the survival rate to 78.9%, 1.0 Gy to 65.2%, and 1.5 Gy to 49.0%, respectively.

2D gel image analysis

Figure 2 shows representative image (control) of silver stained 2D gels obtained from benzene metabolites treatment, and Figure 3 shows representative image (1.5 Gy) of 2D gels from X-ray irradiation. By the image analysis software, over thousand spots per gel were detected in samples, but some of them were too small or faint to be identified, so we manually selected spots enough large and deep to be quantified and identified. As a result, 692 spots per gel were detected in chemical exposure samples and 412 spots per gel were detected in X-ray irradiation samples.

Before the differential analysis, the correlation coefficients between gels were calculated based on normalized volume of each spot in order to check the reproducibility of 2DE. The Calculated values of correlation coefficients are shown in Table 1 and Table 2. Since the total amount of proteins in every gel is equal (50 mg) and the expression levels of most of the proteins remain constant across treatments, so the correlation coefficients between gels are expected to be high. As shown in Table 1 and Table 2, calculated coefficients were large enough (0.695 to 0.930) to enable us to perform differential analysis and to confirm the reproducibility of the experiments.

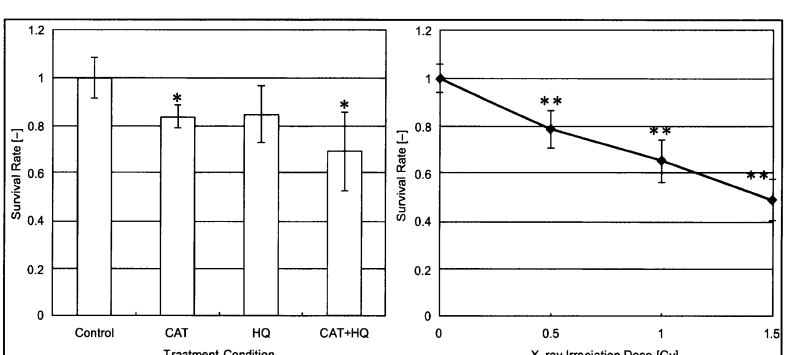

Figure 1: The survival rates of cells in chemical treatment (left) and X-ray irradiation (right).
The marks * and ** mean the significance of difference (*: $P < 0.05$, **: $P < 0.01$) between control and each treatment tested by the Student's t-test.

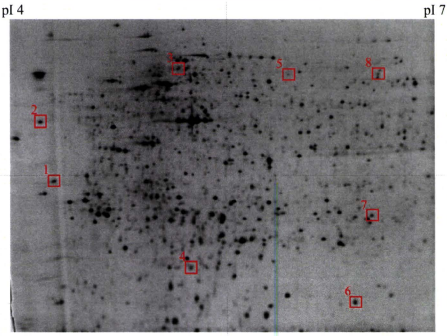

Figure 2: Representative image of gels from benzene metabolites exposure (control). Fifty micrograms of proteins were applied to a pH 3-7 IPG strip (24cm), and with 12.5% constant vertical SDS-PAGE as the second dimension. The gel was visualized by silver staining, and the resulting image was analyzed by PDQuest software. Marked squares show specific spots to exposure.

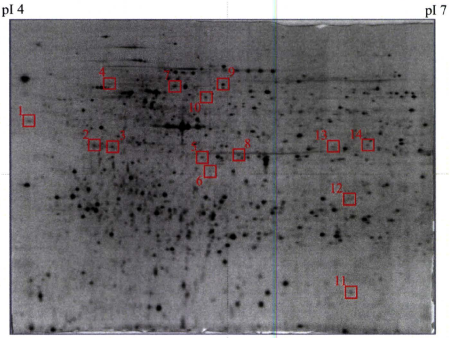

Figure 3: Representative image of gels from X-ray irradiation (1.5Gy). Fifty micrograms of proteins were applied to a pH 3-7 IPG strip (24cm), and with 12.5% constant vertical SDS-PAGE as the second dimension. The gel was visualized by silver staining, and the resulting image was analyzed by PDQuest software. Marked squares show specific spots to exposure.

