増幅を行った。
hTS-1(F): 5'-gtggctcctgcgtttccccc-3'
hTS-2(R): 5'-ccaagcttggctccgagccggccacagg catggcgcgg-3'
GST M1-G5(F): 5'-gaactccctgaaaagctaaagc-3'
GST M1-G6(R): 5'-gttgggctcaaatatacggtgg-3'
GST T1-T1(F) 5'-ttccttactggtcctcacatctc-3'
GST T1-T2(R): 5'-tcaccggatcatggccagca-3'
β-globin (F): 5'-gaagagccaaggacaggtac-3'
β-globin (R): 5'-caacttcatccacgttcacc-3'
PCR の条件は、94 度 1 分、60 度 1 分、72 度 2 分のサイクルを 30 回繰り返した。PCR 産物各 2.5μl をとり、1.5%アガロースゲル上で泳動したのち、UV で可視化して、増幅された遺伝子断片の確認を行った。

【結果 *TS* 遺伝子】Thymidylate synthase (TS; N5,N10-methylenetetra hydolate: dump C-methyltransferase; EC 2.1.1.45) は deoxyuridylate を thymidinlate に変換する酵素であり、がん細胞中の TS 蛋白の発現量が高いほど、特定の抗腫瘍剤が効き難いことが明らかとなっている。また、TS 蛋白の遺伝子発現に関して、プロモーター領域の cap サイトの下流に存在する繰り返し配列に多形が存在し、この配列が 2 回の場合（ Short type, 本邦における多形率 19%）と 3 回の場合（ long type, 同 81%）があり、後者の方が TS 蛋白の遺伝子発現が高いことが報告されている。今回、種々の小児がんの細胞株における上記 TS 遺伝子の多形について PCR により解析を行った結果、この PCR 解析が同遺伝子の多形検出に非常に有用であることが明らかとなった。また、B 前駆細胞性 ALL は、全例いずれか片方の多形のホモ形質であることが明らかとなり、NALM-17 および KM-3 が short type、NALM-6 と HPB-NULL が long type であった。成熟 B 細胞型（バーキット型） ALL では、検討した 5 株中、NAMALWA のみ hetero 形質であり、他の 4 株（ BALM-18, -24, EB-3, P32/ISH ）株から増幅された遺伝子断片は単一であったが、後者はいずれも Short type と long type の中間の泳動度を示した。従来報告されているもの以外の多形が存在する可能性について検討する必要性があると考えられ、またこれらの株がどのような薬剤感受性の傾向を示すのか興味がもたれる。一方、神経芽腫細胞 7 株の検討では、hetero 4 株および Short type1 株、long type2 株、と多様であった。

【結果 GST 遺伝子】種々の腫瘍細胞株を用いて、GST-T1 および-M1 遺伝子の PCR 検出を行い、同遺伝子の欠失のスクリーニング法として、この PCR 法が適していることを確認した。腫瘍細胞株における GST の発現については、骨随単球系白血病の細胞株で GST 遺伝子の欠失率が非常に高く、4 株中 3 株が双方のアイソフォームを欠失しており、残る 1 株も GST-M1 のみの発現であった。これに対して、他の白血病および神経芽腫株では、双方を発現している率が高く、双方の欠失を認めたのは、B 前駆細胞性 ALL、成熟 B 細胞性 ALL 双方でそれぞれ 1 株であった。この GST 欠失の傾向が、骨随単球系白血病が株化する過程で、何らかのバイアスがかかるためなのか、あるいは骨随単球系白血病細胞がもともと有する特性なのか、興味がもたれる。また、腫瘍細胞における GST の欠失が、単純に体細胞の多型を反映した結果なのか、あるいは腫瘍細胞になる過程での付加的な遺伝子変異に伴って欠失が起こる可能性はないのか、といった点についても、今後検討を行いたい。

D. 考察

薬物代謝関連分子の遺伝子多型の頻度は人種により異なることが知られている。また、ALL に対する化学療法は多剤併用で行われることから、それぞれの治療レジメンに特異的な効果、毒性を生じ得ると考えられ、薬物代謝関連分子の遺伝子多型との関連も治療レジメンにより異なる可能性が推測される。現在、国内の小児 ALL の約 1/3 に適用されている TCCSG ALL 治療研究において薬物代謝関連分子の遺伝子多型と治療効果、毒性の関連の検証を行うことは、今後の ALL 治療開発において重要な意義を持つと考えられる。

大部分の小児がん臨床研究は、疾患の頻度、診療施設の受け入れの問題から多施設共同研究として行われている。これまで、国内の多施設共同小児がん臨床研究において、前方視的な胚細胞系列の遺伝子解析研究は行われていない。本研究は、小児がん領域における胚細胞系列遺伝子解析研究の基盤整備にも貢献するものと考えられる。

本研究では小児 ALL に対する前方視的大規模臨床研究における遺伝薬理学研究の整備を試みた。多施設共同研究であることから、さまざまな手続きを要し、さらにそれぞれの手続きに多くの時間を要し、速やかな研究の進行に困難を感じた。

E. 結論

小児 ALL に対する前方視的多施設共同治療研究である TCCSG ALL L07-16-02 を利用して、ALL に対する薬物治療における効果、毒性の個体差と薬物代謝関連分子の遺伝子多型の関連を検証する研究計画を立案し準備を進めたが、本報告書作成時点までに TCCSG 研究審査委員会による本研究の実行の承認は得られず、臨床検体の解析には至らなかった。

G. 研究発表

1. 論文発表

1) Kobayashi R, Yamato K, Tanaka F, Takashima Y, Inada H, Kikuchi A, Kumagai MA, Sunami S, Nakagawa A, Fukano R, Fujita N, Mitsui T, Tsurusawa M, Mori T; Lymphoma Committee, Japanese Pediatric Leukemia/Lymphoma Study Group. Retrospective analysis of non-anaplastic peripheral T-cell lymphoma in pediatric patients in Japan. Pediatr Blood Cancer 2010; 54: 212-5.
2) Mitsui T, Mori T, Fujita N, Inada H, Horibe K, Tsurusawa M; Lymphoma Committee, Japanese Pediatric Leukemia/Lymphoma Study Group. Retrospective analysis of relapsed or primary refractory childhood lymphoblastic lymphoma in Japan. Pediatr Blood Cancer 2009; 52: 591-5.

2. 学会発表

1) 恩田恵子, 清河信敬, 齋藤正博, 森鉄也, 藤本純一郎, 真部淳, 康勝好, 小原明, 林泰秀, 花田良二, 土田昌宏. TCCSG-L1602 治療研究/Day8 末梢血 芽球数のフローサイトメトリー測定. Cytometry Research19 巻抄録集 Page57 (2009.06)
2) 恩田恵子, 平林真介, 清河信敬, 齊藤正博, 森鉄也, 福島敬, 藤本純一郎, 真部淳, 康勝好, 熊谷昌明, 小原明, 林泰秀, 花田良二, 土田昌宏. TCCSG-L1602 治療研究における Day8 末梢血-芽球数のフローサイトメトリー測定についての評価. 小児がん 46 巻プログラム・総会号 Page228 (2009.11)
3) 清河信敬, 恩田恵子, 飯島一智, 長谷川大輔, 加藤元博, 大喜多肇, 齋藤正博, 森鉄也, 真部淳, 康勝好, 小原明, 林泰秀, 花田良二, 土田昌宏, 中川温子, 小川誠司, 藤本純一郎. 小児 B 細胞性リンパ腫のマイクロアレイを用いた molecular karyotyping と網羅的発現遺伝子解析. 臨床血液 50 巻 9 号 Page1268 (2009.09)
4) 土田昌宏, 小原明, 花田良二, 真部淳, 熊谷昌明, 高橋浩之, 金沢崇, 藤村純也, 富澤大輔, 康勝好, 嶋田博之, 森鉄也, 後藤裕明, 福島敬, 小池和俊, 野口靖, 小川千登世, 犬飼岳史, 福島啓太郎, 塩原正明, 加藤陽子, 前田美穂, 菊地陽, 梶原道子, 矢部晋正, 外松学, 太田節雄, 磯山恵一, 金子隆, 林泰秀, 東京小児がん研究グループ ALL 委員会. 東京小児がん研究グループにて 1981 年から 1999 年の 5 つの研究に登録された小児急性リンパ性白血病 2035 例の長期追跡結果. 臨床血液 50 巻 9 号 Page904 (2009.09)
ほか)

H. 知的財産権の出願・登録状況

(予定を含む)

1. 特許取得

なし

2. 実用新案登録

なし

3. その他

なし

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）
分担研究報告書

難治性小児がんの臨床的特性の解析と新規診断・治療法開発

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研究要旨：　難治性小児がんの一つである Ewing 肉腫において EWS/ETS キメラ遺伝子の腫瘍発生における機能を、特にその標的遺伝子である DKK1/DKK2 の Ewing 肉腫における作用を中心に解析した。DKK ファミリーは WNT シグナルを制御する分子で、Ewing 肉腫では DKK 2 が高発現で、DKK1 が低発現であるが、DKK1、DKK2 の下流因子の候補を同定するとともに、DKK2 の発現抑制が Ewing 肉腫細胞に与える影響や EWS/ETS と WNT シグナルの関係を検討した。

A. 研究目的

Ewing 肉腫は、小児期～若年成人期に好発する骨軟部の悪性腫瘍である。近年、化学療法をはじめとする治療法の進歩により治療成績は向上しつつあるものの、治癒率は 50 %程度でありいまだに難治性の腫瘍である。本腫瘍の 90 %以上では、EWS 遺伝子と ETS ファミリーの転写因子が染色体転座によって融合し、キメラ遺伝子が形成される（EWS/ETS キメラ遺伝子）。このキメラ遺伝子は、本腫瘍に極めて特異性が高く、確定診断をつける上で重要であり、かつ、腫瘍発生にも重要な役割を演じている。このキメラ遺伝子の機能を明らかにすることが、Ewing 肉腫の発生機序の解明に役立つばかりでなく、新たな治療法の開発の基盤情報を提供するものとなると考えられる。

そこで、本研究は、Ewing 肉腫やその他の小児腫瘍のトランスクリプトーム解析によってキメラ遺伝子によって転写を調節される標的遺伝子を探索し、その腫瘍発生や進展に関わる機能を明らかにすることを目的とした。特に、昨年度までに同定した Dickkopf family 分子の腫瘍発生における意義ならびに作用メカニズムを明らかにすることを目的とした。

B. 研究方法

昨年度までに Ewing 肉腫や他の小児腫瘍のトランスクリプトーム解析により、キメラ遺伝子の標的遺伝子の候補として同定した Dickkopf-1(DKK1)、Dickkopf-2(DKK2)遺伝子の解析を進めた。

