20141103 8A

厚生労働科学研究費補助金

がん対策推進総合研究事業(革新的がん医療実用化研究事業)

難治癌を標的治療できる完全オリジナルのウイルス遺伝子医薬の実用化のための前臨床研究

平成２６年度　総括研究報告書

研究代表者　小戝　健一郎

平成２７（２０１５）年　５月

目　　次

厚生労働科学研究費補助金（がん対策推進総合研究事業（革新的がん医療実用化研究事業））
総括研究報告書

研究総括

研究代表者　　小賀　健一郎　　鹿児島大学大学院医歯学総合研究科・教授

研究要旨

独自開発の m-CRA（多因子で癌特異化する増殖制御型アデノウイルスベクター）作製技術を基盤に開発した、Surv.m-CRA の医師主導治験開始に向け、３年間の前臨床研究の３年目であった。本年も、GMP 製造、非臨床試験、ならびに当局対応を計画通り行った。

研究分担者

三井　薫　　鹿児島大学大学院医歯学総合研究科・講師
入江　理恵　鹿児島大学大学院医歯学総合研究科・助教
伊地知　暢広鹿児島大学大学院医歯学総合研究科・助教
王　宇清　　鹿児島大学産学連携推進センター・プロジェクト研究員
小宮　節郎　鹿児島大学大学院医歯学総合研究科・教授
永野　聡　　鹿児島大学大学院医歯学総合研究科・助教
夏越　祥次　鹿児島大学大学院医歯学総合研究科・教授
福﨑　好一郎株式会社新日本科学・専務取締役

A．研究目的

この研究にいたる背景として、研究代表者は、まず遺伝子治療研究の黎明期（90年代初頭）に米国専門施設でアデノウイルスベクターによる癌遺伝子治療法の開発に世界に先駆け成功し（***PNAS*** 1995, 1996等）、米国共同研究者が臨床試験にも成功した。帰国後に本邦の自身の研究室で、完全オリジナルの「多因子で同時に精密に癌特異標的治療できる増殖制御型アデノウイルス」(m-CRA)の作製技術の開発に成功し(***Gene Ther*** 2005）、Survivin 依存性 m-CRA (Surv.m-CRA)（第一弾のm-CRA医薬）を開発した（***Cancer Res*** 2005）。さらに癌特異性（安全性）と治療効果を向上した新型Surv.m-CRAの開発を行った(***Cancer Gene Ther*** 2011)。さらに、Surv.m-CRAは既存の治療法が効果を示さない、癌幹細胞を効果的に治療できることを実証した（***J Trans Med*** 2014）。

本研究は、Surv.m-CRA の臨床用のGMP製造、GLP基準での非臨床試験のデータ取得等を3年間で行い、平成27年度よりこの分野で本邦初の医師主導治験を開始することを目的とする。

B．研究方法とC．研究結果

「本研究終了後の翌（平成27）年度に本邦初の癌遺伝子治療の医師主導治験を開始」という当初の研究計画通り、本研究は以下のように順調に進んだ。

1．　医師主導治験に使用する本ウイルスの治験薬の製造と品質・安定性試験など

①　治験薬のGMP原薬の製造

医師主導治験に使用することを目的として、本ウイルスのGMP原薬の製造をSAFC社に委託実施し、製造した。

②　GMP原薬の品質試験

GMP原薬の力価、品質試験をSAFC社及びBioReliance社で実施し、治験に使用する上で必要な品質が確保されていることを確認した。試験項目は以下のとおりである。

- 安全性試験（細菌・真菌、エンドトキシン、マイコプラズマ、混入ウイルス等）
- 製造品の確認試験（制限酵素マッピング、GLP対応の全シークエンスの確認）
- 純度試験（宿主DNA・蛋白、Benzonaseなどの工程由来不純物の残存）

- 強度試験（本ウイルスの力価、粒子数、感染ウイルス純度）
- 活性／能力試験（本ウイルスの癌特異的なウイルス増殖能）
- その他（外観、pH）

③ GMP製剤の安定性試験

GMP製剤を製造し、安定性試験を開始した。

2. 前臨床（非臨床）試験

1) POC (Proof of Concept / Efficacy)試験

① *in vivo*治療試験

マウスの骨肉腫モデルにおいて、治験薬surv.m-CRA-1を用いた治療実験を行い、有効性を確認した。

2) 安全性試験

この分野では実績のある英国のハンティンドンライフサイエンス（HLS）社に委託して行った。

① ハムスター単回投与（静脈投与）による毒性試験（GLP）

PMDAの助言に従い、前年度までの皮下・筋肉投与と合わせて、ワーストケースシナリオを想定した静脈投与の毒性試験を実施した。

② ハムスター単回ならびに反復投与（静脈投与）による薬物動態試験

上記の毒性試験と併せて、薬物動態試験も実施した。

3. 医師主導治験のための準備

当局対応、大学の治験体制整備も以下のように進めた。

1) PMDAとの相談

＜全体の方向性の相談：個別相談、事前相談＞

① 2012年8月2日（個別面談）
② 2012年11月26日（事前相談）
③ 2013年10月10日（事前相談）
④ 2014年6月24日（事前相談）

本研究が３次公募で採択されてすぐに、PMDAに個別面談を申し込み実施した。その後、平成24、25、26年度（毎年）に事前相談を着実に行いながら、製造・品質、非臨床試験、臨床プロトコールについての全体事項を適切に進捗させた。2014年6月24日の事前相談にて最終的な方向性ならびに、正式な対面助言の進め方もPMDAと確認し、製造・品質、非臨床試験、臨床プロトコールの３つを順次進めることとなり、そのスケジュールにしたがって適切に３つの対面助言を以下のように進めた。

＜製造・品質：対面助言等＞

① 2014年10月2日（対面助言）
② 2014年12月24日（事前相談：フォローアップ）

製造・品質についてはPMDAとの意見相違はなく、大きな問題はなかった。生物由来原料は全情報（CMOでの20年前位のMaster cell bank製造時の使用材料の情報等まで含めて）を一旦は情報入手を試みて欲しいこと、そしてその後に物理的に情報入手不可能な部分は適切性を説明していく、という方向性の助言をPMDAより受けた。それに従い、情報収集をして、フォローアップの事前相談も行い、確認をとった。

＜非臨床試験：対面助言等＞

① 2014年11月2日（対面助言）

非臨床試験はPMDAの助言を受け入れて進めているため、問題は何もなかった。

＜臨床プロトコール：対面助言等＞

① 2014年11月11日（事前相談）
② 2015年3月3日（対面助言）

事前相談で方向性などは確認し、3月3日に対面助言を実施し、臨床プロトコールの全体像が確定した。

2) 大学の治験体制整備

本学に臨床研究支援センターを平成26年度4月に設置し、専従スタッフも配置した。よって、協力している京都大学医学部附属病院臨床研究総合センターの支援ももらいながら、本プロジェクトの本学での医師主導治験の支援体制も具体的に整備していった。

D．考察とE．結論

独自開発の m-CRA（多因子で癌特異化する増殖制御型アデノウイルスベクター）作製技術を基盤に開発した、Surv.m-CRA の医師主導治験開始に向け GMP 製造、非臨床試験、ならびに当局対応を計画通り行った。平成 27 年度に医師主導治験を開始できる予定で、順調に進捗している。

F．健康危険情報

特になし。

G．研究発表

1.論文発表

1. Nagano S, Yokouchi M, Setoguchi T, Ishidou Y, Sasaki H, Shimada H, Komiya S.Differentiation of lipoma and atypical lipomatous tumor by ascoring system: implication of increased vascularity on pathogenesis of liposarcoma. *BMC Musculoskelet Disord*. 16:36, 2015

2. Tanoue K, Wang Y, Ikeda M , Mitsui K, Irie R , Setoguchi T, Komiya S, Natsugoe S, Kosai K.:

Survivin-responsive conditionally replicating adenovirus kills rhabdomyosarcoma stem cells more efficiently than their progeny. ***J Trans Med*** 27;12(1):27.doi: 10.1186/1479-5876-12-27,2014