For the chemical exposure samples, the comparison was performed between the expression levels of proteins in control gels and those in treated gels. For the X-ray irradiation samples, the comparison was performed between the expression levels in 0 Gy gels and those in irradiated gels. The expression levels used for the comparison were the average of expression levels in two or three gels of the same treatment. As a result of differential analysis, we found 8 spots showing at least twofold changes in expression levels by treatment of CAT, HQ or the mixture of them. Also, 14 spots showed more than twofold changes in expression levels by X-ray irradiation. These spots were labeled in Figure 2 and Figure 3. Their expression ratios to control were shown in Table 3. We could not identify some spots specific to X-ray shown as "not identified" in Table 3 because the amounts of these proteins were too low.

Among these specific spots, two proteins, protein SET and cofilin-1, changed their expression levels by both benzene metabolites exposure and X-ray irradiation. The expression of protein SET was decreased by HQ and X-ray exposure, and that of cofilin-1 was decreased by CAT and X-ray exposure.

Discussions

In this study, we extracted proteins from human bone marrow $CD34^+$ cells and performed 2-D gel image analysis. As a result, we found 8 proteins specific to benzene metabolites exposure, and 14 to X-ray irradiation. Especially, we found 2 proteins, protein SET and cofilin-1, where changes common to both benzene metabolites and X-ray exposure were identified. The expression of protein SET was decreased by HQ and X-ray exposure, and that of cofilin-1 was decreased by CAT and X-ray exposure.

Cofilin-1, 18 kD phosphoprotein (p18), controls reversibly actin polymerization and depolymerization. In benign prostatic hyperplasia cells (BPH), LIM kinase 1 (LIMK1) was reported to inactivate phosphorylation of cofilin and result in chromosomal abnormalities, which indicated carcinogenicity in prostate (Davila et al., 2007; Nakano et al., 2003; Pope et al., 2004; Sumi et al., 2006).

Protein SET is a multitasking protein, involved in apoptosis, transcription, nucleosome assembly and histone binding. SET also works as a stimulator for DNA replication of the adenovirus genome complexed with viral core proteins. SET is known as a myeloid leukemia-associated protein, and it was reported that a fusion-protein, SET-CAN, was found in cases of acute undifferentiated leukemia (Adachi et al., 1994; Nagata et al., 1995; Tsujio et al., 2005; von Lindern et al., 1992). In addition, this protein is an inhibitor of protein phosphatase 2A (PP2A) and is thought to play a key role in leukemogenesis by its nuclear localization, protein-protein interactions and PP2A inhibitory activity (Minakuchi et al., 2001). PP2A is a serine/threonine phosphatase and it was reported that the activation of PP2A inhibited the activity of c-Jun N-terminal kinase (JNK), which is thought to play a key role in the control of apoptotic cell death (Shanley et al., 2001). It was also suggested that PP2A be involved in the function of cyclin G which controls the cell cycle and is known to be regulated at the transcriptional level by p53 (Li et al., 2009). Therefore, the decrease of the expression level of SET in this study might imply the excess expression of PP2A, resulting in some troubles in cell cycle such as apoptotic process. Moreover, it was reported that SET suppressed activation of ERK following EGF stimulation, which suggested that SET negatively regulates cell growth by inhibiting the G1/S transition and inhibiting the MEK/ERK pathway stimulated by external stimuli and that SET potentially functions as a tumor suppressor (Fukukawa et al., 2005). Hence, we think that there might be some problems in the control of cell cycle of bone marrow cells because of the decrease of SET by benzene metabolites and X-ray exposure, which suggests that the SET-PP2A-JNK pathway might play an important role in basic leukemogenicity.