(1) 昨年度までに作成した DKK1 過剰発現あるいは、DKK2 過剰発現 SK-ES1 細胞（Ewing 肉腫）の網羅的遺伝子発現解析を Affymetrix 社 U133 array を用いて行った。GeneSpring を用いて発現データを解析し、DKK1、DKK2 過剰発現によって、発現が変動する遺伝子群を抽出、特に着目すべき遺伝子に対して定量 PCR によって発現を確認した。

(2) Ewing 肉腫細胞で DKK2 をノックダウンするために、SK-ES1 細胞に DKK2 の shRNA を遺伝子導入し、安定導入したクローンを得た。そのクローンについて DKK2 の発現を定量 PCR 法で確認した。それらのクローンの増殖能を MTT assay、浸潤能を matrigel を用いた invasion assay、移動能を migaration assay、wound healing assay によって検討した。

(3) 293 細胞に TCF Reporter Plasmid である TOPflash、pUSE-Wnt3a、pcDNA-LacZ-His、pcDNA-Flag EWS/FLI1 を同時に遺伝子導入し、ルシフェラーゼアッセイを行い、WNT シグナルの活性化に対して、EWS/FLI1 が与える影響を検討した。

（倫理面への配慮）

動物実験は、関連法規を遵守し、あらかじめ国立成育医療センター動物実験委員会へ申請し、承認を得て行った。

C. 研究結果

昨年度までに、Ewing 肉腫、神経芽腫、その他の小児腫瘍細胞のトランスクリプトーム解析より DKK2 は Ewing 肉腫で高発現であり EWS/ETS の直接の標的遺伝子、DKK1 は Ewing 肉腫において低発現で、EWS/ETS によって発現低下するが必ずしも直接の標的分子ではないとの結果が得られている。DKK は Dickkopf family に属

する分泌型の分子で、DKK1〜DKK4の4種類が報告されており、Wntシグナルを調節あるいは抑制することが報告されている。

まず、昨年度に引き続きDKK1過剰発現SK-ES1細胞のin vitroでの細胞増殖アッセイを行ったが、in vitroでは明らかな増殖抑制効果は認められなかった。そこで、DKK1過剰発現SK-ES1細胞及びDKK2過剰発現SK-ES1細胞を用いて網羅的遺伝子解析発現を行った。DKK1、DKK2によって発現が上昇あるいは低下する遺伝子群を同定し、その中で、G protein familyの一分子が、DKK1によって過剰発現、DKK2によって発現抑制されることが明らかとなった。この分子はDKK2ノックダウンにより発現が上昇し、DKK1/2の下流因子と考えられ、現在、この分子について更に解析中である。

Ewing肉腫細胞で、DKK2をノックダウンすることは可能であるが、一過性導入効率の問題や、DKK2導入による細胞増殖効率低下が解析を困難にする可能性が考えられた。そこで、SK-ES1細胞にDKK2のshRNAを安定遺伝子導入した。定量PCR法でDKK2の発現抑制を確認した。shRNA DKK2 SK-ES1細胞は、増殖能が軽度低下傾向にあった一方で、DKK2発現量と浸潤能、遊走能には強い相関は認められなかった。このことから、EWS/ETS依存的DKK2発現上昇は、Ewing肉腫腫瘍形成に関与している可能性が考えられたが、なお詳細な検討が必要と考えられた。

DKK1/2はWNTシグナルの調節因子であることが知られている。Ewing肉腫細胞でのWNTシグナルについて検討するために、まず、EWS/FLI1がWNT3aによるTCF/LEF活性化能に対して影響を与えるかどうか解析した。その結果、EWS/FLI1がWNT3aによるTCF/LEF活性化を抑制することが示唆された。

D. 考察

EWS/ETSキメラ遺伝子は、RNA結合タンパク質であるEWSとETSファミリーの転写因子が融合した蛋白をコードする。従ってキメラ遺伝子の転写産物は、転写因子として機能することにより腫瘍発生に重要な役割を演じていると考えられており、実際に複数の標的遺伝子が同定されている。DKK1/2は、EWS/ETSの直接的、あるいは間接的な標的遺伝子の一つとして機能していると考えられ、EWS/ETSによる腫瘍発生に少なくとも部分的に関与していると考えられる。

今回、G protein familyの一分子をDKK1/2の下流因子の候補として同定した。更に本分子について解析することにより、DKK1/2がEwing肉腫の発生に関与する経路が解明されることが期待される。また、DKK1がin vivoで腫瘍形成を抑制する一方で、DKK2はEwing肉腫細胞で高発現であることもあり、過剰発現による機能解析には限界がある。現在、DKK2のノックダウン実験を行っており、この結果により、DKK2のEwing肉腫発生における役割が明らかになると期待される。

今回の解析により、EWS/FLI1自身が直接的あるいは間接的にWNTシグナルに影響を与えることが示唆された。DKK1/2が古典的なWNTシグナル経路を解して機能するか、あるいはEwing肉腫では他の経路を介しているかどうかを含めて、今後、検討していく計画である。近年、WNTがEwing肉腫の神経突起伸長を促進するとの報告もあり、WNTの古典的な経路とそれ以外の両面から解析を進める。

E. 結論

我々が、Ewing肉腫特異的キメラ遺伝子EWS/ETSが発現を調節する分子として同定したDKK1、DKK2について、そのEwing肉腫における機能解析を進めた。DKK1、DKK2の下流因子の候補を同定するとともに、DKK2の発現抑制がEwing肉腫細胞に与える影響やEWS/ETSとWNTシグナルの環系を検討した。

G. 研究発表

1. 論文発表

1) Kitamura N, Katagiri YU, Itagaki M, Miyagawa Y, Onda K, Okita H, Mori A, Fujimoto J, Kiyokawa N. The expression of granulysin in systemic anaplastic large cell lymphoma in childhood. Leuk Res. 2009 Jul; 33(7):908-12.

2) Horiuchi Y, Onodera M, Miyagawa Y, Sato B, Onda K, Katagiri YU, Okita H, Okada M, Otsu M, Kume A, Okuyama T, Fujimoto J, Kuratsuji T, Kiyokawa N. Kinetics and Effect of Integrin Expression on Human CD34(+) Cells during Murine Leukemia Virus-Derived Retroviral Transduction with Recombinant Fibronectin for

Stem Cell Gene Therapy. Hum Gene Ther. 2009 Jul;20(7):777-83.
3) Miyagawa Y, Kiyokawa N, Ochiai N, Imadome K-I, Horiuchi Y, Onda K, Yajima M, Nakamura H, Katagiri YU, Okita H, Morio T, Shimizu N, Fujimoto J, Fujiwara S. Ex vivo expanded cord blood CD4 T lymphocytes exhibit distinct expression profile of cytokine-related genes from those of peripheral blood origin. Immunology. 2009 Nov;128(3):405-19.
4) Watanabe N, Okita H, Matsuoka K, Kiyotani C, Fujii E, Kumagai M and Nakagawa A. Vaginal yolk sac (endodermal sinus) tumors in infancy presenting persistent vaginal bleeding. J Obstet Gynaecol Res 36(1) 213-216, 2010
5) Onda K, Iijima1 K, Katagiri YU, Okita H, Saito M, Shimizu T, Kiyokawa N. Differential effects of BAFF on B-cell precursor acute lymphoblastic leukemia and Burkitt lymphoma. Int J Hematol. (in press).

2. 学会発表

1) 宮川世志幸，大喜多肇，佐藤伴，片桐洋子，梅澤明弘，藤本純一郎，清河信敬. Wnt シグナル拮抗因子 DKK の Ewing 肉腫ファミリー腫瘍発生における機能. 第 98 回日本病理学会総会，京都， 5 月 1 ～ 3 日，2009 .
2) 大喜多肇，宮川世志幸，佐藤伴，中島英規，片桐洋子，梅澤明弘，秦順一，藤本純一郎，清河信敬. Ewing family tumor 特異的融合遺伝子 EWS/ETS による Dickkopf1/2 の発現制御. 第 68 回日本癌学会学術総会，横浜，10 月 1 日～3 日，横浜.
3) 清河信敬，恩田恵子，飯島一智，長谷川大輔，加藤元博，大喜多肇，齋藤正博，森鉄也，真部淳，康勝好，小原明，林泰秀，花田良二，土田昌宏. 中川温子，小川誠司，藤本純一郎. 小児 B 細胞性リンパ腫のマイクロアレイを用いた molecular karyotyping と網羅的発現遺伝子解析. 第 71 回日本血液学会学術集会，京都， 10 月 23 日-25 日， 2009 .
4) 森鉄也，熊谷昌明，中川温子，黒田達夫，森川信行，大喜多肇，清河信敬，清谷知賀子，塩田曜子，正木英一，藤本純一郎，小児がん教育プログラムの整備（公開系統講義・診断実習の構築），第 25 回日本小児がん学会，千葉， 11 月 27 日～29 日， 2009 .

H. 知的財産権の出願・登録状況
（予定を含む）

1. 特許取得
   無し
2. 実用新案登録
   無し
3. その他
   無し

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分担研究報告書

小児造血器腫瘍の遺伝子診断と分子モニタリングに関する研究

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研究要旨：　小児急性骨髄性白血病の診断時の検体を用いたキメラ遺伝子のスクリーニングを行った結果、361 例中 157 例（ 43.5%）において検討したキメラ遺伝子のいずれかが検出された。急性骨髄性白血病の代表的な染色体異常である t(8;21)によって生じる AML1-ETO のキメラ遺伝子は 361 例中 99 例（ 27.4%）にみとめられ、高頻度であった。AML1-ETO のキメラ遺伝子を利用した治療後の微小残存病変の解析では、治療終了時の分子寛解例は 20%と少なかったが、治療初期の AML1-ETO のキメラ遺伝子の低下には多様性がみられ、治療反応性の指標として検討が必要である。

A. 研究目的

小児急性骨髄性白血病（ AML ）を対象とした臨床試験である JPLSG AML-05 プロトコールの登録時に、キメラ遺伝子のスクリーニングを実施し、同時に行われる染色体分析結果と比較することで、主要なキメラ遺伝子における遺伝子診断の臨床的意義の検討を行う。診断時に染色体分析不能例や染色体分析では異常を検出されない症例も存在すると予想され、キメラ遺伝子のスクリーニングの実施によって正確な分子診断が可能になると期待される。また診断時にキメラ遺伝子が検出された症例を対象に、同一の治療法における治療早期から治療終了時点まで経時的にキメラ遺伝子の発現量を定量することで、完全寛解例でのいわゆる微小残存病変（ Minimal residual disease, MRD ）の評価を行い、寛解導入療法および寛解後療法に対する治療反応性と再発との関連について検討を行う。今までの AML におけるキメラ遺伝子を用いた MRD 研究は主に再発の予知として検討されてきたが、明確な関連は示されていない。しかし治療反応性の指標としての治療早期の MRD と再発との間に関連が見いだせれば、治療における層別化因子となることが期待できる。