3. Yuge K, Takahashi T, Khai NC, Goto K, Fujiwara T, Fujiwara H, Kosai K.: Intramuscular injection of adenoviral hepatocyte growth factor at a distal site ameliorates dextran sodium sulfate-induced colitis in mice. ***Int J Mol Med*** 33(5):1064-74, 2014
4. Nagao-Kitamoto H, Setoguchi T, Kitamoto S, Nakamura S, Tsuru A, Nagata M, Nagano S, Ishidou Y, Yokouchi M, Kitajima S, Yoshioka T, Maeda S, Yonezawa S,Komiya S. Ribosomal protein S3 regulates GLI2-mediated osteosarcoma invasion ***Cancer Lett*** , 2014
5. Nagao-Kitamoto H, Nagata M, Nagano S, Kitamoto S, Tsuru A, Abematsu M, Fujimoto Y,Yokouchi M, Kitajima S, Yoshioka T, Maeda S, Yonezawa S, Komiya S, Setoguchi T. Gli2 is a novel therapeutic target for metastasis of osteosarcoma. ***Int J Cancer*** , 2014
6. Tanoue K, Wang Y, Ikeda M, Mitsui K, Irie R, Setoguchi T, Komiya S, Natsugoe S, Kosai K. Survivin-responsive conditionally replicating adenovirus killsrhabdomyosarcoma stem cells more efficiently than their progeny. ***J Transl Med***12:27-40, 2014
7. Komiya S. Personal development through research. ***J Orthop Sci*** 19:697-698, 2014
8. Yokouchi M, Ueno Y, Nagano S, Shimada H, Nakamura S, Setoguchi T, Kawamura I, Ishidou Y, Komiya S. Extended curettage and heat ablation for desmoplastic fibromaof the distal femur with a 12-year follow-up period: A case report. ***Oncol Lett*** 8:1103-1106, 2014
9. Shimada H, Setoguchi T, Yokouchi M, Sasaki H, Ishidou Y, Kawamura I, Abematsu M, Nagano S, Komiya S.Metastatic bone tumors: Analysis of factors affecting prognosis and efficacy of CT and (18)F-FDG PET-CT in identifying primary lesions. ***Mol Clin Oncol*** 2:875-881, 2014
10. Yokouchi M, Nagano S, Kijima Y, Yoshioka T, Tanimoto A, Natsugoe S, Komiya S. Solitary breast metastasis from myxoid liposarcoma. ***BMC Cancer*** 14:482, 2014
11. Tofuku K, Koga H, Komiya S. Percutaneous drainage combined with hyperbaric oxygen therapy for pyogenic spondylitis with iliopsoas abscess. ***Asian Spine J*** 8:253-259, 2014
12. Sakamoto Y, Yokouchi M, Nagano S, Shimada H, Nakamura S, Setoguchi T, Kawamura I, Ishidou Y, Tanimoto A,Komiya S. Metastasis of osteosarcoma to the trapeziusmuscle: a case report. ***World J Surg Oncol*** 12:176, 2014
13. Yokouchi M, Nagano S, Shimada H, Nakamura S, Setoguchi T, Kawamura I, Ishidou Y, Komiya S. Early complete remission of osteoid osteoma with conservative medical management. ***Pediatr Rep*** 6:5311, 2014
14. Shimada H, Setoguchi T, Yokouchi M, Sasaki H, Ishidou Y, Kawamura I, Abematsu M, Nagano S, Komiya S, Metastatic bone tumors: Analysis of factors affecting prognosis and efficacy of CT and 18F-FDG PET-CT in identifying primary lesions. ***Mol Clin Oncol,*** 2:875-881,2014
15. Nagano S, Yokouchi M, Setoguchi T, Sasaki H, Shimada H, Kawamura I, Ishidou Y,Kamizono J, Yamamoto T, Kawamura H, Komiya S. Analysis of surgical site infectionafter musculoskeletal tumor surgery: risk assessment using a new scoring system. ***Sarcoma.*** 2014:645496, 2014
16. Nagano S, Yokouchi M, Setoyama T, Sasaki H, Shimada H, Kawamura I, Ishidou Y, Setoguchi T, Komiya S. Elastofibroma dorsi: Surgical indications andcomplications of a rare soft tissue tumor. ***Mol Clin Oncol.*** 2(3):421-424, 2014
17. Nishimura H, Enokida H, Nagano S, Yokouchi M, Hayami H, Komiya S, Nakagawa M. Effects of blood purification therapy on a patient with ifosfamide-induced neurotoxicity and acute kidney injury. ***J Artif Organs***. 17(1):110-3, 2014
18. Kakoi H, Maeda S, Shinohara N, Matsuyama K, Imamura K, Kawamura I, Nagano S, Setoguchi T, Yokouchi M, Ishidou Y, Komiya S.BMP signaling upregulates neutral sphingomyelinase 2 to suppress chondrocyte maturation via the Akt signaling pathway as a negative feedback mechanism. ***J BiolChem*** 289: 8135-8150, 2014
19. Imamura K, Maeda S, Kawamura I, Matsuyama K, Shinohara N, Yahiro Y, Nagano S, Setoguchi T, Yokouchi M, Ishidou Y, Komiya S.Human Immunodeficiency Virus Type I Enhancer Binding Protein 3 is Essential for the Expression of Asparagine-linked Glycosylation 2 in the Regulation of Osteoblast and Chondrocyte Differentiation ***J BiolChem*** 289: 9865-9879, 2014
20. Ikeda R, Nishizawa Y, Tajitsu Y, Minami K, Mataki H, Masuda S, Furukawa T, Akiyama S, Yamada K, Takeda Y.: Regulation of major vault protein expression by upstream stimulating factor 1 in SW620 human colon cancer cells.

Oncol Rep. 31(1):197-201,2014

21. Omoto I, Matsumoto M, Okumura H, Uchikado Y, Setoyama T, Kita Y, Owaki T, Kijima Y, Shinchi H, Ishigami S, Ueno S, Natsugoe S.: Expression of vascular endothelial growth factor-C and vascular endothelial growth factor receptor-3 in esophageal squamous cell carcinoma.***Oncol Lett.***7(4):1027-103, 2014
22. Mizoguchi T, Kijima Y, Hirata M, Kaneko K, Arima H, Nakajo A, Higashi M, Tabata K, Koriyama C, Arigami T, Uenosono Y, Okumura H, Maemura K, Ishigami S, Yoshinaka H, Shinden Y, Ueno S, Natsugoe S.: Histological findings of an autologous dermal fat graft implanted onto the pectoralis major muscle of a rat model.***Breast Cancer,*** 2014 [Epub ahead of print]
23. Kijima Y, Yoshinaka H, Hirata M, Mizoguchi T, Ishigami S, Nakajo A, Arima H, Ueno S, Natsugoe S.: Oncoplastic surgery in a Japanese patient with breast cancer in the lower inner quadrant area: partial mastectomy using horizontal reduction mammoplasty.***Breast Cancer.*** 21(3):375-8, 2014
24. Yoneyama T, Fukukura Y, Kamimura K, Takumi K, Umanodan A, Ueno S, Nakajo M.: Efficacy of liver parenchymal enhancement and liver volume to standard liver volume ratio on Gd-EOB-DTPA-enhanced MRI for estimation of liver function. ***Eur Radiol.*** 24(4):857-65, 2014
25. Arigami T, Uenosono Y, Ishigami S, Matsushita D, Hirahara T, Yanagita S, Okumura H, Uchikado Y, Nakajo A, Kijima Y, Natsugoe S.: Decreased density of CD3+ tumor-infiltrating lymphocytes during gastric cancer progression.***J Gastroenterol Hepatol*** 29(7):1435-41, 2014
26. Omoto I, Matsumoto M, Okumura H, Uchikado Y, Setoyama T, Kita Y, Owaki T, Kijima Y, Shinchi H, Ishigami S, Ueno S, Natsugoe S.: Expression of vascular endothelial growth factor-C and vascular endothelial growth factor receptor-3 in esophageal squamous cell carcinoma. ***Oncol Lett*** 7(4):1027-1032, 2014
27. Omoto I, Matsumoto M, Uchikado Y, Kita Y, Sakurai T, Sasaki K, Setoyama T, Okumura H, Owaki T, Ishigami S, Natsugoe S.: Immunohistochemical evidence of association between ghrelin expression and tumor growth in esophageal carcinoma. ***Anticancer Res*** 34(6):2727-33, 2014
28. Okumura H, Uchikado Y, Omoto I, Kita Y, Sasaki K, Arigami T, Uenosono Y, Matsushita D, Hiraki Y, Owaki T, Ishigami S, Natsugoe S.: The usefulness of neoadjuvant chemoradiation therapy for locally advanced esophageal cancer with multiple lymph-node metastases. ***Ann Surg Oncol*** 21(9):2845-9, 2014
29. Kita Y, Nishizono Y, Okumura H, Uchikado Y, Sasaki K, Matsumoto M, Setoyama T, Tanoue K, Omoto I, Mori S, Owaki T, Ishigami S, Nakagawa H, Tanaka F, Mimori K, Mori M, Natsugoe S.: Clinical and biological impact of cyclin-dependent kinase subunit 2 in esophageal squamous cell carcinoma. ***Oncol Rep*** 31(5):1986-92, 2014

