膵癌に対するサバイビン免疫療法の研究

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ペプチドワクチン療法は，癌免疫療法の中でも，最近，脚光を浴びている治療法の1つである。我々は現在，進行膵癌に対する，サバイビンタンパクを標的とした癌ペプチドワクチン療法の第II相試験を，医師主導治験で行っている。本稿では，本治験の科学的根拠，治験の実際，および本治験の問題点と展望を概説する。

本治験の科学的根拠

1）背景

サバイビン遺伝子は，胎生期の組織において強い発現をみるが，精巣，胸腺，胎盤以外では発現しないことが知られる。一方，サバイビン遺伝子は消化器癌や乳癌など様々な癌腫において高頻度に発現しており，サバイビン遺伝子産物が癌抗原となった場合，正常組織に対する副作用が低く，癌細胞に特異的な免疫療法が樹立できる可能性が考えられた[1)]。そこで次に，大腸癌，乳癌，食道癌，胃癌などの末梢血を用いて，サバイビン遺伝子産物を認識するTリンパ球の存在およびCTLの細胞傷害活性を検討した。サバイビンのアミノ酸配列より，日本人に最も多いHLA-A24と結合親和性の高いSVN-2B（Ala-Tyr-Ala-Cys-Asn-Thr-Ser-Thr-Leu；AYACNTSTL）ペプチドを合成し，HLA-A24陽性の癌患者末梢血を*in vitro*で刺激した結果，22例中17例（78%）においてHLA-A24/SVN-2B特異的CTLが誘導された[1)-3)]（図1）。また，これらのCTLはサバイビンを発現している癌細胞に対し細胞傷害活

性を発揮した。さらにHLA-A24/SVN-2Bテトラマーを作成し，癌患者末梢血におけるHLA-A24/SVN-2B特異的CTLの数を検討した結果，高頻度に特異的CTLを検出することができた[3]。こうして，サバイビン蛋白が癌細胞内で分解され，ヒト白血球抗原HLA class I分子とともに細胞表面に提示されてSVN-2B特異的なCTLにより認識されることが証明された[4]。

一般に8〜11アミノ酸からなる癌抗原ペプチドを皮内または皮下に投与することで，樹状細胞やランゲルハンス細胞表面のHLA class I分子にペプチドが直接結合し[5]，もしくはペプチドが樹状細胞などの抗原提示細胞に貪食されHLA class I分子と複合体を形成して細胞表面に提示されることで[6]，所属リンパ節においてこれら複合体がCTLに認識されるものと考えられている。ペプチド/HLA複合体を認識し種々のシグナルにより活性化したT細胞は，抗原ペプチド特異的CTLとして生体内を巡回し，癌局所において細胞表面に同一のペプチド/HLA複合体をもつ癌細胞を認識・攻撃し，抗腫瘍作用を発揮することが期待される[4]（図2）。一方，樹状細胞やランゲルハンス細胞のような抗原提示細胞（APC）が抗原を捕捉し，CTLに認識されるためには，APCの活性化が重要であることが知られており[7][8]，IFN-αおよびIFN-β（Type Iインターフェロン）は，細胞表面に発現している受容体IFNAR1/2を介してAPCを活性化する作用を持つ。したがって，抗原ペプチドとともにIFN-βを投与することによって，高い抗原ペプチド特異的CTL誘導効果が期待できる。

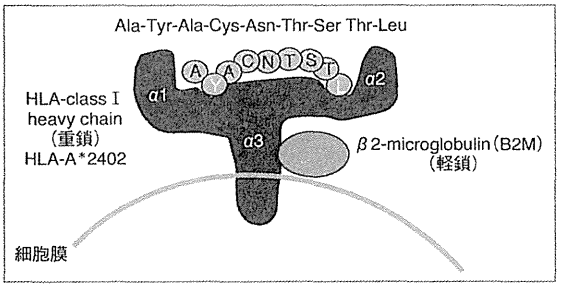

図1 Survivin-2B ペプチド（SVN-2B）の構造
Ala：アラニン，Tyr：チロシン，Cys：システイン，Asn：アスパラギン，Thr：スレオニン，Ser：セリン，Leu：ロイシン