B. 研究方法

診断時の骨髄液あるいは末梢血の有核細胞から RNA 抽出を行い、定量的 RT-PCR 法によって急性骨髄性白血病での代表的な 8 種類のキメラ遺伝子（ AML1-ETO、CBF β -MYH11、MLL-AF6、MLL-AF9、MLL-ELL、TLS/FUS-ERG、PML-RAR α、NUP98-HOXA9 ）の測定を行った。治療後の MRD 解析では、全て骨髄液から RNA を抽出し、該当症例で陽性のキメラ遺伝子の発現を定量した。

（倫理面への配慮）

本研究は、関連法規を遵守し、施設の臨床研究審査委員会ならびに実施機関長の承認を経た上で実施した。全ての検体は、文書によるインフォームドコンセントを得た後に収集された。また検体提出、収集において全て匿名化を行い、検体提供者の人権の保護、個人情報保護に注意を払って実施した。

C. 研究結果

診断時のキメラ遺伝子スクリーニングにおいては、現在までに解析した 361 例中 157 例（ 43.5%）にいずれかのキメラ遺伝子が検出された。その内訳は、AML1-ETO 99 例、CBF β -MYH11 20 例、MLL-AF6 2 例、MLL-AF9 25 例、MLL-ELL 6 例、TLS/FUS-ERG 3 例、PML-RAR α 2 例であった。NUP98-HOXA9 は 1 例も検出されなかった。

治療後のキメラ遺伝子の経時的なモニタリングを行う MRD 解析には、診断時のスクリーニングでキメラ遺伝子が陽性の症例から 69 例が登録され、242 検体の解析を行った。69 例中 46 例が AML1-ETO 陽性の症例であり、現時点で 10 例以上の経時的な検討が可能であった。AML1-ETO の診断時の RNA 1 μ g あたりの発現量は中央値で 3.7×10^5 コピー（範囲； 3.2×10^4 ～ 1.7×10^6 コピー、n=46 ）であっ

たが、寛解導入療法 1 コース後では、中央値 1.3x10^3 コピー（範囲； 8.7x10^1 ～ 9.4x10^5 コピー、n=35）、寛解導入療法第 2 コース後では、中央値 4.7x10^2 コピー（範囲； 0 ～ 1.4x10^5 コピー、n=36）、強化療法 1 コース後では、中央値 3.1x10^2 コピー（範囲； 0 ～ 5.3x10^3 コピー、n=30）、強化療法 2 コース後では、中央値 2.4x10^2 コピー（範囲； 0 ～ 1.2x10^4 コピー、n=23）、強化療法 3 コース後（全治療終了時点）では、中央値 1.6x10^2 コピー（範囲； 0 ～ 3.6x10^5 コピー、n=25）であった。寛解導入療法第 1 コース後の MRD は治療前に比して約 2 log 減少しているが、症例間での差が大きくみられた。寛解導入療法第 2 コース以降の MRD の低下は緩徐な低下を示す傾向がみられた。また治療終了時点で MRD が検出感度以下であった症例は 25 例中 5 例のみであった。

D. 考察

現在までに小児 AML と新規に診断された 361 例のキメラ遺伝子スクリーニングを行い、99 例において t(8;21)(q22;q22)の染色体異常の結果として形成される AML1-ETO のキメラ遺伝子が検出された。その頻度は約 27%であり、欧米の報告と比べて高頻度であるが、我が国の成人においても同様の傾向にあり、人種による違いと考えられる。AML1-ETO に比べて CBFβ-MYH11 はおよそ 5 分の 1 であり、全体の約 5%と低頻度であった。

また AML1-ETO のキメラ遺伝子を対象にした MRD の解析では、寛解導入量法終了時の MRD の残存の程度には症例間でのばらつきがみられるため、個々の症例における治療反応性を判定する指標として用いることが可能と考えているが、現時点で治療研究が終了していないため、臨床成績の解析が行われていないため、本研究でも臨床的結果との解析は今後実施する課題である。治療終了時点でもキメラ遺伝子が検出感度未満になる、いわゆる「分子寛解」を到達した症例は 20%であり、この染色体異常が予後良好で過半数の症例が無再発で経過することを考慮すると、治療終了時に分子寛解を得ることは必ずしも必要でないと予想される。予後との関連については、やはり治療早期での治療反応性の指標としての MRD の位置づけを検討することが必要であることが示唆される。小児 AML では高い完全寛解率を得られるため AML1-ETO 以外のキメラ遺伝子も含めて MRD の検討により、寛解例での治療反応性を更に分類することができれば、予後判定への応用が期待される。

E. 結論

小児 AML 361 例の診断時のキメラ遺伝子スクリーニングにより、AML1-ETO を代表とする主要なキメラ遺伝子が約 44%の症例で陽性であった。染色体分析との比較は、臨床試験の終了後に解析予定である。またこのキメラ遺伝子を利用した MRD の解析により、AML1-ETO 陽性例では治療終了時点で MRD としてキメラ遺伝子の発現が検出感度未満になる症例は多くなく、分子寛解は治癒に必須の条件ではないと考えられた。AML1-ETO の MRD と予後との関連については、臨床試験終了後にデータが固定された後に検討する必要がある。

G. 研究発表

1. 論文発表

1) Ohashi H, Arita K, Fukami S, Oguri K, Nagai H, Yokozawa T, Hotta T, Hanada S. Two rare MPL gene mutations in patients with essential thrombocythemia. Int J Hematol. 2009 Oct;90(3):431-432.

2) Nishiwaki S, Terakura S, Yasuda T, Imahashi N, Sao H, Iida H, Kamiya Y, Niimi K, Morishita Y, Kohno A, Yokozawa T, Ohashi H, Sawa M, Kodera Y, Miyamura K. Outcome of allogeneic bone marrow transplantation from unrelated donors for adult Philadelphia chromosome-negative acute lymphocytic leukemia in first complete-remission. Int J Hematol. 2010 Feb 10.

2. 学会発表

1) Imoto N, Yokozawa T, Suzuki Y, Kihara R, Aoki E, Kato C, Ohashi H, Hamaguchi M, Hotta T, Nagai H . Clinical outcome of Hyperleukocytosis in adult acute myeloid leukemia: Single institute experience . 第 71 回日本血液学会総会，京都， 10 月 23 日～ 10 月 25 日， 2009 .

2) Ishokawa Y, Kiyoi H, Miyamura K, Nakano Y, Kitamura K, Kohno A, Sugiura I, Yokozawa T, Hnamura A, Yamamoto K, Iida H, Emi N, Suzuki R, Ohnishi K, Naoe T . Imtinib trough level reflects BCR/ABL inhibitory activity and is associated with clinical response . 第 71 回日本血液学会総会，京都， 10 月 23 日～ 10 月 25

日， 2009．
3) Kihara R, Aoki E, Imoto N, Suzuki Y, Kato C, Yokozawa T, Ohashi H, Hamaguchi M, Hotta T, Nagai H . Bloodstream infections in patients with lymphoid malignancy．第 71 回日本血液学会総会，京都， 10 月 23 日～10 月 25 日，2009．
4) Ohashi H, Arita K, Oguri K, Yokozawa T, Nagai H, Hamaguchi M, Hanada S, Hotta T . Mutation analysis of the MPL gene in patients with essential thrombocythemia．第 71 回日本血液学会総会，京都， 10 月 23 日～10 月 25 日， 2009．
5) Suzuki Y, Moritani S, Imoto N, , Kihara R, Aoki E, Kato C, Yokozawa T, Ohashi H, Hamaguchi M, Ichihara S, Hotta T, Nagai H . The difference of clinical outcome between Burkitt lymphoma and intermediate BL/DLBCL．第 71 回日本血液学会総会，京都， 10 月 23 日～10 月 25 日， 2009．
6) 木原里香、井本直人、鈴木康裕、青木恵津子、加藤千明、横澤敏也、大橋春彦、濱口元洋、堀田知光、永井宏和．マントル細胞リンパ腫の治療成績の後方視的解析．第 49 回日本リンパ網内系学会総会，兵庫， 7 月 9 日～7 月 11 日， 2009．
7) 蓮尾隆博、井本直人、鈴木康裕、木原里香、青木恵津子、加藤千明、横澤敏也、大橋春彦、森谷鈴子、市原周、濱口元洋、堀田知光、永井宏和．濾胞性リンパ腫病変を伴った intermediate BL/DLBCL の一例．第 49 回日本リンパ網内系学会総会，兵庫， 7 月 9 日～7 月 11 日， 2009．

H. 知的財産権の出願・登録状況
（予定を含む）

1. 特許取得
   無し
2. 実用新案登録
   無し
3. その他

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ

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
Kitamura N, Katagiri YU, Itagaki M, Miyagawa Y, Onda K, Okita H, Mori A, Fujimoto J, Kiyokawa N.	The expression of granulysin in systemic anaplastic large cell lymphoma in childhood.	Leukemia Research.	33 (7)	908-12	2009
Horiuchi Y, Onodera M, Miyagawa Y, Sato B, Onda K, Katagiri YU, Okita H, Okada M, Otsu M, Kume A, Okuyama T, Fujimoto J, Kuratsuji T, Kiyokawa N.	Kinetics and Effect of Integrin Expression on Human CD34(+) Cells during Murine Leukemia Virus-Derived Retroviral Transduction with Recombinant Fibronectin for Stem Cell Gene Therapy.	Hum Gene Ther.	20 (7)	777-83	2009
Miyagawa Y, Kiyokawa N, Ochiai N, Imadome K-I, Horiuchi Y, Onda K, Yajima M, Nakamura H, Katagiri YU, Okita H, Morio T, Shimizu N, Fujimoto J, Fujiwara S.	Ex vivo expanded cord blood CD4 T lymphocytes exhibit distinct expression profile of cytokine-related genes from those of peripheral blood origin.	Immunology.	128 (3)	405-19	2009
Onda K, Iijima1 K, Katagiri YU, Okita H, Saito M, Shimizu T, Kiyokawa N.	Differential effects of BAFF on B-cell precursor acute lymphoblastic leukemia and Burkitt lymphoma.	Int J Hematol.	in press		
Yang L, Fujimoto J, Qiu D, Sakamoto N.	Trends in cancer mortality in Japanese adolescents and young adults aged 15 to 29 years, 1970-2006.	Ann Oncol	20 (4)	758-66	2009
Tsuchida M, Ohara A,Manabe A, Kumagai M, Shimada H, Kikuchi A, Mori T, Saito M, Akiyama M, FukushimaT, Koike K, Shiobara M, Ogawa C, Kanazawa T, Noguchi Y, OotaS, Okimoto Y, Yabe H, Kajiwara M, Tomizawa D, Ko K, Sugita K, Kaneko T, Maeda M, Inukai T, Goto H,Takahashi H, IsoyamaK, Hayashi Y, HosoyaR, Hanada R.	Long-term results of Tokyo Children's Cancer Study Group trials for childhood acute lymphoblastic leukemia, 1984-1999.	Leukemia	24	383-396	2010
Ono R, Kumagai H, Nakajima H, Hishiya A, Taki T, Horikawa K,Takatsu K, Satoh T, Hayashi Y, Kitamura T, Nosaka T.	Mixed-lineage-leukemia (MLL) fusion protein collaborates with Ras to induce acute leukemia through aberrant Hox expression and Raf activation.	Leukemia.	23	2197-2209	2009
Kuroiwa M, Sakamoto J, Shimada A, Suzuki N, Hirato J, Park MJ,Sotomatsu M, HayashiY.	Manifestation of alveolar rhabdomyosarcoma as primary cutaneous lesions in a neonate with Beckwith-Wiedemann syndrome.	J Pediatr Surg.	44	31-35	2009