2.学会発表

1. 三井 薫、井手佳菜子、高山明子、和田忠久、小 賊 健 一 郎：独自開発の増殖制御型アデノウイルスベクターによる新たな ES/iPS 細胞の腫瘍化細胞 治療技術の開開発． 第 14 回日本再生医療学会総会．口頭発表．2015 年 3 月 21 日（横浜）
2. 井手佳菜子、三井 薫、松下洋平、小賊健一郎：ES/iPS 細胞の腫瘍化細胞を可視化・標的殺傷するレンチウイルスベクターの効 率 的作 製法の開発． 第 14 回日本再生医療学会総会．口頭発表．2015 年 3 月 21 日（横浜）
3. 井手佳菜子，三井 薫，松下洋平，小賊健一郎：ES/iPS 細胞の腫瘍化細胞を可視化・標的殺傷するレンチウイルスベクターの効率的作製法の開発（国内・口頭発表）第 8 回桜ヶ丘地区基礎系研究発表会．2015 年 1 月 28 日（鹿児島）
4. 三井 薫，井手佳菜子，王 宇清，入江理恵，小賊健一郎：独自開発の増殖制御型アデノウイルスベクターを用いた新たな ES/iPS 細胞の腫瘍化細胞除去方法の開発（国内・口頭発表）第 8 回桜ヶ丘地区基礎系研究発表会．2015 年 1 月 28 日（鹿児島）
5. 小賊健一郎：増殖制御型アデノウイルスによる革新的癌治療法の独自開発と臨床応用への展望．（国内・特別講演）第 29 回日本整形外科基礎学術集会 2014 年 10 月 9-10 日（鹿児島）
6. 小賊健一郎: 癌への遺伝子治療の独自開発と本学での医師主導治験を目指して．市民公開講座・鹿児島大学公開講座 2014 年 7 月 19 日(鹿児島)

H．知的財産権の出願・登録状況（予定を含む。）

【特許出願・取得】

1. 幹細胞における腫瘍化原因細胞の新たな標識法と治療法
   発明者：小賀健一郎、三井薫、井手佳菜子
   出願人：鹿児島大学
   国際出願：2015年1月14日 (PCT/JP2015/000138)

2. 血管新生抑制剤
   発明者：小賀健一郎、坂本泰二、上笹貫太郎
   出願人：鹿児島大学
   【国内特許取得】 2014年8月15日（特許第5594695号）
3. ヘパリン結合性上皮増殖因子様増殖因子の新規医薬用途
   発明者：小賀健一郎、ニン・チン・カイ、高橋知之　　出願人：鹿児島大学
   【欧州特許取得】: 2014年7月2日（特許番号: EP 1949907）(ドイツ登録 No.60 2006 042 158.3)
4. サービビン(Survivin)プロモーターを含む増殖型ベクターを有効成分とする癌治療薬
   発明者：小賀健一郎、神囿純一、永野聡
   出願人：小賀健一郎
   【国内特許取得】2014年7月11日（特許第5574284号）
5. Drug Comprising As The Active Ingredient Proliferative Vector Containing Survivin Promoter
   発明者：小賀健一郎、神園純一、永野聡
   出願人：小賀健一郎
   【米国特許取得】2014年4月29日（特許番号：US 8,709,812）

厚生労働科学研究費補助金（難病・がん等の疾患分野の医療の実用化研究事業(がん関係研究分野)）
分担研究報告書

研究分担者　三井　薫　鹿児島大学大学院医歯学総合研究科（遺伝子治療・再生医学）・講師
研究分担者　入江　理恵　鹿児島大学大学院医歯学総合研究科（遺伝子治療・再生医学）・助教
研究分担者　伊地知　暢広　鹿児島大学大学院医歯学総合研究科（遺伝子治療・再生医学）・助教
研究分担者　王　宇清　鹿児島大学大学院医歯学総合研究科（遺伝子治療・再生医学）・特任研究員
研究分担者　小宮　節郎　鹿児島大学大学院医歯学総合研究科（整形外科学）・教授
研究分担者　夏越　祥次　鹿児島大学大学院医歯学総合研究科（消化器・乳腺甲状腺外科）・教授
研究分担者　永野　聡　鹿児島大学大学院医歯学総合研究科（整形外科学）・助教
研究分担者　福﨑　好一郎　株式会社新日本科学・専務取締役

上記研究者の分担報告はすべて研究総括報告書に記載している。

別紙４

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
Nagano S, Yokouchi M, Setoguchi T, Ishidou Y, Sasaki H, Shimada H, Komiya S	Differentiation of lipoma and atypical lipomatous tumor by ascoring system: implication of increased vascularity on pathogenesis of liposarcoma	*BMC Musculoskelet Disor*	16	36	2015
Yuge K, Takahashi T, Khai NC, Goto K, Fujiwara T, Fujiwara H, Kosai K	Intramuscular injection of adenoviral hepatocyte growth factor at a distal site ameliorates dextran sodium sulfate-induced colitis in mice.	***Int J Mol Med.***	33(5)	1064-1074	2014
Tanoue K, Wang Y, Ikeda M , Mitsui K, Irie R , Setoguchi T, Komiya S, Natsugoe S, Kosai K	Survivin-responsive conditionally replicating adenovirus kills rhabdomyosarcoma stem cells more efficiently than their progeny.	***J Trans Med***	12		2014
Nagao-Kitamoto H, Setoguchi T, Kitamoto S, Nakamura S, Tsuru A, Nagata M, Nagano S, Ishidou Y, Yokouchi M, Kitajima S, Yoshioka T, Maeda S, Yonezawa S, Komiya S.	Ribosomal protein S3 regulates GLI2-mediated osteosarcoma invasion.	***Cancer Lett***			2014

Shimada H, Setoguchi T, Yokouchi M, Sasaki H, Ishidou Y, Kawamura I, Abematsu M, Nagano S, Komiya S.	Analysis of factors affecting prognosis and efficacy of CT and (18)F-FDG PET-CT in identifying primary lesions.	***Mol Clin Oncol***	2	875-881	2014
Imamura K, Maeda S, Kawamura I, Matsuyama K, Shinohara N, Yahiro Y, Nagano S, Setoguchi T, Yokouchi M, Ishidou Y, Komiya S.	Human Immunodeficiency Virus Type 1 Enhancer-binding Protein 3 Is Essential for the Expression of Asparagine-linked Glycosylation 2 in the Regulation of Osteoblast and Chondrocyte Differentiation.	***J Biol Chem.***	289(14)	9865-79	2014
Kakoi H, Maeda S, Shinohara N, Matsuyama K, Imamura K, Kawamura I, Nagano S, Setoguchi T, Yokouchi M, Ishidou Y, Komiya S	Bone Morphogenic Protein (BMP) Signaling Up-regulates Neutral Sphingomyelinase 2 to Suppress Chondrocyte Maturation via the Akt Protein Signaling Pathway as a Negative Feedback Mechanism.	***J Biol Chem.***	289(12)	8135-50	2014
Omoto I, Matsumoto M, Okumura H, Uchikado Y, Setoyama T, Kita Y, Owaki T, Kijima Y, Shinchi H, Ishigami S, Ueno S, Natsugoe S.	Decreased density of CD3+ tumor-infiltrating lymphocytes during gastric cancer progression	***J Gastroenterol Hepatol***	29(7)	1435-41	2014
Uenosono Y, Arigami T, Kozono T, Yanagita S, Hagihara T, Haraguchi N, Matsushita D, Hirata M, Arima H, Funasako Y, Kijima Y, Nakajo A, Okumura H, Ishigami S, Hokita S, Ueno S, Natsugoe S	Expression of vascular endothelial growth factor-C and vascular endothelial growth factor receptor-3 in esophageal squamous cell carcinoma.	***Oncol Lett***	7(4)	1027-1032	2014
Omoto I, Matsumoto M, Uchikado Y, Kita Y, Sakurai T, Sasaki K, Setoyama T, Okumura H, Owaki T, Ishigami S, Natsugoe S.	Immunohistochemical evidence of association between ghrelin expression and tumor growth in esophageal carcinoma.	***Anticancer Res***	34(6)	2727-33	2014