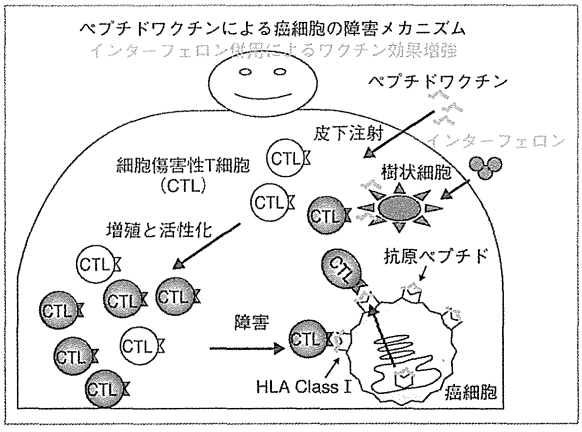

図2 癌ワクチンの作用メカニズム

2）治験前臨床試験

十分な非臨床試験により安全を確認した後に，HLA-A2402遺伝子陽性でサバイビンを発現している進行癌患者を対象として，平成14年度から平成19年度にかけて，SVN-2B単独投与および，IFN-α併用プロトコルの自主臨床研究を行い，SVN-2Bの免疫効果を高めるためにはIFNを併用することの有用性を確認してきた。大腸癌を対象に本剤単独および本剤をIFAエマルジョンとして投与した症例では，約20%程度の症例で腫瘍抑制効果が確認されたが，IFN-α併用により50%まで上昇した。膵臓癌に対してIFN-α併用投与した症例では，腫瘍抑制効果は67%であった。また，IFN-α併用によりSVN-2Bペプチド特異的CTL数の上昇が認められた（表1）。

また本治験の前相の第Ⅰ相臨床試験では，HLA-

表1 SVN-2Bペプチドの自主臨床研究成績

癌腫	プロトコル	評価症例数	腫瘍抑制症例数*1	評価症例数	CTL数増加症例数*2
膵臓癌	SVN-2B+IFA+IFN-α併用	6	4（67%）	6	3（50%）
大腸癌	SVN-2B+IFA	5	1（20%）	5	0（0%）
	SVN-2B+IFA+IFN-α併用	8	4（50%）	8	2（25%）

*1：腫瘍抑制症例数：RECISTに基づく評価でSD以上の症例数
*2：CTL数増加症例数：テトラマー対数差分が1.00を超えた症例数

A2402遺伝子陽性でサバイビンを発現している進行消化器癌患者(大腸癌，膵臓癌，胃癌)を対象として，平成24年度から平成25年度にかけて，SVN-2B単独(0.3mg群，1.0mg群，3.0mg群)の皮下投与を行った。その結果，SVN-2Bと因果関係がありと判断された副作用は4例6件(0.3mg群：2例3件，1.0mg群：1例1件，3.0mg群：1例2件)認められた。内訳は，SVN-2Bの投与に伴う皮膚硬結(Grade 1)，注入に伴う反応(Grade 1)，注射部位の血管外漏出(Grade 2)の他，発熱(Grade 1)であり，いずれも軽度であった。また，重篤な有害事象は10例12件(0.3mg群：3例3件，1.0mg群：4例5件，3.0mg群：3例4件)認められたが，いずれもSVN-2Bとの因果関係は否定された。有効性としてSVN-2B特異的CTL数(テトラマー解析)の評価を行った結果，1.0mg群が有意に高値を示した。また，治療開始前と4回目終了後の画像診断による病巣の評価をRECISTガイドラインに準拠して行った結果，50%がSDであった。

以上の臨床試験の結果から，SVN-2BにIFNを併用した場合，膵臓癌と大腸癌で最も腫瘍抑制効果が期待できると考えられた。特に進行膵臓癌は，現在有効とされている治療法がBest Supportive Careに比べ，生命予後を少しだけしか改善できないことより，SVN-2BとIFNの併用療法は新たな作用機序を有し全身の副作用が少ない新規膵臓癌治療薬となりうると考えられた。SVN-2B単独投与の第Ⅱ相臨床試験の結果，1回あたりの投与量として1.0mgが至適用量と推定された。以上の科学的根拠をもとに，今回，SVN-2BにIFN-βを併用した際の有効性および安全性を検討するため，進行膵臓癌患者を対象として国内での第Ⅱ相臨床試験を医師主導治験として計画するに至った。

治験の実際

1)治験課題名：SVN-2B(第Ⅱ相臨床試験)

有効な治療法のない進行膵臓癌患者におけるプラセボ，SVN-2B単独投与を対照SVN-2B/STI-01併用療法の無作為化二重盲検群間比較試験。

2)目的

本治験は，有効な治療法のない進行膵臓癌患者を対象としてプラセボ投与群，SVN-2B単独投与群あるいはSVN-2B/STI-01併用投与群のいずれかに無作為化割付し，無増悪生存期間を比較する。副次的に免疫学的評価，RECISTガイドラインに基づく腫瘍縮小効果，安全性プロファイルを検討する。探索的にirRC(Immune-related Response Criteria)に基づく無増悪生存期間を検討する。

3)デザイン

本治験は多施設で二重盲検下にて実施する中央登録方式による無作為化群間比較試験である。被験者をプラセボ群，SVN-2B群，SVN-2B/STI-01群に1：2：2の割合で無作為に割り付ける。