Kurosawa H, Okuya M, Matsushita T, Kubota T, Endoh K, Kuwashima S, Hagisawa S,Sato Y, Fukushima K, Sugita K, Okada Y, Park MJ, Hayashi Y, Arisaka O.	JAK2V617F mutation-positive childhood essential thrombocythemia associated with cerebral venous sinus thrombosis.	J Pediatr Hematol Oncol.	31	678-680	2009
Sanada M, Suzuki T, Shih LY, Otsu M, Kato M, Yamazaki S, Tamura A, Honda H, Sakata-Yanagimoto M, Kumano K, Oda H, Yamagata T, Takita J, Gotoh N, Nakazaki K, Kawamata N, Onodera M, Nobuyoshi M, Hayashi Y, Harada H, Kurokawa M, Chiba S, Mori H, Ozawa K, Omine M, Hirai H, Nakauchi H, Koeffler HP, Ogawa S.	Gain-of-function of mutated C-CBL tumour suppressor in myeloid neoplasms.	Nature.	460	904-908	2009
Takita J, Motomura A, Koh K, Ida K, Taki T, Hayashi Y, Igarashi T.	Acute megakaryoblastic leukemia in a child with the MLL-AF4 fusion gene.	Eur J Haematol.	83	149-153	2009
Kato M, Sanada M, Kato I, Sato Y, Takita J, Takeuchi K, Niwa A, Chen Y, Nakazaki K, Nomoto J, Asakura Y, Muto S, Tamura A, Iio M, Akatsuka Y, Hayashi Y, Mori H, Igarashi T, Kurokawa M, Chiba S, Mori S, Ishikawa Y, Okamoto K, Tobinai K, Nakagama H, Nakahata T, Yoshino T, Kobayashi Y, Ogawa S.	Frequent inactivation of A20 in B-cell lymphomas.	Nature.	459	712-716	2009
Taketani T, Taki T, Nakamura H, Taniwaki M, Masuda J, Hayashi Y.	NUP98-NSD3 fusion gene in radiation-associated myelodysplastic syndrome with t(8;11)(p11;p15) and expression pattern of NSD family genes.	Cancer Genet Cytogenet.	190	108-112	2009
Watanabe-Okochi N, Oki T, Komeno Y, Kato N, Yuji K, Ono R, Harada Y, Harada H, Hayashi Y, Nakajima H, Nosaka T, Kitaura J, Kitamura T.	Possible involvement of RasGRP4 in leukemogenesis.	Int J Hematol.	89	470-481	2009
Mizoguchi Y, Fujita N, Taki T, Hayashi Y, Hamamoto K.	Juvenile myelomonocytic leukemia with t(7;11)(p15;p15) and NUP98-HOXA11 fusion.	Am J Hematol.	84	295-297	2009
Park MJ, Taki T, Oda M, Watanabe T, Yumura-Yagi K, Kobayashi R, Suzuki N, Hara J, Horibe K, Hayashi Y.	FBXW7 and NOTCH1 mutations in childhood T cell acute lymphoblastic leukaemia and T cell non-Hodgkin lymphoma.	Brit J Haematol 1	45	198-206	2009
Jo A, Tsukimoto I, Ishii E, Asou N, Mitani S, Shimada A, Igarashi T, Hayashi Y, Ichikawa H.	Age-associated difference in gene expression of pediatric acute myelomonocytic lineage leukemia (FAB M4 and M5 subtypes) and its correlation with prognosis.	Brit J Haematology.	144	917-929	2009
Kitoh T, Taki T, Hayashi Y, Nakamura K, Irino T, Osaka M.	Transient abnormal myelopoiesis in a Down syndrome newborn followed by acute myeloid leukemia: identification of the same chromosomal abnormality in both stages.	Cancer Genet Cytogenet.	188	99-102	2009
Masuda S, Kumano K, Suzuki T, Tomita T, Iwatsubo T, Natsugari H, Tojo A, Shibutani M, Mitsumori K, Hanazono Y, Ogawa S, Kurokawa M, Chiba S.	Dual antitumor mechanisms of Notch signaling inhibitor in a T-cell acute lymphoblastic leukemia xenograft model.	Cancer Sci.	100 (12)	2444-50	2009

Akagi T, Shih LY, Ogawa S, Gerss J, Moore SR, Schreck R, Kawamata N, Liang DC, Sanada M, Nannya Y, Deneberg S, Zachariadis V, Nordgren A, Song JH, Dugas M, Lehmann S, Koeffler HP.	Single nucleotide polymorphism genomic arrays analysis of t(8;21) acute myeloid leukemia cells.	Haematologica.	94.	1301-6	2009
Identified hidden genomic changes in mantle cell lymphoma using high-resolution single nucleotide polymorphism genomic array.	Kawamata N, Ogawa S, Gueller S, Ross SH, Huynh T, Chen J, Chang A, Nabavi-Nouis S, Megrabian N, Siebert R, Martinez-Climent JA, Koeffler HP.	Exp Hematol.	37	937-946	2009
Kawamata N, Ogawa S, Seeger K, Kirschner-Schwabe R, Huynh T, Chen J, Megrabian N, Harbott J, Zimmermann M, Henze G, Schrappe M, Bartram CR, Koeffler HP.	Molecular allelokaryotyping of relapsed pediatric acute lymphoblastic leukemia.	Int J Oncol.	34	1603-1612	2009
Haraguchi K, Suzuki T, Koyama N, Kumano K, Nakahara F, Matsumoto A, Yokoyama Y, Sakata-Yanagimoto M, Masuda S, Takahashi T, Kamijo A, Takahashi K, Takanashi M, Okuyama Y, Yasutomo K, Sakano S, Yagita H, Kurokawa M, Ogawa S, Chiba S.	Notch activation induces the generation of functional NK cells from human cord blood CD34-positive cells devoid of IL-15.	J Immunol.	182 (10)	6168-6178	2009
Lee SY, Kumano K, Nakazaki K, Sanada M, Matsumoto A, Yamamoto G, Nannya Y, Suzuki R, Ota S, Ota Y, Izutsu K, Sakata-Yanagimoto M, Hangaishi A, Yagita H, Fukayama M, Seto M, Kurokawa M, Ogawa S, Chiba S.	Gain-of-function mutations and copy number increases of Notch2 in diffuse large B-cell lymphoma.	Cancer Sci.	100	920-6	2009
Yin D, Ogawa S, Kawamata N, Tunici P, Finocchiaro G, Eoli M, Ruckert C, Huynh T, Liu G, Kato M, Sanada M, Jauch A, Dugas M, Black KL, Koeffler HP.	High-resolution genomic copy number profiling of glioblastoma multiforme by single nucleotide polymorphism DNA microarray.	Mol Cancer Res.	7	665-77	2009
Akagi T, Shih LY, Kato M, Kawamata N, Yamamoto G, Sanada M, Okamoto R, Miller CW, Liang DC, Ogawa S, Koeffler HP.	Hidden abnormalities and novel classification of t(15;17) acute promyelocytic leukemia (APL) based on genomic alterations.	Blood.	113	1741-8	2009
Akagi T, Ogawa S, Dugas M, Kawamata N, Yamamoto G, Nannya Y, Sanada M, Miller CW, Yung A, Schnittger S, Haferlach T, Haferlach C, Koeffler HP.	Frequent genomic abnormalities in acute myeloid leukemia/myelodysplastic syndrome with normal karyotype.	Haematologica.	94	213-23	2009
Ogawa S, Matsubara A, Onizuka M, Kashiwase K, Sanada M, Kato M, Nannya Y, Akatsuka Y, Satake M, Takita J, Chiba S, Saji H, Maruya E, Inoko H, Morishima Y, Kodera Y, Takehiko S.	Exploration of the genetic basis of GVHD by genetic association studies. Japan Marrow Donation Program (JMDP).	Biol Blood Marrow Transplant.	15 (1Suppl)	39-41	2009
Yokoyama Y, Suzuki T, Sakata-Yanagimoto M, Kumano K, Higashi K, Takato T, Kurokawa M, Ogawa S, Chiba S.	Derivation of functional mature neutrophils from human embryonic stem cells.	Blood.	113	6584-6592	2009
Nowak D, Le Toriellec E, Stern MH, Kawamata N, Akagi T, Dyer MJ, Hofmann WK, Ogawa S, Koeffler HP.	Molecular allelokaryotyping of T-cell prolymphocytic leukemia cells with high density single nucleotide polymorphism arrays identifies novel common genomic lesions and acquired uniparental disomy.	Haematologica.	94	518-527	2009