Okumura H, Uchikado Y, Omoto I, Kita Y, Sasaki K, Arigami T, Uenosono Y, Matsushita D, Hiraki Y, Owaki T, Ishigami S, Natsugoe S.	The usefulness of neoadjuvant chemoradiation therapy for locally advanced esophageal cancer with multiple lymph-node metastases.	***Ann Surg Oncol***	21(9)	2845-9	2014
Kita Y, Nishizono Y, Okumura H, Uchikado Y, Sasaki K, Matsumoto M, Setoyama T, Tanoue K, Omoto I, Mori S, Owaki T, Ishigami S, Nakagawa H, Tanaka F, Mimori K, Mori M, Natsugoe S.	Clinical and biological impact of cyclin-dependent kinase subunit 2 in esophageal squamous cell carcinoma.	***Oncol Rep***	21(7)	1986-92	2014
Kawasaki Y, Okumura H, Uchikado Y, Kita Y, Sasaki K, Owaki T, Ishigami S, Natsugoe S.	Nrf2 is useful for predicting the effect of chemoradiation therapy on esophageal squamous cell carcinoma.	***Ann Surg Oncol***	21(7)	2347-52	2014

Nagano et al. BMC Musculoskeletal Disorders (2015) 16:36
DOI 10.1186/s12891-015-0491-8

BMC Musculoskeletal Disorders

RESEARCH ARTICLE Open Access

Differentiation of lipoma and atypical lipomatous tumor by a scoring system: implication of increased vascularity on pathogenesis of liposarcoma

Satoshi Nagano[1*], Masahiro Yokouchi[1], Takao Setoguchi[2], Yasuhiro Ishidou[3], Hiromi Sasaki[1], Hirofumi Shimada[1] and Setsuro Komiya[1,2]

Abstract

Background: Well-differentiated liposarcoma (WDL)/atypical lipomatous tumor (ALT) is considered a low-grade malignancy that rarely metastasizes but should be carefully followed because recurrence or dedifferentiation may occur. It is recognized that WDL and ALT are essentially synonymous, describing lesions that are identical both morphologically and karyotypically, and that site-specific variations in behavior relate only to surgical resectability. Preoperative differential diagnosis between lipoma and ALT has been well studied because their clinical and image characteristics are very similar. We evaluated the factors that may differentiate ALTs from lipomas, and validated a tentative scoring system for the diagnosis of the 2 tumor types.

Methods: Forty-eight lipomas and 12 ALTs were included. The mean age, location and depth of the tumor as well as the compartment were not significantly different between the 2 groups. To evaluate the vascularity of the tumors, the average number of intratumoral vessels on pathological sections was calculated and compared between cases of lipoma and ALT.

Results: The tumor size was significantly larger in ALT cases than in lipoma cases ($P < 0.001$). Magnetic resonance imaging (MRI) revealed septal structures in 91.6% of ALTs, whereas 20.8% of lipomas showed septa. Contrast enhancement in MRI was found significantly more often in ALTs (81.2%) than in lipomas (18.8%) ($P < 0.001$). We created a "ALT score" to discriminate between lipoma and ALT (0–6 points). ALT cases gave significantly higher point values (average 5.1 points) than lipoma cases (average 1.7 points) ($P < 0.001$). We found a significantly increased number of vessels in cases of ALT than in cases of lipoma ($P = 0.001$).

Conclusions: Our ALT score may help surgeons to differentiate a suspected ALT from a lipoma and could recommend a marginal resection in cases of suspected ALT. Increased intratumoral vascularity in ALT is reflected in the MRI findings and may play a key role in the acquisition of a malignant phenotype in adipocytic tumors.

Keywords: Atypical lipomatous tumor, Magnetic resonance imaging, Scoring system, Tumor angiogenesis, Dedifferentiation

* Correspondence: naga@m2.kufm.kagoshima-u.ac.jp
[1]Department of Orthopaedic Surgery, Graduate School of Medical and Dental Sciences, Kagoshima University, 8-35-1 Sakuragaoka, Kagoshima-city, Kagoshima 890-8520, Japan
Full list of author information is available at the end of the article

Table 1 Summary of patient characteristics

	Lipoma	ALT	P-value
	Average (range)		
Age	59 (27–77)	62 (44–80)	0.27
Gender	Cases (%)		
Male	25 (52.1)	7 (58.3)	0.70
Female	23 (47.9)	5 (41.7)	
Location			
Extremity	29 (60.4)	8 (66.7)	0.70
Trunk	19 (39.6)	4 (33.3)	
Depth			
Superficial	24 (50.0)	4 (33.3)	0.30
Deep	24 (50.0)	8 (66.7)	
Compartment			
Intracompartment	5 (16.1)	2 (16.7)	0.97
Extracompartment	26 (83.9)	10 (83.7)	

Background

Adipocytic tumors are the soft tumors most frequently encountered by orthopaedic physicians in clinics. Benign adipocytic tumors, lipomas, can be conservatively observed unless patients experience symptoms due to the presence of the mass. However, tumors that are preoperatively suspected to be lipomas, can sometimes be intermediate (locally aggressive)-type adipocytic tumors or well-differentiated liposarcoma (WDL)/atypical lipomatous tumors (ALTs). WDL is considered a low-grade malignancy that rarely metastasizes but should be carefully followed because recurrence or dedifferentiation may occur [1]. It is recognized that WDL and ALT are essentially synonymous, describing lesions that are identical both morphologically and karyotypically, and that site-specific variations in behavior relate only to surgical resectability [2]. The term WDL is now used for tumors of the retroperitoneum, mediastinum, and deep pelvis, whereas the term ALT includes tumors of the extremities and superficial sites. Preoperative differential diagnosis between lipoma and WDL/ALT has been well studied because their clinical and image characteristics are very similar [3,4]. Magnetic resonance imaging (MRI) is currently the most popular modality for the screening and diagnosis of soft tissue tumors, including adipocytic tumors. MRI findings of lipomas usually show high intensity in both T1- and T2-weighted images, reflecting their uniform structure with fatty tissue. In contrast, high-grade liposarcomas, including myxoid, round cell, pleomorphic, and dedifferentiated liposacrcoma, show low intensity in T1-weighted images. The MRI features of WDL/ALT are similar to those of lipoma, which makes differentiation between them difficult. In general, a larger size, deeper localization, or enhancement with contrast medium in MRI is suggestive of malignant soft tissue tumors. In this study, we evaluated the factors that may differentiate ALTs from lipomas and aimed to establish a feasible scoring system to help in the diagnosis of the 2 tumor types. Furthermore, we examined if increased vascularity in the surgical specimen could be a finding that pathologically differentiates ALTs from lipomas, and affect the clinical behavior of ALT.

Methods

We retrospectively reviewed the records of 48 patients with lipomas and 12 patients with ALT. According to the definitions of WDL and ALT, tumors of the extremities and superficial trunk come under the term ALT [2]. In this series, we aimed to study tumors of the extremities and superficial trunk treated in our department of

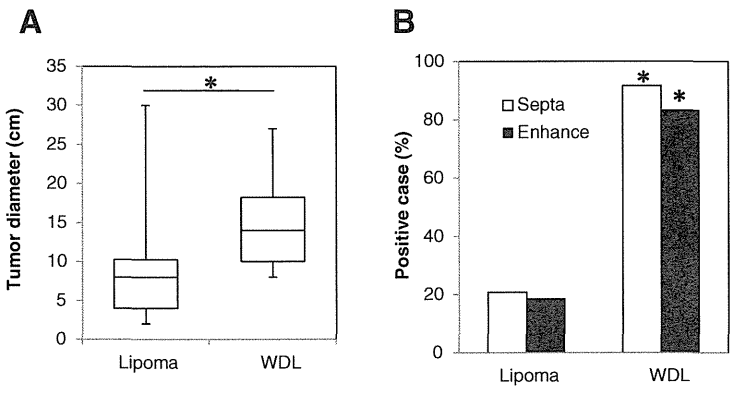

Figure 1 Comparison of magnetic resonance imaging (MRI) findings in cases of lipoma and well-differentiated lipoma (ALT). (A) The tumor diameter was significantly larger in ALTs than lipomas (*$P < 0.001$). **(B)** Septa formation and contrast enhancement were found in most of the ALT cases and rarely in lipoma cases (*$P < 0.001$).