本治験は，ゲムシタビンまたはS-1が無効もしくは不耐となった進行膵臓癌の患者を対象として，SVN-2B/STI-01併用群，SVN-2B単独群，プラセボ群の有効性および安全性をと比較する。本治験は，STEP 1とSTEP 2の二段階の構成になっている(図3)。STEP 1は，RECISTガイドラインに基づきPDと判定されるまで投与を継続する。STEP 1においてRECISTガイドラインに基づきPDと判定された場合，STEP 1を終了する。STEP 1において32週以内にPDと判定された場合，さらなる投与継続を希望する被験者には同意取得後にSTEP 2に移行し，irRC[3)8)]に基づくPDと判定されるまで，もしくは最大7ヵ月(30週目)まで投与を継続する。ただし，STEP 2で全例がirRCに基づく評価でirPD(Immune-related Progressive Disease)となるかSTEP 2の最終評価被験者の評価が終了した際，STEP 1で投与を継続している被験者は，次回投与予定の投与を行わず，来院予定日に最終評価を行い，治験を終了する。

本治験は，プラセボ群の救済として，PDと判定された被験者には盲検性を担保したままSVN-2B/STI-01の併用投与を受ける機会を設けることとした(STEP 2)。これは本治験の大きな特徴の1つである。また，癌ワクチンのような免疫系の活性化に基づく悪性腫瘍に対する治療では，腫瘍抑制効果が遅れて発現することが知られている。したがって，従来のRECIST基準では免疫学的腫瘍抑制効果を正確に判断

することが難しい。この問題を解決する新基準としてirRCが米国において提唱され，癌免疫治療の治験に採用されている。irRCにおいては，4週間以上の間隔をおいた2回の画像検査において最低25%以上の腫瘍量の増加が認められた場合にはじめてirPDと判断されるため，従来のRECIST基準では有効性が認められず排除されていた症例の観察も可能であることが報告されている。

4)治験薬およびその投与方法

SVN-2B注1.0mg：1バイアル中に生理食塩水1mLあたり1.0mgのSVN-2Bを含有する注射剤

STI-01：1バイアル中にIFN-β 3×10^6IUを含有

SVN-2B：SVN-2B注1.0mg，1.0mLと乳化用の添加剤モンタナイド0.8mLを混合して乳化。調整した投与用薬液1.8mLを2ヵ所(1ヵ所0.9mL)に分けて原則14日ごとに皮下投与する。

STI-01：STI-01 3×10^6IUを1.0mLの生理食塩水に溶解し，2ヵ所に分けてSVN-2Bと同部位に皮下投与する。最初の8週間は原則7日ごとに投与し，8週目の投与以降は原則14日ごと，32週目の投与以降は原則28日ごとにSVN-2BまたはSVN-2B(プラセボ)と同じ日に投与する。