Suzuki I, Takenouchi T, Ohira M, Oba S, Ishii S.	Robust Model Selection for Classification of Microarrays.	Cancer Informatics	7	141-57	2009
Yu M, Ohira M, Li Y, Niizuma H, Oo ML, Zhu Y, Ozaki T, Isogai E, Kamijo T, Nakamura Y, Koda T, Oba S, Yu B, Nakagawara A.	High expression of ncRAN, a novel non-coding RNA mapped to 17q25.1, is associated with poor prognosis in neuroblastoma.	Int J Oncol.	34 (4)	931-8	2009
Ochiai H, Takenobu H, Nakagawa A, YamaguchiY, Kimura M, Ohira M, Okimoto Y, Fujimura Y, Koseki H, Kohno Y, Nakagawara A, Kamijo T.	Bmi1 is a MYCN target gene that regulates tumorigenesis through repression of KIF1Bb and TSLC1 in neuroblastoma	Oncogene.	in press		
Kusuki S, Hashi Y, Fukushima N, Takizawa S, Tokimasa S, Kogaki S, Ohta H, Tsuda E, Nakagawa A, Ozono K.	Pediatric post-transplant diffuse large B cell lymphoma after cardiac transplantation.	Int J Hematol.	89	209-213	2009
Watanabe N, Okita H, Matsuoka K, Kiyotani C, Fujii E, Kumagai M and Nakagawa A.	Vaginal yolk sac (endodermal sinus) tumors in infancy presenting persistent vaginal bleeding.	J Obstet Gynaecol Res.	36 (1)	213-216	2010
Kobayashi R, Yamato K, Tanaka F, Takashima Y, Inada H, Kikuchi A, Kumagai MA, Sunami S, Nakagawa A, Fukano R, Fujita N, Mitsui T, Tsurusawa M, Mori T; Lymphoma Committee, Japanese Pediatric Leukemia/Lymphoma Study Group.	Retrospective analysis of non-anaplastic peripheral T-cell lymphoma in pediatric patients in Japan.	Pediatr Blood Cancer.	54	212-5	2010
Mitsui T, Mori T, Fujita N, Inada H, Horibe K, Tsurusawa M; Lymphoma Committee, Japanese Pediatric Leukemia/Lymphoma Study Group.	Retrospective analysis of relapsed or primary refractory childhood lymphoblastic lymphoma in Japan.	Pediatr Blood Cancer.	52	591-5	2009
Ohashi H, Arita K, Fukami S, Oguri K, Nagai H, Yokozawa T, Hotta T, Hanada S.	Two rare MPL gene mutations in patients with essential thrombocythemia.	Int J Hematol.	90 (3)	431-432	2009
Nishiwaki S, Terakura S, Yasuda T, Imahashi N, Sao H, Iida H, Kamiya Y, Niimi K, Morishita Y, Kohno A, Yokozawa T, Ohashi H, Sawa M, Kodera Y, Miyamura K.	Outcome of allogeneic bone marrow transplantation from unrelated donors for adult Philadelphia chromosome-negative acute lymphocytic leukemia in first complete-remission.	Int J Hematol.	in press		

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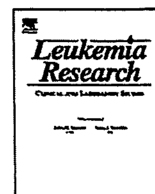

The expression of granulysin in systemic anaplastic large cell lymphoma in childhood

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ABSTRACT

The expression of granulysin, a cytolytic protein produced by activated T and NK cells, has been revealed to be correlated with the prognosis of some adult cancer patients. By examination on various childhood lymphoma tissues, we found that granulysin level was especially high in systemic anaplastic large cell lymphoma (ALCL) cases, whereas no close correlation with the expression of CD96, a marker for activated T and NK cells, was observed. We further demonstrated that both ALCL cells in biopsy specimens and cell lines established from ALCL express granulysin, indicating some correlation of granulysin with biological features of ALCL.

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1. Introduction

Granulysin is a cytolytic protein colocalized with other cytotoxic effectors in the granules of human activated T and NK cells [1,2]. Granulysin causes defects in negatively charged cholesterol-free membranes, a membrane composition typically found in bacteria, leading to both an elevation in the intracellular Ca^{2+} concentration and an increase in ceramide. As a consequent, mitochondria are damaged, causing the release of cytochrome c and apoptosis is induced [3–6]. In contrast, granulysin is able to bind to lipid rafts in eukaryotic cell membranes, where it is taken up by the endocytotic pathway, leaving the cell intact [6]. To date, two actions of granulysin have been described [2,7]. First, granulysin has an antibacterial effect and its antimicrobial activity against *Mycobacterium tuberculosis* as well as *Cryptococcus neoformans* has been shown to be dependent on the expression level of granulysin in cytotoxic T cells [8–12]. Second, granulysin reveals antitumoral activity. Many studies suggest that granulysin is an effective antitumor protein [2,7]. In experiments using an animal model, human granulysin promoted survival in transgenic mice with tumors [13]. Granulysin protein levels in NK cells were correlated with the prognosis or progression of disease in adult cancer patients [14,15]. However, the correlation between granulysin and clinicopathological features of childhood cancers, including lymphoma, is yet to be clarified.

There are four major types of childhood non-Hodgkin lymphoma; lymphoblastic lymphoma (LBL), Burkitt lymphoma, diffuse large B cell lymphoma and systemic anaplastic large cell lymphoma (ALCL), each with its own prognosis. ALCL is characterized by specific large lymphoma cells expressing CD30 frequently with chromosomal translocations that generate chimeric gene NPM-ALK in which the anaplastic lymphoma kinase (ALK) gene is involved [16–18]. ALCL has a T cell- or null cell-like phenotype and in some cases perforin and other cytotoxic effectors are expressed, suggesting ALCL originates from cytotoxic T or NK cells [19,20]. ALCL in childhood generally has a relatively good prognosis, however, clinicopathologic differences exist and some patients do poorly. Biological variation including the presence of NPM-ALK-positive and -negative cases indicates ALCL to be a heterogeneous disease type [17].

In an attempt to clarify the expressional state of granulysin in childhood lymphoma, we examined the mRNA expression of granulysin in tissues obtained from various cases of childhood lymphoma including ALCL. In this paper, we present a difference in granulysin expression among the types of childhood lymphomas.

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Notably, ALCLs had high levels of granulysin. The biological effect of granulysin in ALCL is discussed.

2. Materials and methods

2.1. Materials

Biopsy specimens from pediatric patients, including ten patients with ALCL, four with precursor B (B)-LBL, six with Burkitt lymphoma, five with diffuse large B cell lymphoma, five with Hodgkin's lymphoma and eight with precursor T (T)-LBL, were selected from files between 1985 and 2001 at our laboratory. In each case, the initial diagnosis was based on morphological observations and the immunophenotypic characteristics. The specimens are now kept under conditions of anonymity and all of the experiments included in this study followed the tenets of the Declaration of Helsinki and were performed with the approval of the local ethics committee.

The human ALCL cell lines Karpas-299 (a gift from Dr. K. Kikuchi of Sapporo Medical College, Sapporo, Japan) and SUDHL-1, the human Burkitt lymphoma cell line BALM-24 (gifts from Cell Biology Institute, Research Center, Hayashibara Biochemical Laboratories, Inc., Okayama, Japan), and the human megakaryoblastic cell lines CMK (a gift from Dr. T. Sato of Chiba University, School of Medicine, Chiba, Japan), Dami (American Type Culture Collection, Manassas, VA) and Meg-01 (Institute of Fermentation, Osaka, Japan) were used. Cells were maintained in RPMI1640 medium supplemented with 10% fetal calf serum at 37 °C in a humidified 5% CO_2 atmosphere. Mononuclear cells obtained from the peripheral blood of healthy volunteers by Ficoll-Paque centrifugation were cultured for 24 h in the presence of 5 nM of 12-myristate 13 acetate (PMA, Sigma–Aldrich Fine Chemical Co., St. Louis, MO) and 1 μM of ionomycin (Sigma) and collected for the extraction of total RNA.

2.2. RT-PCR and real-time PCR

Each tissue was embedded in optimal cutting temperature (O.C.T.) compound under rapid freezing conditions. The frozen tissues were sliced up 5-μm thick by cryostat, homogenized in Isogen (Nippon gene, Toyama, Japan). Total RNA was extracted according to the manufacturer's protocol. Using 1 μg of total RNA, cDNA was synthesized by transcriptase (Amersham Biosciences, Buckinghamshire, UK) and expression of granulysin mRNA was determined by quantitative real-time PCR using Taqman MGB probe with ABI 7900 systems (ABI, Foster City, CA).

2.3. Flow cytometry and immunohistochemical staining

To detect the cytoplasmic expression of granulysin, cells sequentially fixed with 4% paraformaldehyde followed by 70% ethanol were stained with fluorescein isothiocyanate (FITC)-labeled mouse anti-granulysin monoclonal antibody (RC8, Medical & Biological Laboratories, Co., Ltd., MBL, Nagoya, Japan) and examined by flow cytometry (EPICS-XL, Beckman Coulter, Inc., Fullerton, CA).

For immunohistochemical staining, the formalin-fixed, paraffin-embedded tissue specimens were deparafinized, treated using the heat-induced epitope retrieval method in 10 mM of citrate buffer, pH 6.0, stained with a combination of Alexa Fluor™546 (Invitrogen, Co., Carsbad, CA)-labeled rabbit anti-granulysin polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Crus, CA) and Alexa Fluor™488-labeled anti-ALK monoclonal antibody (ALK-C, Beckman) and examined by immunofluorescence microscopy (Olympus, Co., Tokyo, Japan). Photographs were taken with a CCD Camera (Cascade, Roper Scientific, Inc., Tucson, AZ).

The peripheral blood-derived mononuclear cells cultured for 5 days as described above were cytocentrifuged on slide glasses by using Cytospin II (Shandon, Inc., Pittsburgh, PA) and fixed with 4% paraformaldehyde. Cytochemical staining was performed by using either mouse monoclonal anti-granulysin antibody (RC8) or rabbit anti-granulysin polyclonal antibody as described above.

2.4. Statistical analysis

The statistical analysis was performed with a nonparametric Mann–Whitney test and correlations were determined using nonparametric statistics. A p-value less than 0.05 was considered to be statistically significant.

3. Results

First, to verify any differences in the level of granulysin expression between the types of lymphoma in childhood, we performed a quantitative real-time PCR analysis with total RNA extracted from the tumor tissues obtained from pediatric patients with ALCL, B-LBL, Burkitt, diffuse large B cell lymphoma, Hodgkin, and T-LBL. As shown in Fig. 1, the pattern of granulysin expression differed in each type of lymphoma. When compared with the value normalized to be expression of GAPDH, the level of granulysin was particularly high in ALCL (mean intensity normalized with GAPDH = 1.066),

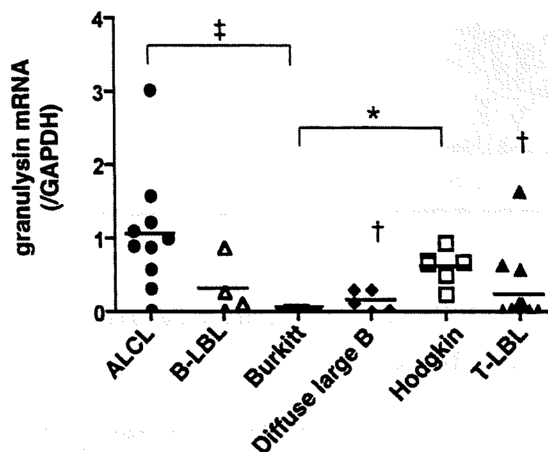

Fig. 1. Expression of granulysin mRNA in childhood lymphoma cases. The granulysin mRNA levels in extirpated tissues were determined with real-time quantitative PCR. Each value is normalized to GAPDH expression in the sample. ALCL, systemic anaplastic large cell lymphoma; B-LBL, precursor B lymphoblastic lymphoma; T-LBL, precursor T lymphoblastic lymphoma. The Burkitt cases (‡$p < 0.001$), diffuse large B cases (†$p < 0.01$) and T-LBL cases (†$p < 0.01$) exhibited significantly low levels of granulysin mRNA in comparison with the ALCL cases. The Burkitt cases (*$p < 0.01$) showed significantly low levels in comparison with the Hodgkin cases.

while the level in Burkitt lymphoma was very low (mean intensity = 0.012) and that in Hodgkin lymphoma was moderate (mean intensity = 0.625). Some significant differences between the types were observed: ALCL vs. Burkitt, $p < 0.001$; ALCL vs. Diffuse large B- or T-LBL, $p < 0.01$; Burkitt vs. Hodgkin, $p < 0.01$.