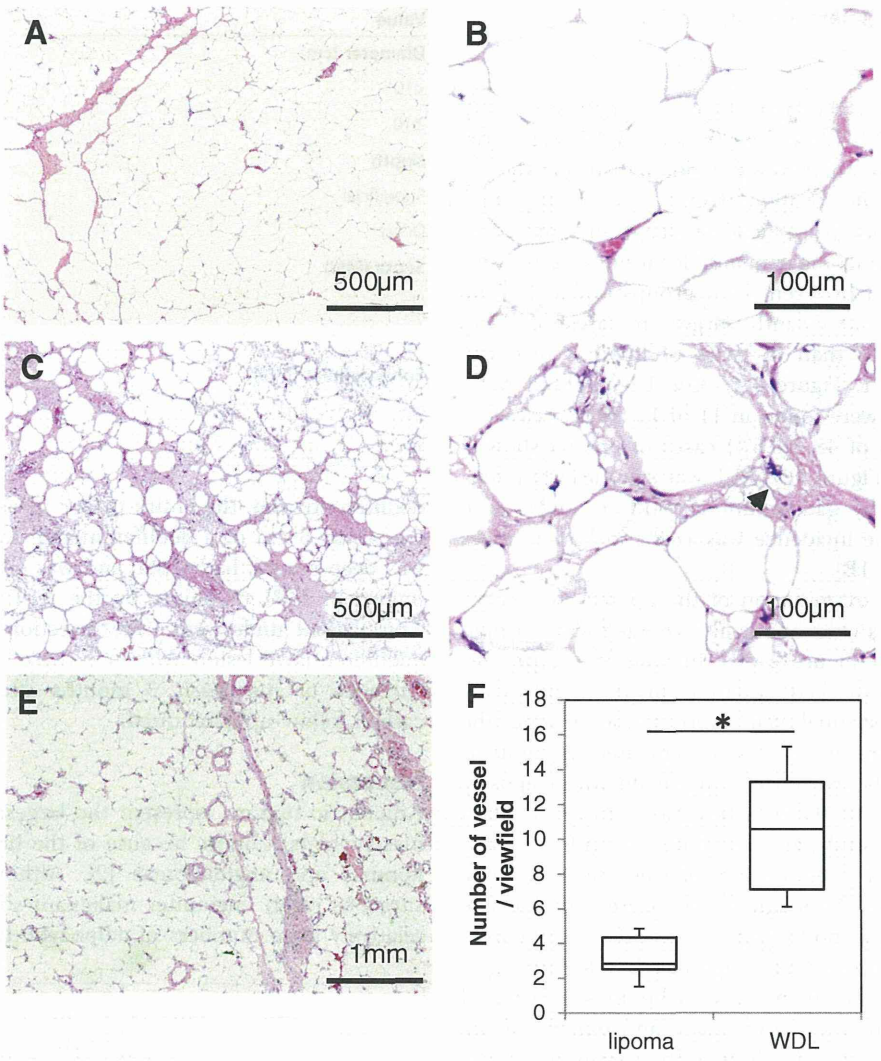

Figure 2 Pathological analysis and evaluation of intratumoral vascularity. In lipoma specimens, mature adipocytes were uniformly observed without high variation in size **(A, B)**. By contrast, adipocytes in ALT showed significant variation in size **(C)**, and lipoblasts with cytoplasmic vacuoles were occasionally found around the thick septa **(D)**. Septa in ALTs were thicker than those in lipomas, and vessel formation was observed both inside and outside of the thick septa **(E)**. Vascular formation was found significantly more often in ALTs than in lipomas **(F)** (*$P = 0.001$). Original magnification, ×20 **(E)**, ×40 **(A, C)**, ×100 **(B, D)**.

orthopedic surgery. Therefore, no cases of WDL were included in this study. All patients underwent surgical excision of the tumor, and a pathologist established the pathological diagnosis. Age, sex, tumor location (limb or trunk), size (diameter in MRI), and depth (superficial, subcutaneous or deep, or under the fascia), and intracompartmental or extracompartmental location were evaluated in all cases. In the MRI analysis, the presence of septal structures (more than 2 mm thick) was assessed. On fat-suppressed T1-weighted images after the administration of contrast-enhancing medium, enhancement of intratumoral lesions was evaluated in all cases. All tumors were resected by marginal resection, and pathological diagnosis was established by pathologists.

To evaluate the vascularity of the tumors, the number of vessels was counted in 10 randomly taken microscopic pictures of hematoxylin and eosin stained sections. The average number of intratumoral vessels was calculated and compared between cases of lipoma and ALT.

The average value of age and tumor size was analyzed with a Student's *t*-test. All other factors were analyzed using the Chi-square test. A P value of less than 0.05 was considered significant.

The ethical committee in Kagoshima University approved the study (reference number, 353).

Results

The mean ages of the patients with lipoma and ALT were 59 (range, 27–77) and 62 (range, 44–78) years, respectively, and the difference was not statistically significant ($P = 0.22$). The location (trunk or extremity) and depth of the tumor (superficial or deep) and intracompartmental or extracompartmental location were not significantly different between the 2 groups (Table 1). The tumor size was significantly larger in cases of ALTs (average, 15.3 cm) than in cases of lipomas (average, 8.9 cm) ($P < 0.001$, Figure 1A). On T2-weighted MRI, septal structures were found in 11 of 12 (91.6%) cases of ALT, whereas 10 of 48 (20.8%) cases of lipoma showed septa ($P < 0.001$, Figure 1B). ALT was significantly intensively enhanced by gadolinium in MRI in 81.2% ALT cases, whereas the incidence was 18.8% in lipoma cases ($P < 0.001$, Figure 1B).

In pathological examination of the lipoma specimens, mature adipocytes were uniformly arranged without high variations in size (Figure 2A,B). In contrast, adipocytes in ALT showed marked variation in size, and many hyperchromatic stromal cells were found around the thick septa (Figure 2C,D). Monovacuolated or multivacuolated lipoblasts are considered a hallmark of liposarcoma, although WDL/ALTs do not always contain lipoblasts. In our study, atypical lipoblasts with cytoplasmic vacuoles and scalloped nuclei were seen in some cases (Figure 2D). Although vessels were found inside the fibrous septa in both lipomas and ALTs, septa were much less frequently found in lipomas. In addition, septa in ALTs were thicker than those in lipomas, and vessel formation was observed both inside and outside of the thick septa (Figure 2E). Analysis of the vascularity in the tumors revealed that ALTs had significantly more vessels (average 11.1/view field) than lipomas (average 3.82/view field) ($P = 0.001$, Figure 2F).

In order to develop a new diagnostic tool for adipocytic tumors, we created a scoring system to discriminate between lipomas and ALTs by considering the tumor size, depth, septa, and enhancement on MRI (Table 2). Total points for the scoring system ranged from 0 to 6 depending on the positivity of those findings, and we expected that a high number of points would suggest the increased probability of the diagnosis of ALT. Almost all lipoma cases gave low scores (average 1.7 points), whereas ALT cases gave significantly high point values (average of 5.1 points) ($P < 0.001$, Figure 3). Based on this scoring system, the diagnosis of ALT was possible with 100% sensitivity and 77% specificity.

One of the 12 ALT patients had a recurrence with dedifferentiation 4 years after resection. At the time of dedifferentiation, the entire tumor measured 28 cm and contained 5 cm of a dedifferentiated lesion. The tumor was deep-seated, had septa, and was enhanced by gadolinium in MRI (6 points by the ALT scoring system). This patient underwent wide resection of the ALT and dedifferentiated lesion. Although there was no sign of recurrence or metastasis 3 months after the resection, careful follow-up is required.