5)主要評価項目

無増悪生存期間とする。

6)副次的評価項目

免疫学的効果として，①SVN-2Bペプチド特異的CTL数(テトラマー解析)，②SVN-2Bペプチド特異的CTL活性(ELISPOT解析)を評価。

7)治験実施予定期間

2013年10月～2016年12月(登録期間：24ヵ月)。

8)対象

対象の選択基準，および除外基準は表2，3に示す。

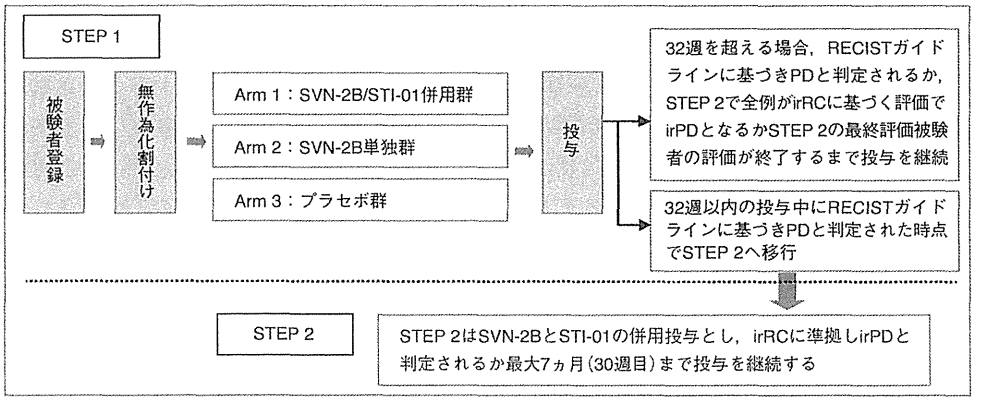

図3 本治験のデザイン

表2 選択基準

1）組織学的に膵臓原発腺癌，膵臓腺管癌と確定診断された患者。
2）癌細胞にサバイビン蛋白質の発現が確認された患者。
3）以下の基準をすべて満たす患者。 a）根治手術が不可能である(遠隔転移例，再発例，局所進行例等)患者。 b）ゲムシタビンまたはテガフール・ギメラシル・オテラシルカリウム配合剤に対し不応例，不耐容例の患者。 C）ゲムシタビンまたはS-1のいずれかしか投与していない場合，投与していない薬剤の投与不適応患者または投与を拒否した患者。
4）前観察期のCTまたはMRIでRECISTに基づく測定可能評価病変がある患者。
5）HLA遺伝子型がHLA-A*2402である患者。
6）Eastern Cooperative Oncology Group(ECOG)Performance Statusが0または1の患者。
7）登録前30日以内に重篤な臓器不全がないことが確認された患者(好中球≧1,500/μL，ヘモグロビン値≧8.0g/dL，血小板数≧$75\times10^3/\mu$L，血清クレアチニン値≦正常上限値の1.5倍，血清総ビリルビン値≦正常上限値の3倍，AST，ALT≦正常上限値の3倍)。
8）同意取得時の年齢が20歳以上85歳以下の患者。
9）本治験内容について十分な説明を受け，本人の文書による同意が得られている患者。

表3 除外基準

1）登録前90日以内のHIV抗体検査で陽性の患者。
2）NYHA分類ⅢまたはⅣの心疾患，登録180日以内の心筋梗塞の既往，治療を要する不整脈，活動性の狭心症，または重度の閉塞性肺疾患を有するなど，本治験治療に支障を来す疾患を有する患者。
3）コントロール不能な糖尿病または高血圧の患者。
4）ドレナージが必要な胸水，心嚢水または腹水を有する患者(登録時点においてドレーン抜去後，増悪がないと判断された患者は許容する)。
5）症状を伴う脳転移を有する患者。
6）活動性の重複癌を有する患者。
7）自己免疫疾患またはその疑いがある患者。
8）CRP値が15.0以上の重度な炎症疾患が疑われる患者。
9）間質性肺炎の既往歴のある患者。
10）生命予後に影響を及ぼすような他の疾患に罹患している患者。
11）過去に癌に対する免疫細胞療法を受けた患者。
12）登録時に以下のいずれかの前治療または処置を行い，治療または処置の終了時から以下の期間が経過していない患者。 a）手術療法，放射線療法　21日間 b）化学療法(分子標的薬を含む)　14日間 c）内分泌療法，免疫療法(BRM療法を含む)　14日間 d）輸血，造血因子製剤　14日間 e）免疫抑制剤　28日間 f）他の治験薬，未承認薬　28日間
13）小柴胡湯，ワルファリン，テオフィリンを投与中の患者。
14）ステロイド剤の全身投与(経口，静注)を必要としている患者。ただし，治療に伴う急性アレルギー症状の処置用に投与した場合を除く。
15）過去に重大な薬物アレルギーの既往がある患者。
16）ウシ由来物質に対し，過敏症の既往歴のある患者。
17）ワクチン等生物学的製剤に対し，過敏症の既往歴のある患者。
18）重度の精神病または精神症状を合併しており治験への参加が困難と判断される患者。
19）妊娠している，もしくは授乳中である患者。治験期間中に被験者本人およびパートナーの妊娠を希望する患者。効果の高い避妊法を行えない患者。
20）その他，治験責任医師または治験分担医師が不適切と判定した患者。

表4 投与期間，最終観察時/中止時スケジュール(0～32週目)

Week	0	1	2	3	4	5	6	7	8	10	12	14	16	18	20	22	24	26	28	30	32	中止時[e]
Day	1	8	15	22	29	36	43	50	57	71	85	99	113	127	141	155	169	183	197	211	225	
許容範囲(日)	−3～1	±3	±3	±3	±3	±3	±3	±3	±3	±7	±7	±7	±7	±7	±7	±7	±7	±7	±7	±7	±7	
治験薬投与 SVN-2B[a]	●		●		●		●		●	●	●	●	●	●	●	●	●	●	●	●	[d]	
治験薬投与 STI-01[b]	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	[d]	
バイタルサイン，PS	●	●	●		●		●		●	●	●	●	●	●	●	●	●	●	●	●	●	●
血液学的検査	●	●	●		●		●		●	●	●	●	●	●	●	●	●	●	●	●	●	●
血液生化学的検査	●	●	●		●		●		●	●	●	●	●	●	●	●	●	●	●	●	●	●
尿検査	●	●	●		●		●		●	●	●	●	●	●	●	●	●	●	●	●	●	●
画像評価[c]									●			●			●			●			●	▲[e]
腫瘍マーカー[c]									●			●			●			●			●	▲[e]
免疫学的評価(CTL数，CTL活性)									●			▲			●			▲			●	▲
有害事象	←———																					———→
併用薬・併用療法	←———																					———→