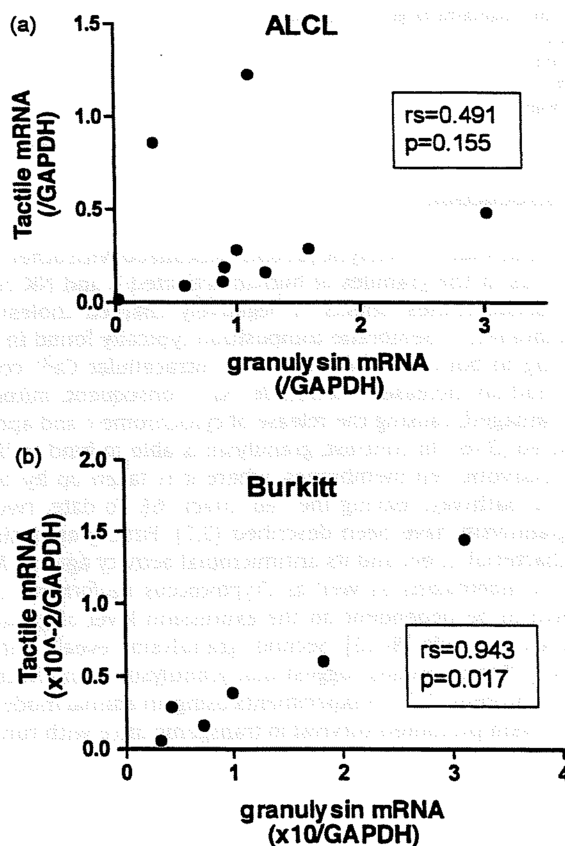

Fig. 2. Analysis of the correlation between granulysin and CD96 mRNA expression in childhood lymphoma cases. The mRNA expression of CD96 in the ALCL cases (a) and Burkitt cases (b) was examined and the correlation between granulysin and CD96 mRNA expression was evaluated.

The difference in granulysin expression was possibly influenced by the number of activated cytotoxic T and/or NK cells that had infiltrated into the tissues. Therefore, we examined the expression of a marker gene for cytotoxic T and NK cells and compared it with that of granulysin. CD96, also called T cell-activated increased late expression (TACTILE), is involved in the adhesion of activated T and NK cells late in an immune response and one of the several markers of activated T or NK cells [21]. In fact, its expression has been demonstrated to be well correlated with that of granulysin in some cancer patients [1,21]. Thus, we selected CD96 to confirm from where the granulysin mRNA was derived. Although the expression of CD96 mRNA was observed to be closely correlated with the expression of granulysin in Burkitt cases (rs = 0.943, $p < 0.05$) (Fig. 2b), no significant correlation between the expressions of these two molecules in ALCL cases (rs = 0.491) was obtained (Fig. 2a).

In some cases, ALCL cells express cytotoxic molecules such as perforin and granzyme B and thus have been suggested to be directed from cytotoxic T or NK cells. Considering the above results, we hypothesized that ALCL cells themselves express granulysin. Therefore, we used the ALCL-derived cell lines Karpas-299 and SUDHL-1 and determined the expression of granulysin in these cells. As shown in Fig. 3, granulysin mRNA was expressed in these cell lines as well as activated peripheral blood mononuclear cells and the megakaryoblastic cell line CMK. However, the mRNA was not detected in the megakaryoblastic cell lines Daudi and Meg-01 (Fig. 3a) and Burkitt lymphoma cell lines including BALM-24 (data

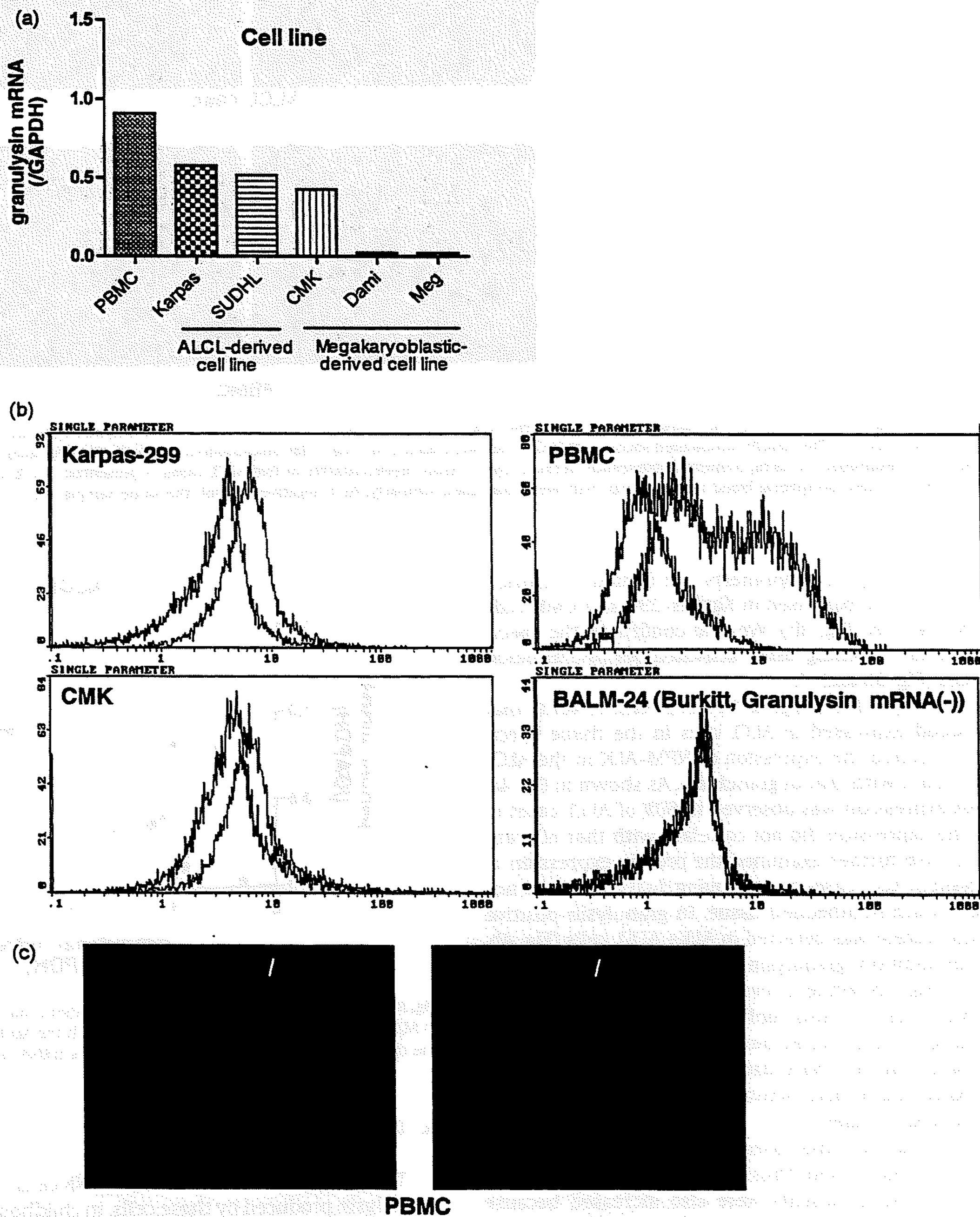

Fig. 3. Expression of granulysin in ALCL cell lines. (a) The levels of granulysin mRNA in ALCL-derived cell lines (Karpas-299 and SUDHL-1) and megakaryoblastic-derived cell lines (CMK, Dami and Meg-01) were determined. As a positive control, activated peripheral blood mononuclear cells (PBMC) were also examined. (b) Cytoplasmic expression of granulysin in Karpas-299, CMK, Burkitt lymphoma BALM-24 and PBMC was examined by using flow cytometry. (c) Cytoplasmic localization of granulysin in PBMC was also confirmed by cytochemical staining. As a negative control, the same sample specimen was also stained with control mouse IgG.

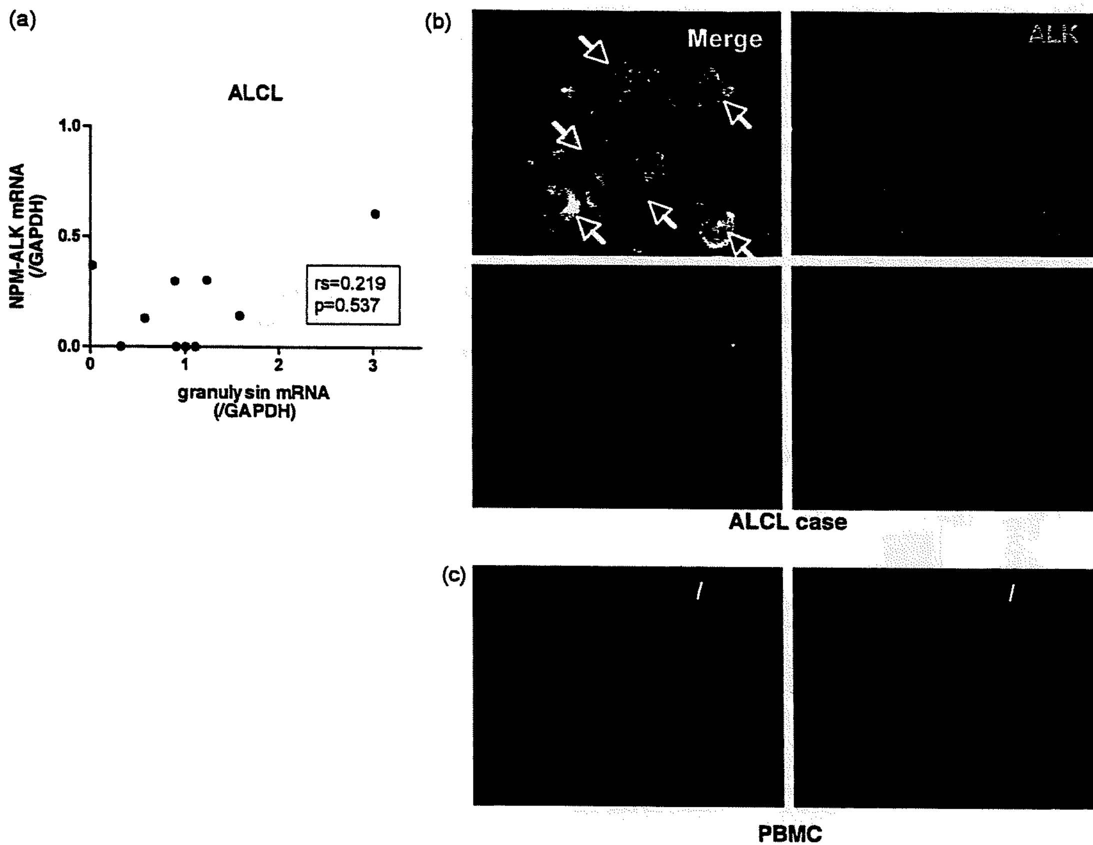

Fig. 4. Expression of granulysin in ALCL cases. (a) The mRNA expression of NPM-ALK in the ALCL cases was examined and the correlation between granulysin and NPM-ALK mRNA expression was evaluated. (b) The paraffin-embedded sections of ALCL cases were tested for dual color immunohistochemical staining using anti-granulysin antibody and anti-ALK antibody and examined by using immunofluorescence microscopy. A result representative of five ALCL cases is presented. (c) To confirm the specificity of anti-granulysin antibody, activated peripheral blood mononuclear cells were examined similarly. As a negative control, the same sample specimen was also stained with control rabbit IgG.

not shown). By employing flow cytometry, we further confirmed that granulysin protein is expressed in Karpas-299 and CMK cells but not in BALM-24 cells (Fig. 3b). We also confirmed the specificity of the granulysin staining using activated peripheral blood mononuclear cells (Fig. 3b and c).