In addition, we should pay attention to another two proteins. One is the serine/threonine-protein kinase PAK2, which was specific to the exposure of benzene metabolites, and the other is the COP9 signalosome subunit 5 (COPS5), which was specific to the X-ray exposure. PAK2 is an activated kinase acts on a variety of targets such as phosphorylates ribosomal protein S6, histone H4

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gels	CON1	CON2	CON3	CAT1	CAT2	CAT3	HQ1	HQ2	HQ3	MIX1	MIX2	MIX3
CON1		0.796	0.799	0.743	0.802	0.729	0.781	0.704	0.695	0.834	0.815	0.768
CON2			0.874	0.780	0.878	0.826	0.813	0.807	0.789	0.867	0.857	0.855
CON3				0.846	0.883	0.848	0.830	0.841	0.804	0.868	0.851	0.912
CAT1					0.802	0.784	0.747	0.783	0.754	0.775	0.749	0.829
CAT2						0.847	0.829	0.821	0.788	0.877	0.861	0.858
CAT3							0.792	0.855	0.770	0.831	0.798	0.829
HQ1								0.764	0.776	0.851	0.836	0.824
HQ2									0.808	0.779	0.758	0.825
HQ3										0.777	0.763	0.797
MIX1											0.907	0.857
MIX2												0.839
MIX3												

* 'CON' means control (non-treated), 'MIX' means CAT+HQ.

Table 1: Correlation coefficients between gels of chemical exposure samples.

gels	CON1	CON2	CON3	CAT1	CAT2	CAT3	HQ1	HQ2	HQ3	MIX1	MIX2	MIX3
CON1		0.796	0.799	0.743	0.802	0.729	0.781	0.704	0.695	0.834	0.815	0.768
CON2			0.874	0.780	0.878	0.826	0.813	0.807	0.789	0.867	0.857	0.855
CON3				0.846	0.883	0.848	0.830	0.841	0.804	0.868	0.851	0.912
CAT1					0.802	0.784	0.747	0.783	0.754	0.775	0.749	0.829
CAT2						0.847	0.829	0.821	0.788	0.877	0.861	0.858
CAT3							0.792	0.855	0.770	0.831	0.798	0.829
HQ1								0.764	0.776	0.851	0.836	0.824
HQ2									0.808	0.779	0.758	0.825
HQ3										0.777	0.763	0.797
MIX1											0.907	0.857
MIX2												0.839
MIX3												

Table 2: Correlation coefficients between gels of X-ray irradiation samples.

Spot in Fig 2	Spot in Fig 3	Protein	ratio of expression level							
			CON	CAT	HQ	MIX	0Gy	0.5Gy	1.0Gy	1.5Gy
1	-	Clathrin, light polypeptide A	1	0.50	0.55	0.75	1	not specific		
2	1	Protein SET	1	0.71	0.38[c]	0.80	1	0.47	0.46	0.44
3	-	Keratin-9	1	3.06	2.42	1.50	1	not specific		
4	-	Chromobox homolog 3	1	0.79	0.23[b]	0.81	1	not specific		
5	-	Serine/threonine-protein kinase PAK2	1	1.84	4.47	1.43	1	not specific		
6	11	Cofilin-1	1	0.51	0.85	1.62	1	1.21	0.93	0.32
7	-	Proteasome subunit alpha type-6	1	1.71	2.58[c]	1.38	1	not specific		
8	-	T-complex protein 1 subunit zeta	1	1.81	2.58[a]	0.98	1	not specific		
-	2	Isoform 1 of Protein SET	1	not specific			1	0.49	0.70	0.52
-	3	40s ribosomal protein SA	1	not specific			1	0.99	0.70	0.54
-	4	not identified	1	not specific			1	0.48	0.56	0.49
-	5	highly similar to Actin, cytoplasmic 1	1	not specific			1	0.60	0.55	0.55
-	6	not identified	1	not specific			1	0.36	0.30	0.43
-	7	not identified	1	not specific			1	0.73	0.34[d]	0.69
-	8	CAPZA2 20kDa protein	1	not specific			1	0.56	0.50	0.32[c]
-	9	not identified	1	not specific			1	1.08	0.89	0.38
-	10	not identified	1	not specific			1	1.37	2.04	4.08[a]
-	12	not identified	1	not specific			1	0.33	0.72	0.44
-	13	COP9 signalosome subunit 5 variant	1	not specific			1	0.43	0.36	0.65
-	14	not identified	1	not specific			1	0.69	0.72	0.67