●：必須，▲：可能な限り実施

a）SVN-2Bは，原則14日ごとに投与する。許容範囲は±7日とし，4日以上の間隔を空けること。ただし，祝日，休診等の理由により投与が定期的に行えない場合は，連日投与を避け，この限りではない。

b）STI-01は，最初の8週目まで原則7日ごとに1回投与する。その後，原則14日ごとにSVN-2BまたはSVN-2B(プラセボ)と同じ日に投与する。許容範囲は8週目までは±3日とし，2日以上の間隔を空けること。その後の許容範囲は±7日とし，4日以上の間隔を空けること。ただし，祝日，休診等の理由により投与が定期的に行えない場合は，連日投与を避け，この限りではない。

c）画像評価，腫瘍マーカー，免疫学的評価(CTL数、CTL活性)の許容範囲は，8週目が±7日，14，20，26週目，32週目が±14日とする。

d）STEP2においては，治験薬の投与を行わない。

e）画像評価，腫瘍マーカーの結果により，治験薬投与中止を決定した場合，投与中止を決定した画像評価および腫瘍マーカーの検査を中止時の検査とすることができる。

HLA-A*2402は日本人では最も多い遺伝子型ではあるが，60%に過ぎず，遺伝子型が合わないため，治験薬投与前に3分の1以上が脱落となる。また，膵癌は組織学的に確定診断がされない(できない)状況で化学療法が施行されている場合があるため，EUS-FNA等の侵襲的検査を選択基準を満たすために追加しなければならない場合がある。

目標症例数は71例(プラセボ投与群：15例，SVN-2B投与群:28例，SVN-2B/STI-01併用投与群:28例)である。

9)治験スケジュール

表4に示す。初回の効果判定は，8週後となる。

本治験の問題点と展望

現在，治験開始より約9ヵ月が経過した。治験実施症例数は目標数の3分の1を超えた。最初は札幌医科大学第一外科および東京大学医科学研究所附属病院外科で実施されたが，2014年8月より神奈川県立がんセンターでも実施予定である。

この臨床試験の最も大きな問題は，プラセボ投与が8週間にも及ぶ点であろう。膵臓癌は進行が早く，8週間の間に，急速に病状が進行する例もあり，救済策であるSTEP 2への移行が行うことができない症例も散見される。

しかし，本治験によりサバイビンワクチンの有効性が証明されれば，今後既存の化学療法との併用や，膵臓癌以外の固形癌に対する使用において有効な治療となる可能性が期待できる。

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ORIGINAL ARTICLE

Detection of *APC* mosaicism by next-generation sequencing in an FAP patient

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Familial adenomatous polyposis (FAP) of the colon is characterized by multiple polyps in the intestine and extra-colonic manifestations. Most FAP cases are caused by a germline mutation in the tumor-suppressor gene *APC*, but some cases of adenomatous polyposis result from germline mutations in *MUTYH*, *POLD1* or *POLE*. Although sequence analysis of *APC* by the Sanger method is routinely performed for genetic testing, there remain cases whose mutations are not detected by the analysis. Next-generation sequencing has enabled us to analyze the comprehensive human genome, improving the chance of identifying disease causative variants. In this study, we conducted whole-genome sequencing of a sporadic FAP patient in which we did not find any pathogenic *APC* mutations by the conventional Sanger sequencing. Whole-genome sequencing and subsequent deep sequencing identified a mosaic mutation of c.3175G>T, p.E1059X in ~12% of his peripheral leukocytes. Additional deep sequencing of his buccal mucosa, hair follicles, non-cancerous mucosa of the stomach and colon disclosed that these tissues harbored the *APC* mutation at different frequencies. Our data implied that genetic analysis by next-generation sequencing is an effective strategy to identify genetic mosaicism in hereditary diseases.
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INTRODUCTION

Approximately 5% of colorectal cancer is caused by hereditary tumor syndromes including Lynch syndrome and familial polyposis of the colon. Their diagnosis is crucial not only for the treatment of patients, but also the healthcare of their relatives. Genetic testing is, therefore, of great importance for the diagnosis and the identification of the mutation carriers in the relatives.[1] Familial adenomatous polyposis (FAP) is an autosomal-dominant colorectal cancer predisposition syndrome that accounts for ~1% of newly diagnosed colorectal cancer cases. FAP is characterized by the development of multiple adenomatous polyps ranging from hundreds to thousands in the large intestine. A majority of the patients carry a germline mutation in the tumor-suppressor gene *APC* (adenomatous polyposis coli), but a small number of adenomatous polyposis cases are caused by germline mutations in *MUTYH*, *POLD1* or *POLE*. Genetic tests are performed for the screening of *APC*, but pathogenic mutations are not detected in some FAP cases by conventional direct sequencing owing to several reasons; (i) the region of testing is often restricted to 5′-half in the coding region and does not usually include the 3′-half, promoter region, or the 5′- or 3′-UTR. (ii) Structural alteration comprising large deletions/insertions and inversions are hard to identify by the conventional sequencing method and other testing methods are required. (iii) Some cases of adenomatous polyposis are caused by mutations in other genes such as *MUTYH*, *POLD1* and *POLE*.[2,3] (iv) Some FAP cases are caused by somatic mosaicism of *APC*.[4–7]