Table 2 Scoring for the diagnosis of ALT

Value	Points
Diameter (cm)	
<10	0
≥10	1
Depth	
Superficial	0
Deep	1
Septa (MRI)	
No	0
Yes	2
Enhancement (MRI)	
No	0
Yes	2

Discussion

Adipocytic tumors represent the largest single group of mesenchymal tumors because of the high prevalence of lipomas and angiolipomas [2]. Although orthopaedic surgeons rarely encounter malignant soft tissue tumors, relatively large numbers of adipocytic tumors are found

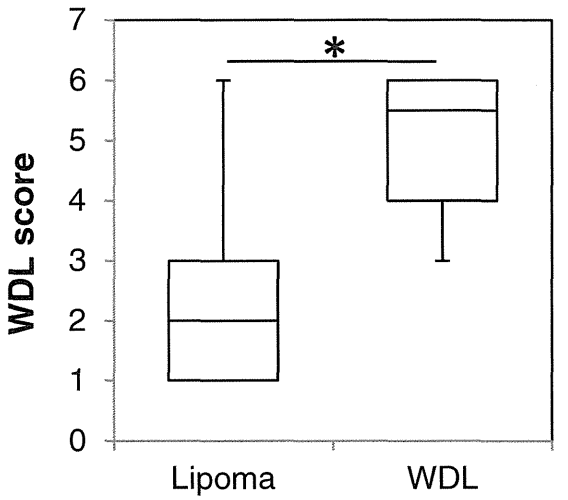

Figure 3 Comparison of the ALT score (0–6 points) in lipoma and ALT groups. The majority of lipoma cases showed a ALT score of less than 2 points, whereas the ALT cases showed significantly high points values (average of 5.1 points) (*$P < 0.001$).

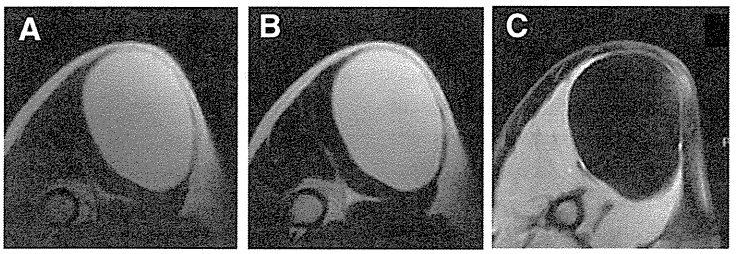

Figure 4 A 34-year-old male patient with a lipoma measuring 12 cm in diameter. Axial T1- and T2- weighted MRIs showed a homogenously high intramuscular mass **(A, B)**. T1-weighted fat saturation gadolinium-enhanced MRI showed no enhancement of the tumor **(C)**. This case obtained 2 points using the ALT score.

in outpatient clinics. It is well-known that adipocytic tumors, regardless of their benign or malignant status, could be large in size without inducing any symptoms. In this study, 31 out of 48 patients with lipomas had tumors larger than 5 cm. Patients with tumors less than 5 cm in size and superficially located may undergo resectional biopsy for diagnosis; however, diagnostic imaging studies are usually performed preoperatively. If computed tomography or MRI examinations reveal a soft tumor with lipomatous content in the majority of the tumor volume, lipoma or WDL/ALT is suspected rather than a high-grade soft tissue sarcoma (Figures 4 and 5). Even though the tumor may be large in size, asymptomatic lipomas do not necessarily require surgical resection. On the other hand, treatment of WDL/ALT is still controversial because of its very low malignancy potential [1,2,5,6]. Because WDL/ALTs have no potential for metastasis unless they undergo dedifferentiation, some pathologists suggest that the term "atypical lipomatous tumor" is more appropriate to use rather than "liposarcoma" [1]. The rate of dedifferentiation of WDL/ALT was previously reported to be 1–4% [6-8]. However, dedifferentiated liposarcoma (DDL) shows much more malignant potential than conventional WDL/ALT with a 5-year survival rate of 60–70% [9,10]. Okada et al. reviewed 18 cases of primary (*de novo*) DDL in the extremities and reported that the duration of the symptoms was an average of 38 months, and 9 patients showed rapid growth of long-standing tumors [10]. This result suggests that if preoperative diagnosis of WDL/ALT is easily made, surgeons could recommend resection of the tumor before it dedifferentiates.

Previously, other researchers reported on the significance of septal structures in WDL/ALT [4,11]. Gaskin et al. tried to differentiate WDL/ALTs from lipomas based upon the viewpoint that simple lipomas may contain thin, discrete septa, whereas WDL/ALTs usually contain thick or nodular septa or enhancement [12]. MRI analysis of 126 fatty masses by musculoskeletal radiologists reached the correct diagnosis in all 6 WDL/ALT cases (sensitivity, 100%); however, 10 of the suspected ALT tumor cases turned out to be variants of benign lipomas, such as chondroid lipoma, osteolipoma, or angiolipoma. The differential diagnosis of lipomatous tumors largely depended on the decisions made by the musculoskeletal radiologists. It would be useful for non-oncologist orthopedic surgeons if simplified diagnostic criteria were available. Therefore, we have created a scoring system to discriminate between lipoma and ALT by the combination of 4 values (Table 2). The score can be measured if enhanced MRI is performed (Figures 4 and 5). Based on this score, diagnosis of ALT is possible with 100% sensitivity and 77% specificity. This result is superior to MRI findings of intratumoral septa alone as a diagnostic finding, which showed 91.7% sensitivity and 74.2% specificity. Although the prevalence of hibernoma

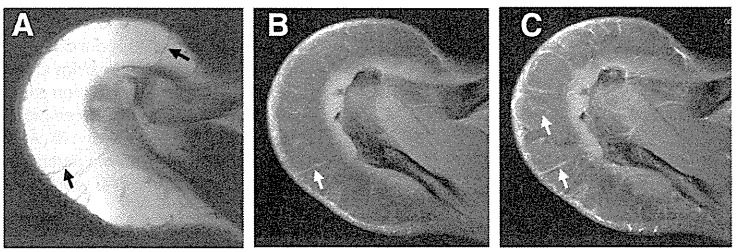

Figure 5 A 40-year-old male patient with ALT measuring 15 cm in diameter. Axial T1-weighted image **(A)** and T1- weighted fat saturation image **(B)** clearly demonstrated thick septa (arrows). T1-weighted fat saturation after gadolinium administration demonstrated enhancement of these septa **(C)**. This case obtained 5 points using the ALT score.

is very low, its MRI findings are similar to those of WDL/ALT. Vassos recently reported that hibernomas show spotty areas of contrast enhancement as well as prominent fibrovascular septa on MRI [13]. Hibernomas exhibit very high standard uptake values (SUVs) on [18F]fluorodeoxyglucose (FDG)-based positron emission tomography (PET) because they contain abundant mitochondria and are highly metabolically active [14]. One of our cases of hibernoma showed an SUV >40, suggesting that PET might be useful to distinguish hibernomas from WDL/ALTs (manuscript in preparation).

Treatment for WDL/ALT is still controversial because the recurrence rate after surgical resection of WDL/ALT is variable, ranging from 0–69% [15-17]. The recurrence of ALT in our series was seen in only 1 case (8.3%), similar to the findings in the report by Sommerville et al. showing an 8% local recurrence rate after marginal resection of 61 cases of ALT [6]. We agree with Sommerville et al. and Kubo et al. in the idea of "conservative" surgery for ALT to preserve the major vessels or nerves [18]. However, for recurrent ALT cases, we recommend as wide of a resection as possible because tumor margins are not usually clear and there is an increased chance of dedifferentiation. An increased number of intratumoral angiogenic vessels was revealed to be a significant factor that differentiates ALTs from lipomas in this study. Because angiolipomas are characterized by rich vasculature in mature adipose tissue, vascularity alone is not useful for the differentiation of ALTs from lipomas. As Folkmann's group proposed, angiogenesis could be a switch that turns on the malignant phenotype in adipocytic tumors [19]. In our study, the highest number of intratumoral vessels (21.4 vessels per field) was observed in the case of ALT recurrence, which eventually dedifferentiated. Contrast enhancement MRI definitely reflects the vascular supply in the tumor and also supports the theory.

Conclusion

Our ALT score (0–6 points) can be used to differentiate ALTs from lipomas based on MRI. If the score is equal to or higher than 3, we recommend marginal resection of the tumor to confirm the pathological diagnosis. Cut-off value should be validated by the future study because of the number of the case is not large in this study. Once the diagnosis of ALT is established, careful follow-up is recommended, especially for cases with increased vascularity.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

SN, MY, HSa and HSh participated in the diagnosis and surgical treatment of the patient. TS and YI helped draft and finalize the manuscript. SK performed the pathological examination and proof reading of the manuscript. All authors read and approved the manuscript.