NPM-ALK is a specific chimera gene for ALCL cells. To verify that granulysin is indeed expressed in ALCL cells in the tissue specimens, next we examined the expression of NPM-ALK in the ALCL cases and compared it with that of granulysin. As shown in Fig. 4a, NPM-ALK mRNA expression was observed in 60% of ALCL cases in this study, but the expression did not correlate with that of granulysin (rs = 0.219). We further examined the protein expression of ALK and granulysin in ALCL cases by employing dual color immunohistostaining in paraffin-embedded tissue. In granulysin-positive cases, granulysin protein was detected in ALK+ ALCL cells (Fig. 4b, yellow arrow). In addition, granulysin was also detected in ALK− small round cells (Fig. 4b, white arrow), most likely activated T or NK cells, in some cases. We also confirmed the specificity of the granulysin staining using activated peripheral blood mononuclear cells (Fig. 4c). Based on the above data, we concluded that granulysin can be expressed in both activated T or NK cells and ALCL cells themselves in ALCL cases.

It was reported that it some cases of ALCL, perforin, another cytotoxic molecule, is expressed. Thus, next we examined whether levels of perforin in ALCL patients were also increased because granulysin and perforin colocalized in activated T and NK cell granules. Though perforin mRNA expression was seen in 90% of cases, no correlation with granulysin was observed in ALCL (rs = 0.382) (Fig. 5).

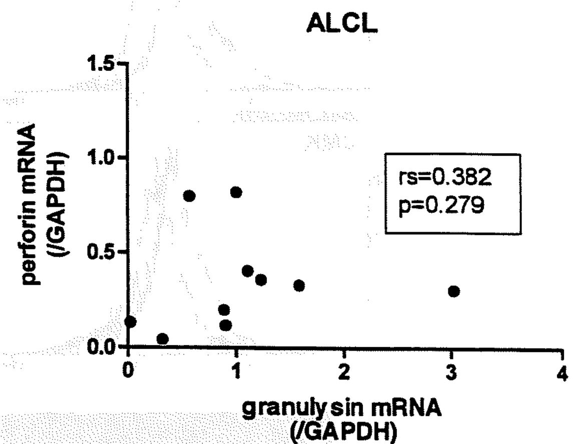

Fig. 5. Analysis of the correlation between granulysin and perforin mRNA expression in ALCL cases. The mRNA expression of perforin in the ALCL cases was examined and the correlation between granulysin and perforin mRNA expression was evaluated.

4. Discussion

The significance of activated T and NK cells, as well as the effect of granulysin produced by these cells, in childhood lymphoma tissues has yet to be clarified. In this study, we investigated the expression of granulysin in childhood cases of different lymphomas and found that the pattern of expression varied. As demonstrated in Fig. 1, level of granulysin was especially high in ALCL cases. Since the major

source of granulysin is generally thought to be activated T and NK cells [1,2], the expression in lymphomas might be dependent on the number of these cells that have infiltrated into tumor tissues. However, we observed that granulysin expression was not necessary correlated with CD96 expression in ALCL tissues (Fig. 2), suggesting that the granulysin expressed in ALCL tissues is not derived only from activated T or NK cells.

Since ALCLs often express cytotoxic effectors such as perforin and granzyme B, it has been postulated that they originate from cytotoxic lymphocytes [19,20]. Therefore, we hypothesized that the ALCL cells themselves express granulysin. Indeed, as we described above, the ALCL-derived cell lines Karpas-299 and SUDHL-1 expressed granulysin mRNA and protein (Fig. 3). Moreover, we detected granulysin protein in ALCL cells in clinical samples with immunohistochemical staining. As shown in Fig. 4b, the protein was detected both in ALK+ ALCL cells and in ALK− small round cells. Therefore, it is suggested that not only activated T and NK cells but also the ALCL cancers themselves express granulysin.

In activated T and NK cells, granulysin is colocalized with perforin in toxic granules, thus we also examined the mRNA expression of perforin in ALCL, but found that it was not correlated with the mRNA expression of granulysin. Therefore, it is suggested that the granulysin in ALCL cells is expressed independent of perforin. Since the expression of granzyme B was not correlated with that of perforin in ALCL cells either, the regulatory mechanism for the production of cytotoxic effectors in ALCL cells may be disorganized. Further investigation to elucidate the significance and molecular basis of granulysin expression in ALCL cells should provide information on the biological characteristics of ALCL, including a clue as to the cellular origin of this lymphoma.

Childhood ALCL has a relatively good prognosis and the relapse-free survival rate with first-line chemotherapy is 60–80% [18,22,23]. However, variation in clinicopathological features is recognized and in some cases the prognosis is poor. Since a way to predict the likely course of ALCL has yet to be established [17], searching for new markers that have a co-relation with prognosis is important. As we discussed above, it would be worth investigating the correlation between prognosis and the level of granulysin in ALCL cases. The question of how the expression of granulysin in the ALCL cells themselves affects the clinical course of ALCL is of particular interest.

In conclusion, we have investigated the expression of granulysin in childhood lymphomas and observed especially high levels in ALCL cases. Besides the activated T and NK cells, our findings indicated that the ALCL cells themselves expressed granulysin. Further elucidation of the biological significance of granulysin expression in ALCL should contribute to a better understanding of the biological features as well as underlying pathogenic mechanisms of this lymphoma.

Conflict of interest

None.

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Contributions. N. Kitamura and N. Kiyokawa contributed to the concept and design, interpreted and analyzed the data, provided drafting of the article, gave final approval, and obtained funding sources. K. Onda, Y.U. Katagiri, M. Itagaki, and Y. Miyagawa provided administrative support. H. Okita, A. Mori, and J. Fujimoto provided critical revisions and important intellectual content.

References

[1] Peña SV, Hanson DA, Carr BA, Goralski TJ, Krensky AM. Processing, subcellular localization, and function of 519 (granulysin), a human late T cell activation molecule with homology to small, lytic, granule proteins. J Immunol 1997;158:2680–8.
[2] Peña SV, Krensky AM. Granulysin, a new human cytolytic granule-associated protein with possible involvement in cell-mediated cytotoxicity. Semin Immunol 1997;9:117–25.
[3] Gamen S, Hanson DA, Kaspar A, Naval J, Krensky AM, Anel A. Granulysin-induced apoptosis. I. Involvement of at least two distinct pathways. J Immunol 1998;161:1758–64.
[4] Kaspar AA, Okada S, Kumar J, Poulain FR, Drouvalakis KA, Kelekar A, et al. A distinct pathway of cell-mediated apoptosis initiated by granulysin. J Immunol 2001;167:350–6.
[5] Okada S, Li Q, Whitin JC, Clayberger C, Krensky AM. Intracellular mediators of granulysin-induced cell death. J Immunol 2003;171:2556–62.
[6] Barman H, Walch M, Latinovic-Golic S, Dumrese C, Dolder M, Groscurth P, et al. Cholesterol in negatively charged lipid bilayers modulates the effect of the antimicrobial protein granulysin. J Membr Biol 2006;212:29–39.
[7] Wang Z, Choice E, Kaspar A, Hanson DA, Okada S, Lyu SC, et al. Bactericidal and tumoricidal activities of synthetic peptides derived from granulysin. J Immunol 2000;165:1486–90.
[8] Stenger S, Hanson DA, Teitelbaum R, Dewan P, Niazi KR, Froelich CJ, et al. An antimicrobial activity of cytolytic T cells mediated by granulysin. Science 1998;282:121–5.
[9] Ernst WA, Thoma-Uszynski S, Teitelbaum R, Ko C, Hanson DA, Clayberger C, et al. Granulysin, A T cell product. Kills bacteria by altering membrane permeability. J Immunol 2000;165:7102–8.
[10] Ma LL, Spurrell JC, Wang JF, Neely GG, Epelman S, Krensky AM, et al. CD8 T cell-mediated killing of *Cryptococcus neoformans* requires granulysin and is dependent on CD4 T cells and IL-15. J Immunol 2002;169:5787–95.
[11] Stegelmann F, Bastian M, Swoboda K, Bhat R, Kiessler V, Krensky AM, et al. Coordinate expression of CC chemokine ligand 5, granulysin, and perforin in CD8+ T cells provides a host defense mechanism against *Mycobacterium tuberculosis*. J Immunol 2005;175:7474–83.
[12] Sahiratmadja E, Alisjahbana B, Buccheri S, Di Liberto D, de Boer T, Adnan I, et al. Plasma granulysin levels and cellular interferon-gamma production correlate with curative host responses in tuberculosis, while plasma interferon-gamma levels correlate with tuberculosis disease activity in adults. Tuberculosis 2007;87:312–21.
[13] Huang LP, Lyu S, Clayberger C, Krensky AM. Granulysin-mediated tumor rejection in transgenic mice. J Immunol 2007;178:77–84.
[14] Kishi A, Takamori Y, Ogawa K, Takano S, Tomita S, Tanigawa M, et al. Differential expression of granulysin and perforin by NK cells in cancer patients and correlation of impaired granulysin expression with progression of cancer. Cancer Immunol Immunother 2002;50:604–14.
[15] Muris JJ, Meijer CJ, Cillessen SA, Vos W, Kummer JA, Bladergroen BA, et al. Prognostic significance of activated cytotoxic T-lymphocytes in primary nodal diffuse large B-cell lymphomas. Leukemia 2004;18:589–96.
[16] Stein H, Mason DY, Gerdes J, O'Connor N, Wainscoat J, Pallesen G, et al. The expression of the Hodgkin's disease-associated antigen Ki-1 in reactive and aneoplastic lymphoid tissue; evidence that Reed-Sternberg cells and histiocytic malignancies are derived from activated lymphoid cells. Blood 1985;66:848–58.
[17] Nakamura S, Shiota M, Nakagawa A, Yatabe Y, Kojima M, Motoori T, et al. Anaplastic large cell lymphoma: a distinct molecular pathologic entity: a reappraisal with special reference to p80 (NPM/ALK) expression. Am J Surg Pathol 1997;21:1420–32.
[18] Drexler HG, Gignac SM, von Wasielewski R, Werner M, Dirks WG. Pathology of *NPM-ALK* and variant fusion genes in anaplastic large cell lymphoma and other lymphomas. Leukemia 2000;14:1533–59.
[19] Foss H, Anagnostopoulos I, Araujo I, Assaf C, Demel G, Kummer JA, et al. Anaplastic large-cell lymphomas of T-cell and Null-cell phenotype express cytotoxic molecules. Blood 1996;88:4005–11.
[20] Krenacs L, Wellmann A, Sorbara L, Himmelmann AW, Bagdi E, Jaffe ES, et al. Cytotoxic cell antigen expression in anaplastic large cell lymphomas of T- and null cell type and Hodgkin's disease: evidence for distinct cellular origin. Blood 1997;89:980–9.
[21] Wang PL, O'Farrell S, Clayberger C, Krensky AM. Identification and molecular cloning of Tactile. A novel human T cell activation antigen that is a member of the Ig gene superfamily. J Immunol 1992;148:2600–8.
[22] Mori T, Kiyokawa N, Shimada H, Miyauchi J, Fujimoto J. Anaplastic large cell lymphoma in Japanese children: retrospective analysis of 34 patients diagnosed at the National Research Institute for Child Health and Development. Br J Haematol 2003;121:94–6.
[23] Mori T, Takimoto T, Katano N, Kikuchi A, Tabuchi K, Kobayashi R, et al. Recurrent childhood anaplastic large cell lymphoma: a retrospective analysis of registered cases in Japan. Br J Haematol 2006;132:594–7.