Large-scale genome sequencing, also known as next-generation sequencing (NGS), is applicable to germline genomic sequencing, as well as other purposes including sequencing of tumors, sequencing of mRNA to analyze gene expression (RNA-seq), sequencing of DNA enriched by chromatin immunoprecipitation to characterize elements in protein-DNA interactions (ChIP-seq) and others. The entire genome of an individual can be sequenced in less than 1 week for 5000 to 10 000 dollars.[8] Cost reduction, together with advanced bioinformatics capabilities, have led to increased opportunities for NGS usage in various clinical applications including the detection of rare hereditary mutations, individualized therapy, pharmacogenomics, preconception/prenatal screening and population screening for disease risk.[9,10]

Here we show that genetic testing by NGS facilitates to identify mosaic *APC* mutations in patients with attenuated polyposis. NGS will improve genetic diagnosis of hereditary diseases whose mutations have been overlooked by conventional direct sequencing.

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MATERIALS AND METHODS

Patients and ethical approval

This project was approved by the ethical committee of Institute of Medical Science, the University of Tokyo (IMSUT-IRB, 23-18-0929 and 23-19-0929). Written informed consent was obtained from the patient in this study.

Genetic testing

Genomic DNA was extracted from peripheral blood of the patient according to the standard phenol extraction/purification procedure. *APC* coding exons were amplified with M13-tailed target specific primers, and the PCR products were sequenced on the Applied Biosystems 3730×l DNA Analyzer (Thermo Fisher Scientific, Waltham, MA, USA) using the BigDye Direct Cycle Sequencing Kit (Thermo Fisher Scientific). The primer sequences used for sequencing are available on request.

Whole-genome sequencing

We prepared insert libraries of 250–350 bp from 1.0 μg of genomic DNA from lymphocytes and sequenced them using the HiSeq 2000 platforms with paired-end reads of 101 bp according to the manufacturer's instructions (Illumina, San Diego, CA, USA). For the data processing, fastq files were aligned to human reference sequence (hg19) by BWA[11] (ver. 0.5.10) and a bam file was created. For the detection of variants, we compared the bam file with the reference sequence by a Bayesian approach. We used a beta non-informative prior for representing the probability of existence of variant and the posterior distribution was obtained using the information of observed reads at each candidate position. We supposed that a random variable 'X' follows the posterior distribution representing the probability of variant. We used $Pr(X \geqslant 0.05)$ as the score, and regarded the candidate positions whose scores were greater than 0.9 as statistically significant.

Validation of variants detected by NGS

To confirm the mutation, DNA of peripheral blood was amplified independently by PCR (KOD-Plus kit, TOYOBO, Osaka, Japan) with a set of primers encompassing the possible pathogenic mutation, and direct sequence was performed by the Sanger method. The primer sequences used for amplification are shown in Supplementary Table 1. To confirm mosaicism, genomic DNA was extracted from hair follicles, two sites of buccal mucosa, three sites of non-tumorous stomach mucosa, five sites of non-tumorous colonic mucosa and five adenomatous polyps from the patient by the standard phenol extraction/purification procedure. Deep sequencing was carried out using IonPGM Sequencing 200 kit and Sequencing 400 kit (Thermo Fisher Scientific) with libraries of PCR products prepared using Ion Plus Fragment Library Kit (Thermo Fisher Scientific). Variants were identified using the Variant Caller deployed with Torrent Suite (Thermo Fisher Scientific).

RESULTS

In clinical genetic testing, we encountered a male, 41 years of age, who suffered from multiple polyps in his large intestine. He earlier visited a hospital for the secondary screening of his intestine because of occult blood in his fecal test. Colonoscopy detected <100 adenomatous polyps in his large intestine and subsequent histological examination of the polyps diagnosed adenomatosis. As he had no family history of polyposis or colorectal cancer, he was suspected to be a *de novo* case of FAP or a patient of MUTYH-associated polyposis. Direct sequencing of *APC* was performed using DNA extracted from his lymphocytes to examine the 5′-half of the coding region where most of the *APC* mutations occur; however, no pathogenic mutations were detected. Although structural analysis of *APC* by Multiplex Ligation-dependent Probe Amplification, or sequencing of *MUTYH*, *POLD* and *POLE* by the Sanger method were available for the second screening, we performed whole-genome sequencing to test the usefulness of NGS in clinical testing.

The average depth of sequence coverage was ~26x, and a total of 4.6 million variants were identified in the patient. Because three to four million variants are generally detected by whole-genome sequencing in an individual,[9] we considered that the number of variants was reasonable. Among the 4.6 million, 30 501 variants were located in the exonic regions, and 8 were detected in the exons or splicing regions of *APC* (Table 1). Importantly, one of the eight variants was a nonsense mutation (c.3175G>T p.E1059X) that was determined in 6 of 50 reads (12%). Although the frequency was much lower than 50%, we suspected that it may be the causative mutation of his polyposis because the nonsense mutation truncates the APC protein leading to the loss of domains involved in β-catenin degradation. We re-sequenced the region by the Sanger method, and found a low peak of mutant allele compared with the wild allele in his DNA from peripheral blood. Taken together with his family history and the number of polyps, we suspected the possibility of *APC* mosaicism. The Sanger method is apparently less sensitive for the detection of mosaicism, compared with NGS (Figure 1). Notably, NGS identified no deleterious mutation in *MUTYH*, *POLD1* or *POLE* (data not shown). The remaining seven *APC* variants had been detected in the initial screening.