Acknowledgements

We thank Shinichi Kitajima and Akihide Tanimoto for performing pathological analysis. This work was supported by JSPS KAKENHI Grant Number 24592237.

Author details

[1]Department of Orthopaedic Surgery, Graduate School of Medical and Dental Sciences, Kagoshima University, 8-35-1 Sakuragaoka, Kagoshima-city, Kagoshima 890-8520, Japan. [2]The Near-Future Locomotor Organ Medicine Creation Course (Kusunoki Kai), Graduate School of Medical and Dental Sciences, Kagoshima University, Kagoshima, Japan. [3]Department of Medical Joint Materials, Graduate School of Medical and Dental Sciences, Kagoshima University, Kagoshima, Japan.

Received: 2 May 2013 Accepted: 2 February 2015
Published online: 22 February 2015

References

1. Laurino L, Furlanetto A, Orvieto E, Dei Tos AP. Well-differentiated liposarcoma (atypical lipomatous tumors). Semin Diagn Pathol. 2001;18:258–62.
2. Fletcher CDM, World Health Organization., International Agency for Research on Cancer. WHO classification of tumours of soft tissue and bone. 4th ed. Lyon: IARC Press; 2013.
3. Doyle AJ, Pang AK, Miller MV, French JG. Magnetic resonance imaging of lipoma and atypical lipomatous tumour/well-differentiated liposarcoma: observer performance using T1-weighted and fluid-sensitive MRI. J Med Imaging Radiat Oncol. 2008;52:44–8.
4. Ohguri T, Aoki T, Hisaoka M, Watanabe H, Nakamura K, Hashimoto H, et al. Differential diagnosis of benign peripheral lipoma from well-differentiated liposarcoma on MR imaging: is comparison of margins and internal characteristics useful? AJR Am J Roentgenol. 2003;180:1689–94.
5. Lucas DR, Nascimento AG, Sanjay BK, Rock MG. Well-differentiated liposarcoma. The Mayo Clinic experience with 58 cases. Am J Clin Pathol. 1994;102:677–83.
6. Sommerville SM, Patton JT, Luscombe JC, Mangham DC, Grimer RJ. Clinical outcomes of deep atypical lipomas (well-differentiated lipoma-like liposarcomas) of the extremities. ANZ J Surg. 2005;75:803–6.
7. Bassett MD, Schuetze SM, Disteche C, Norwood TH, Swisshelm K, Chen X, et al. Deep-seated, well differentiated lipomatous tumors of the chest wall and extremities: the role of cytogenetics in classification and prognostication. Cancer. 2005;103:409–16.
8. Kooby DA, Antonescu CR, Brennan MF, Singer S. Atypical lipomatous tumor/well-differentiated liposarcoma of the extremity and trunk wall: importance of histological subtype with treatment recommendations. Ann Surg Oncol. 2004;11:78–84.
9. Coindre JM, Pedeutour F, Aurias A. Well-differentiated and dedifferentiated liposarcomas. Virchows Arch. 2010;456:167–79.
10. Okada K, Hasegawa T, Kawai A, Ogose A, Nishida J, Yanagisawa M, et al. Primary (de novo) dedifferentiated liposarcoma in the extremities: a multi-institution Tohoku Musculoskeletal Tumor Society study of 18 cases in northern Japan. Jpn J Clin Oncol. 2011;41:1094–100.
11. Arkun R, Memis A, Akalin T, Ustun EE, Sabah D, Kandiloglu G. Liposarcoma of soft tissue: MRI findings with pathologic correlation. Skeletal Radiol. 1997;26:167–72.
12. Gaskin CM, Helms CA. Lipomas, lipoma variants, and well-differentiated liposarcomas (atypical lipomas): results of MRI evaluations of 126 consecutive fatty masses. AJR Am J Roentgenol. 2004;182:733–9.
13. Vassos N, Lell M, Hohenberger W, Croner RS, Agaimy A. Deep-seated huge hibernoma of soft tissue: a rare differential diagnosis of atypical lipomatous tumor/well differentiated liposarcoma. Int J Clin Exp Pathol. 2013;6:2178–84.
14. Robison S, Rapmund A, Hemmings C, Fulham M, Barry P. False-positive diagnosis of metastasis on positron emission tomography-computed tomography imaging due to hibernoma. J Clin Oncol. 2009;27:994–5.
15. Evans HL, Soule EH, Winkelmann RK. Atypical lipoma, atypical intramuscular lipoma, and well differentiated retroperitoneal liposarcoma: a reappraisal of 30 cases formerly classified as well differentiated liposarcoma. Cancer. 1979;43:574–84.
16. Rozental TD, Khoury LD, Donthineni-Rao R, Lackman RD. Atypical lipomatous masses of the extremities: outcome of surgical treatment. Clin Orthop Relat Res. 2002;398:203–11.
17. Weiss SW, Rao VK. Well-differentiated liposarcoma (atypical lipoma) of deep soft tissue of the extremities, retroperitoneum, and miscellaneous sites.

A follow-up study of 92 cases with analysis of the incidence of "dedifferentiation". Am J Surg Pathol. 1992;16:1051–8.
18. Kubo T, Sugita T, Shimose S, Arihiro K, Ochi M. Conservative surgery for well-differentiated liposarcomas of the extremities adjacent to major neurovascular structures. Surg Oncol. 2006;15:167–71.
19. Naumov GN, Folkman J, Straume O. Tumor dormancy due to failure of angiogenesis: role of the microenvironment. Clin Exp Metastasis. 2009;26:51–60.

Intramuscular injection of adenoviral hepatocyte growth factor at a distal site ameliorates dextran sodium sulfate-induced colitis in mice

KENTARO YUGE[1,2], TOMOYUKI TAKAHASHI[1,3], NGIN CIN KHAI[1,3,4], KAZUKO GOTO[1], TAKAKO FUJIWARA[6], HISAYOSHI FUJIWARA[1,5,7] and KEN-ICHIRO KOSAI[1,3,4]

[1]Department of Gene Therapy and Regenerative Medicine, Graduate School of Medicine, Gifu University, Gifu 502-1194; [2]Division of Respirology, Neurology and Rheumatology, Department of Medicine, Kurume University School of Medicine, [3]Division of Gene Therapy and Regenerative Medicine, Cognitive and Molecular Research Institute of Brain Diseases, Kurume University, Kurume 830-0011; [4]Department of Gene Therapy and Regenerative Medicine, Kagoshima University Graduate School of Medical and Dental Sciences, Kagoshima 890-8544; [5]Department of Cardiology, Graduate School of Medicine, Gifu University, Gifu 502-1194; [6]Department of Food Science, Kyoto Women's University, Kyoto 605-8501; [7]Department of Cardiology, Hyogo Prefectural Amagasaki Hospital, Amagasaki, Hyogo 660-0828, Japan

Received November 4, 2013; Accepted February 19, 2014

DOI: 10.3892/ijmm.2014.1686

Abstract. Inflammatory bowel disease (IBD) severely affects the quality of life of patients. At present, there is no clinical solution for this condition; therefore, there is a need for innovative therapies for IBD. Hepatocyte growth factor (HGF) exerts various biological activities in various organs. However, a clinically applicable and effective HGF-based therapy for IBD has yet to be developed. In this study, we examined the therapeutic effect of injecting an adenoviral vector encoding the human HGF gene (Ad.HGF) into the hindlimbs of mice with dextran sodium sulfate (DSS)-induced colitis. Plasma levels of circulating human HGF (hHGF) were measured in injected mice. The results showed that weight loss and colon shortening were significantly lower in Ad.HGF-infected mice as compared to control (Ad.LacZ-infected) colitic mice. Additionally, inflammation and crypt scores were significantly reduced in the entire length of the colon, particularly in the distal section. This therapeutic effect was associated with increased cell proliferation and an antiapoptotic effect, as well as a reduction in the number of $CD4^+$ cells and a decreased CD4/CD8 ratio. The levels of inflammatory, as well as Th1 and Th2 cytokines were higher in Ad.HGF-infected mice as compared to the control colitic mice. Thus, systemically circulating hHGF protein, produced by an adenovirally transduced hHGF gene introduced at distal sites in the limbs, significantly ameliorated DSS-induced colitis by promoting cell proliferation (i.e., regeneration), preventing apoptosis, and immunomodulation. Owing to its clinical feasibility and potent therapeutic effects, this method may be developed into a clinical therapy for treating IBD.