* 'CON' means control (non-treated), 'MIX' means CAT+HQ.

** a, b, c and d mean the significance of difference (a: $P < 0.01$, b: $P < 0.02$, c: $P < 0.05$, d: $P < 0.1$) between control and each treatment tested by the Student's t-test.

*** 'not identified' means the spots which we could not identify because the expression levels of them were too low.

Table 3: Expression levels of specific proteins and results of identification.

and myelin basic protein. This protein stimulates cell survival and cell growth. The process is, at least in part, mediated by phosphorylation and inhibition of pro-apoptotic BAD. Caspase-activated PAK2 is involved in cell death response, probably involving the JNK signaling pathway (Benner et al., 1995; 38. Rudel and Bokoch, 1997; Jakobi et al., 2003; Vilas et al., 2006). COPS5 is a probable protease subunit of the COP9 signalosome complex (CSN), a complex involved in various cellular and developmental processes. The CSN complex is an essential regulator of the ubiquitin (Ubl) conjugation pathway by mediating the deneddylation of the cullin subunits of the SCF-type E3 ligase complexes, leading to decrease the Ubl ligase activity of SCF-type complexes sugh as SCF, CSA or DDB2. The complex is also involved in phosphorylation of p53/TP53, c-jun/JUN, IkappaBalpha/NFKBIA, ITPK1 and ICSBP, possibly via its association with CK2 and PKD kinases. CSN-dependent phosphorylation of TP53 and JUN promotes and protects degradation by the Ubl system, respectively (Dechend et al., 1999; Bech-Otschir et al., 2001; Groisman et al., 2003; Uhle et al., 2003; Kim et al., 2004; Fang et al., 2008). As above, both PAK2 and COPS5 have a possible connection with the JNK signaling pathway as well as SET. Therefore, the changes of their expression levels might also suggest an important role of the SET-PP2A-JNK pathway in leukemogenicity.

While we obtained some proteins that might shed light on the leukemogenesis and might be candidates for biomarkers of leukemia, there were also some problems to be solved. Some of specific proteins detected did not show significant difference of expression levels. Moreover, in X-ray irradiated samples, there were no dose-response decrease of expression levels of proteins detected. These problems occurred mainly because the sample size was small and the variability was still a little large. To solve these problems, additional experiments with larger sample size should be conducted. Besides, more quantitative analysis such as Western blot helps to validate the change of protein expression.

Conclusions

In this study we performed 2-D gel electrophoresis using human bone marrow $CD34^+$ cells for the purpose of finding proteins specific to benzene metabolites and/or X-ray. As a result, we found 8 proteins specific to benzene metabolites exposure, and 14 to X-ray irradiation, which suggest that the SET-PP2A-JNK pathway might play a key role in the mechanisms of the leukemia.

Indeed comprehensive proteomic analyses using 2DE such as this study are useful in obtaining information for the better understanding of the unclear mechanisms of various diseases. However, it is also true that 2DE lacks the quantitative accuracy and that the validation of the results is necessary. Therefore, more quantitative analyses such as Western blot are needed in order to validate the changes of protein expressions identified in this study. In addition, further approaches such as dose-response analysis designed to include more than two exposure conditions, proteomic analysis on other leukemogens, and gene analysis as represented by DNA microarray will shed more light on the leukemogenesis.

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