We further performed a deep sequencing of his DNA isolated form peripheral blood, hair follicles and two sites of oral mucosa. The average depth of coverage achieved with amplicon sequencing was 34 699x ranging from 3726 to 87 133. As shown in Table 2, c.3175G>T mutation was observed in 453 of 3726 reads (12.2%) in peripheral blood, 3774 of 83 679 (4.5%) in hair follicles, and 2099 of 69 169 (3.0%) and 4860 of 66 557 (7.3%) in buccal mucosa. Because the patient underwent gastric endoscopy and total colectomy, we also examined the mutation in three spots of non-tumorous gastric mucosa, five spots of non-tumorous colonic mucosa and five adenomatous polyps in the colon. Interestingly, we found different frequencies of c.3175G>T mutation in non-tumorous gastric (18.9, 22.7 and 27.7%) and colonic (9.2, 3.4, 12.3, 5.8 and 9.0%) tissues (Table 2). In addition, the mutation was found at higher frequencies (32.3, 28.6, 29.8, 32.5 and 24.7%) in the colonic polyps

Table 1 Summary of variations in all *APC* exons detected by WGS

Type	*Mutation*	*Protein alteration*	*dbSNP131*	*Ref (No of reads)*	*Obs (No of reads)*	*Mismatch ratio*
Synonymous	c.T1458C	p.Y486Y	rs2229992	T (0)	C (75)	1
Synonymous	c.G1635A	p.A545A	rs351771	G (0)	A (35)	1
Nonsense	c.G3175T	p.E1059X	—	G (44)	T (6)	0.12
Synonymous	c.G4479A	p.T1493T	rs41115	G (0)	A (55)	1
Synonymous	c.G5034A	p.G1678G	rs42427	G (0)	A (67)	1
Synonymous	c.T5268G	p.S1756S	rs866006	T (0)	G (43)	1
Non synonymous	c.T5465A	p.V1822D	rs459552	T (0)	A (39)	1
Synonymous	c.G5880A	p.P1960P	rs465899	G (0)	A (23)	1

Abbreviations: APC, adenomatous polyposis coli; No, number; Obs, observed nucleotide; Ref, reference nucleotide; WGS, whole-genome sequencing.