Correspondence to: Dr Ken-Ichiro Kosai, Department of Gene Therapy and Regenerative Medicine, Kagoshima University Graduate School of Medical and Dental Sciences, 8-35-1 Sakuragaoka, Kagoshima 890-8544, Japan
E-mail: kosai@m2.kufm.kagoshima-u.ac.jp

Key words: hepatocyte growth factor, inflammatory bowel disease, gene therapy, adenoviral vector, antiapoptosis, immunomodulation, regeneration, cytokine, clinical application

Introduction

The breakdown of normal mucosal immunity causes the development of inflammatory bowel disease (IBD), which can be classified as Crohn's disease (CD) and ulcerative colitis (UC) (1). IBD is a chronically relapsing and remitting condition of unknown origin that exhibits various features of immunological inflammation and affects at least 1 in 1,000 people in western countries. IBD is characterized by inflammation in the intestine, and is associated with diarrhea, occult blood, abdominal pain, weight loss, anemia and leukocytosis. IBD primarily affects young adults, and the disease initially manifests in childhood in 15-25% of cases. Therefore, IBD patients often develop severe symptoms that decrease their quality of life (2). Consequently, there is a need for innovative therapies for IBD.

Current treatments for IBD focus on suppressing inflammation or modulating the immune response using corticosteroids, mercaptopurines, 5-ASA, or monoclonal antibodies against inflammatory cytokines, e.g., the anti-tumor necrosis factor (TNF)-α antibody infliximab (3). However, despite the wide variety of pharmacologic options for patients with IBD, consistent cures and prolonged remissions have yet to be achieved.

Hepatocyte growth factor (HGF) was originally identified (4-7) and cloned (8,9) as a potent mitogen for hepatocytes, but has since been established as a multifunctional cytokine

that exhibits mitogenic, motogenic, morphologic, angiogenic, antiapoptotic and organotrophic effects in a variety of tissues (10). HGF is upregulated in inflamed colonic mucosal tissue in patients with CD or UC (11-13), and plasma HGF levels are elevated in animal models of acute colitis (14). *In vitro*, HGF modulates intestinal epithelial cell proliferation and migration (15), thereby enhancing epithelial cell restitution, which is the initial step of gastrointestinal wound healing. In addition, administration of recombinant human HGF (hHGF) protein reduces the severity of colitis and accelerates colonic mucosal repair in models of TNBS-induced and DSS-induced colitis (16-19), as well as in HLA-B27 transgenic rats with colitis (20). Mukoyama *et al* (21) showed that the intrarectal administration of an adenoviral (Ad) vector carrying the HGF gene prevented TNBS-induced colitis. Additionally, Hanawa *et al* (22) demonstrated the attenuation of mouse DSS colitis by naked gene transfer of rat HGF into the liver, and Kanbe *et al* (23) reported the amelioration of mucosal damage in DSS colitis by the intrarectal administration of the naked HGF gene. In their study, Kanayama *et al* (24) demonstrated the promotion of colonic epithelial regeneration by HGF gene transfer through electroporation. Findings by those authors suggest that HGF is potentially an important new treatment modality for promoting the repair of intestinal mucosa in patients with IBD.

In the majority of previous studies, HGF was provided in the form of recombinant hHGF protein. However, due to the rapid clearance of the HGF protein, large doses and frequent administration of recombinant hHGF were required. Naked gene transfer is a simple and easy method, but the efficiency of gene transduction is extremely low, possibly leading to insufficient clinical effectiveness in human patients. By contrast, the intrarectal administration of an Ad carrying the HGF gene is considered to be extremely stressful for patients. Therefore, in this study we injected an Ad carrying the hHGF gene in single rounds of injections into both hindlimbs of mice 1 day after administration of DSS. We then investigated the therapeutic effects and mechanisms of systemically circulating HGF protein, produced by a gene introduced into the limbs, in the DSS-induced acute colitis model.

Materials and methods

Recombinant Ad. The Ad expressing hHGF under the transcriptional control of the cytomegalovirus immediate-early enhancer and a modified chicken β-actin promoter (Ad.HGF) was generated as described previously (25). The Ad.HGF and the control Ad expressing the LacZ gene (Ad.LacZ) were amplified in HEK-293 cells, purified twice on CsCl gradients, and desalted as described previously (26-29).

Animal studies. Six- to 7-week-old female BALB/c mice weighing 17-20 g (Japan SLC, Inc., Hamamatsu, Japan) were housed in cages in a temperature-controlled environment under a 12-h light-dark cycle with free access to food and water. The animal studies were performed in accordance with the National Institutes of Health guidelines, as specified by the Animal Care Facility at Gifu University School of Medicine.

To induce dextran sodium sulfate (DSS) colitis, the mice were provided with distilled drinking water containing 5% (w/v) DSS (MW, 36,000-50,000; ICN Biomedicals Inc., Aurora, OH, USA) for 7 days. Subsequently, colitis was maintained by feeding the mice 1% DSS (30-32) in the drinking water.

One day after the administration of DSS, Ad.HGF was injected into both hindlimbs of each mouse for a total dose of $1x10^{11}$ particles/mouse (i.e., $5x10^{10}$ particles each into the left and right thigh muscles) (n=8). Ad.LacZ was injected in a similar manner into control mice (n=8). These groups were followed until day 15 (i.e., 8 days after the end of the 7-day period of 5% DSS administration). To evaluate the severity of colitis, body weight was examined on a daily basis. On day 15, all the mice were sacrificed by inhaled anesthetics, and colon samples were collected for examination. In other experiments, on day 5 of 5% DSS administration, 5-bromo-2'-deoxyuridine (BrdU, 100 mg/kg) was administered intraperitoneally to mice (n=8) infected with Ad.HGF or Ad.LacZ, and the animals were sacrificed by inhaled anesthetics 2 h later. These samples were used for analyses of HGF signal transduction, cell proliferation, apoptosis, cytokines and lymphocyte surface markers. The concentration of exogenous hHGF in serum was analyzed using the same dose (i.e., $1x10^{11}$ particles/mouse) of Ad.LacZ or Ad.HGF in intact mice (n=16).

Enzyme-linked immunosorbent assay. The plasma concentration of hHGF following adenoviral intramuscular gene transduction (IMGT) was measured in mice at each time point (n=4) using the Quantikine human HGF Immunoassay kit (R&D Systems, Inc., Minneapolis, MN, USA). TNF-α, interleukin (IL)-1β, IL-6, interferon (IFN)-γ, IL-2, IL-4 and IL-5 levels in the colons of colitic mice were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits (BioSource International, Inc., Camarillo, CA, USA) according to the manufacturer's instructions.

Immunoprecipitation and c-Met receptor phosphorylation assay. The phosphorylation and activation of the c-Met receptor in colon tissues were detected by immunoprecipitation, as described previously (33,34). In brief, 1 g of colon tissue was homogenized in 4 ml of lysis buffer [1% Triton X-100, 150 mM NaCl, 50 mM Tris-HCl (pH 7.6), 10% glycerol, 1 mM vanadate, and 1 mM phenylmethylsulfonyl fluoride] with a protease-inhibitor cocktail (Sigma-Aldrich, Tokyo, Japan). Following centrifugation, the supernatant was incubated with 0.5 μg/ml anti-mouse c-Met antibody (sc-162; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) for 4 h, and then sequentially incubated with 5 μl of protein G-Sepharose beads for 3 h. After washing, proteins bound to the beads were dissolved in sample buffer and subjected to SDS-PAGE. Phosphorylated c-Met was immunoblotted using the anti-phosphotyrosine antibody PY20 (Transduction Laboratories, Lexington, KY, USA).

Histopathological analysis. After each mouse was sacrificed, the intestine was dissected from the anus to the cecum and rinsed with physiological saline. The colon length was measured, and the colon sample was divided into three sections (cecum, proximal colon and distal colon), with the cecum being separated first, and then the remaining part of the colon being divided into two equal segments (proximal colon and distal colon). The cecum, proximal colon and distal colon were opened longitudinally, and the proximal and distal colon