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Kinetics and Effect of Integrin Expression on Human CD34$^+$ Cells During Murine Leukemia Virus-Derived Retroviral Transduction with Recombinant Fibronectin for Stem Cell Gene Therapy

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Abstract

The CH-296 recombinant fragment of human fibronectin is essential for murine leukemia virus (MLV)-derived retroviral transduction of CD34$^+$ cells for the purpose of stem cell gene therapy. Although the major effect of CH-296 is colocalization of the MLV-derived retrovirus and target cells at specific adhesion domains of CH-296 mediated by integrins expressed on CD34$^+$ cells, the precise roles of the integrins are unclear. We examined the kinetics of integrin expression on CD34$^+$ cells during the course of MLV-derived retrovirus-mediated gene transduction with CH-296. Flow cytometry revealed that the levels of both very late activation protein (VLA)-4 and VLA-5 on CD34$^+$ cells freshly isolated from cord blood were insufficient for effective MLV-derived retroviral transduction. However, increases were achieved during culture for preinduction and MLV-derived retrovirus-mediated gene transduction in the presence of a cocktail of cytokines. In addition, we confirmed by using specific antibodies that inhibition of the cell adhesion mediated by the integrins significantly reduced transduction efficiency, indicating that integrin expression is indeed important for CH-296-based MLV-derived retroviral transduction. Only a few cytokines are capable of inducing integrin expression, and stem cell factor plus thrombopoietin was found to be the minimal combination that was sufficient for effective transduction of an MLV-derived retrovirus based on CH-296. Our findings should be useful for improving the culture conditions for CH-296-based MLV-derived retroviral transduction in stem cell gene therapy.

Introduction

Stem cell gene therapy in which a defective gene in some hematopoietic stem and progenitor cells (HSPCs) is replaced with a functional copy can reconstitute the immune system and is therefore expected to be an effective treatment for inherited immunodeficiencies. Indeed, clinical trials in children with severe combined immunodeficiency (SCID), adenosine deaminase (ADA) deficiency, and chronic granulomatous disease (CGD) have been successful (Onodera *et al.*, 1998; Cavazzana-Calvo *et al.*, 2000; Ariga *et al.*, 2001; Ott *et al.*, 2006).

Murine leukemia virus (MLV)-derived retroviral vectors are used to place a functional copy of a gene in HSPCs. Although MLV-derived retroviruses have the ability to introduce and express genes stably in the host cell genome, applicable protocols in humans have been limited because of the low gene transfer efficiency achieved (Bodine *et al.*, 1998). However, the development and application of a truncated form of recombinant human fibronectin (CH-296

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[RetroNectin]; Takara Bio, Otsu, Shiga, Japan) have significantly improved the efficiency of transduction of MLV-derived retroviral vectors into reconstituting hematopoietic stem cells (Kimizuka *et al.*, 1991).

CH-296 is a recombinant fragment of human fibronectin composed of the cell-binding domain (C domain), heparin-binding domain II (H domain), and CS1 site. The H domain was found to bind MLV-derived retroviral particles, and the RGD sequence in the C domain and the LDV sequences in the CS1 site are recognized by a variety of cell types through integrins $\alpha_5\beta_1$ (VLA-5) and $\alpha_4\beta_1$ (VLA-4), respectively. Consequently, CH-296 is capable of enhancing MLV-derived retrovirus-mediated gene transduction by binding to both MLV-derived retroviral particles and target cells that express VLA-5 and/or VLA-4 (Hanenberg *et al.*, 1996, 1997).

The practical effects of CH-296 on MLV-derived retrovirus-mediated gene transduction into HSPCs have been well established in both basic biological experiments and clinical trials of stem cell gene therapy using MLV-derived retroviral vectors (Ariga *et al.*, 2001). However, the actual effects of integrins expressed on HSPCs on MLV-derived retrovirus-mediated gene transduction with CH-296 have not been well characterized. Although differences in integrin expression on HSPCs should significantly affect the efficiency of MLV-derived retroviral gene transduction and thus greatly influence the success of clinical trials of stem cell gene therapy, the details remain unclear, and investigating the kinetics of integrin expression on HSPCs during their manipulation for gene transduction should help to improve stem cell gene therapy.

In this paper we present evidence that CD34$^+$ cells freshly isolated from cord blood express only small amounts of integrins, but that they accumulate integrins during culture for preinduction and MLV-derived retrovirus-mediated gene transduction in the presence of a cocktail of cytokines. We also confirmed the effect of integrin levels on CH-296-based MLV-derived retrovirus-mediated gene transduction in human CD34$^+$ cells and attempted to identify the minimal combination of cytokines that would allow effective integrin expression sufficient for MLV-derived retrovirus-mediated gene transduction.

Materials and Methods

Reagents, cells, and cell culture

The cord blood was distributed by the Tokyo Cord Blood Bank (Tokyo, Japan) and had originally been collected and stored for stem cell transplantation. Stocks inappropriate for transplantation because they contained too few cells were distributed for research use with the informed consent of the donor and the permission of the ethics committee of the Tokyo Cord Blood Bank. All the experiments in which cord blood was used in this study were performed with the approval of the local ethics committee. After isolating mononuclear cells from the cord blood by density gradient centrifugation with Ficoll-Hypaque, immunomagnetic enrichment of CD34$^+$ cells was performed with a magnetic-activated cell-sorting (MACS) system according to the instructions of the manufacturer (Miltenyi Biotec, Bergisch Gladbach, Germany).

X-VIVO 10 (Lonza Walkersville, Walkersville, MD) containing 1% human albumin and a cytokine cocktail consisting of stem cell factor (SCF, 50 ng/ml), thrombopoietin (TPO, 50 ng/ml), Flt3 ligand (FL, 300 ng/ml), interleukin-6 (IL-6, 100 ng/ml), and soluble IL-6 receptor (sIL-6R, 500 ng/ml) was used for pretransduction and maintenance of the CD34$^+$ cells *ex vivo*. In some experiments the cytokines were used alone or in combinations of fewer cytokines than in the complete cocktail, as indicated in the figures. All the cytokines used in this study were purchased from PeproTech EC (London, UK).

Viral supernatants were obtained from P13/DNEGFP cells developed from PG13 packaging cells transfected with pGCDNsap (DNEGFP), as described previously (Suzuki *et al.*, 2002), and used as the MLV-derived retroviral vector for enhanced green fluorescent protein (EGFP) expression. PG13 is a retrovirus-packaging cell line derived from TK-NIH/3T3 cells and stably expresses the gibbon ape leukemia virus (GALV) envelope glycoprotein of this virus for pseudotyping of the MLV vectors that are transfected in these cells. The viral titer was determined with HT-1080 cells (American Type Culture Collection [ATCC], Manassas, VA).

The monoclonal antibodies (mAbs) used for immunofluorescence analysis were as follows: phycoerythrin (PE)-conjugated anti-CD49d and anti-CD49e from BD Biosciences (San Jose, CA), and PE-conjugated anti-CD29 and PE-cyanine 5 (PC-5)-conjugated anti-CD34 from Beckman Coulter (Fullerton, CA).

Transduction of CD34$^+$ cells

CD34$^+$ cells isolated from cord blood were suspended at a concentration of 1×10^6 cells/ml in X-VIVO 10 and cultured for 48 hr, as preinduction, in the presence of the cytokines described previously. To transduce CD34$^+$ cells with the EGFP expression vector, 1×10^5 cells were resuspended in 1 ml of diluted P13/DNEGFP supernatant supplemented with the cytokines used for preinduction and plated in retronectin-precoated 35-mm dishes. After incubation for 12 hr the cells were collected by centrifugation ($200\times g$ for 5 min) and washed once with X-VIVO 10 medium. The cells were then cultured for 48 hr in X-VIVO 10 supplemented with the same cytokines as used for induction. In this experiment, only a single induction was performed to identify the effect of the reagents.

TABLE 1. RESULTS OF COLONY ASSAYS

	SCF	*TPO*	*Flt3 ligand*	*IL-6, sIL-6R*	*SCF, TPO*	*SCF, TPO, Flt3 ligand, IL-6, sIL-6R*
All colonies	48.7 ± 1.2	43.0 ± 1.4	36.3 ± 5.3	33.0 ± 5.0	67.3 ± 1.7	71.7 ± 3.9
GFP positive	0	0	0	0	8 ± 4.2	9.7 ± 1.5
Large colonies	15.3 ± 1.7	13.3 ± 2.1	13.7 ± 3.1	10.0 ± 0.8	16.7 ± 1.2	25.7 ± 1.2
GFP positive	0	0	0	0	3.3 ± 1.7	5.3 ± 0.9

Abbreviations: IL-6, interleukin 6; SCF, stem cell factor; sIL-6R, soluble IL-6 receptor; TPO, thrombopoietin.