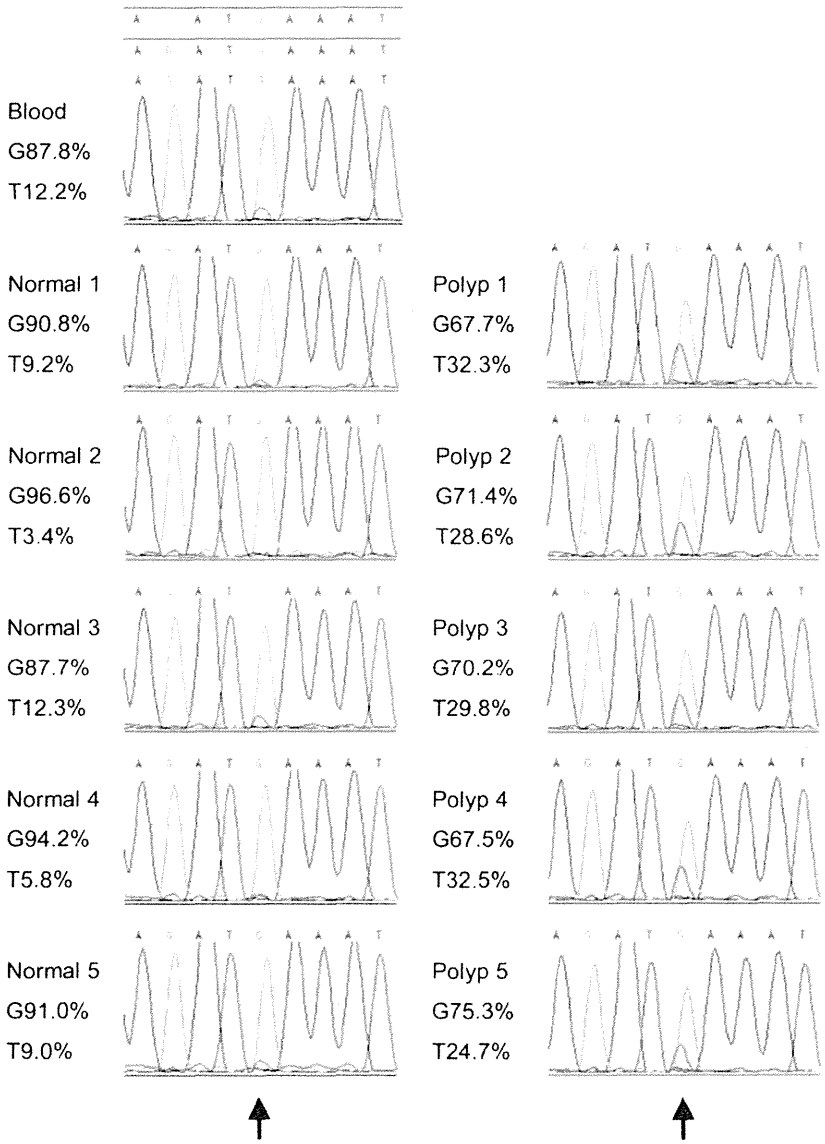

Figure 1 Direct sequencing of the mutant allele (c.3175G>T) in peripheral blood, non-cancerous colonic mucosae and adenomas of the colon. The arrows indicate the position of mutation. The corresponding results obtained from deep sequencing are shown on the left.

than the non-tumorous mucosa (Table 2). These data implied that the c.3175G>T mutation has a crucial role in the development of adenoma, and are consistent with the view of a clonal expansion of cells carrying *APC* mutation. As the average frequency of *APC* mutation in the polyps is ~30%, we can estimate that between 50 and 60% of the polyps are composed of tumor cells if loss of heterozygosity is not involved in the tumorigenesis.

DISCUSSION

In this report, we have shown that NGS is a powerful tool to identify mosaicism of *APC* mutation in a patient with polyposis. Although reports of somatic *APC* mosaicism are limited, recent studies have demonstrated that the mosaicism (10–20%) is present in a significant number of FAP patients harboring *de novo* germline mutation of *APC*.[4,6] Therefore, *APC* mosaicism may have been underestimated compared with other tumor-suppressor genes such as *RB1*, *TSC1* and *TSC2*.[12–14]

According to the number of polyps and age of onset, FAP is divided into three clinical subtypes: attenuated (mild), classical (typical) and severe.[15,16] A genotype–phenotype correlation has been well known in FAP. A recent study confirmed that patients with *APC* mutations between codon 1249–1549 certainly developed polyposis at an early age and exhibited a worse survival. On the other hand, patients with *APC* mutations in codon <178 or 312–412 have a later onset of polyposis and exhibited an improved survival.[17] Aretz *et al.*[4] previously reported eight cases with *APC* mosaic mutations. Although the eight mutations were located between codons 216–1464 associated with classical or severe phenotype, most of the patients exhibited mild phenotype. As the cells carrying *APC* mutation are scattered at a lower frequency in the epithelia of colonic mucosa compared with the classical FAP, the patients with mosaic mutations likely have a milder

Table 2 Frequency of the mutation (c.3175G>T) in various tissues

			No of reads		
Tissue	*Subject*	*Frequency (%)*	*Total*	*Ref allele (G)*	*Variant allele (T)*
Blood(control)	1	0.0	64 489	31 023+ 33 386−	6+ 0−
Blood	1	12.2	3726	1705+ 1566−	302+ 151−
Hair follicles	1	4.5	83 679	39 527+ 40 249−	1918+ 1856−
Buccal mucosa	1	3.0	69 169	32 605+ 34 368−	1047+ 1052−
	2	7.3	66 557	30 510+ 31 082−	2477+ 2383−
Gastric mucosa	1	18.9	81 041	29 167+ 36 446−	7025+ 8228−
	2	22.7	74 101	27 245+ 29 871−	8089+ 8654−
	3	27.7	84 151	27 281+ 33 392−	11 009+ 12 284−
Normal colonic mucosa	1	9.2	5149	2419+ 2252−	290+ 182−
	2	3.4	4698	2248+ 2291−	83+ 76−
	3	12.3	4321	1978+ 1807−	327+ 206−
	4	5.8	4514	2226+ 2026−	165+ 96−
	5	9.0	4097	2123+ 1602−	267+ 103−
Polyp	1	32.3	4797	1687+ 1557−	1013+ 536−
	2	28.6	4543	1883+ 1356−	937+ 362−
	3	29.8	4402	1537+ 1551−	841+ 469−
	4	32.5	4554	1628+ 1442−	981+ 494−
	5	24.7	4166	1581+ 1549−	685+ 344−

+, − indicate strand reads.

polyposis phenotype than the expected phenotype based on the site of the mutation.[4,6] In agreement with this notion, our case showed an attenuated form of polyposis demonstrating less than 100 polyps in the intestine, although the mutation of *APC* c.3175G>T, p.E1059X was reported to exhibit a florid form of adenomatous polyps in the large intestine at a young age and additional extra-colonic manifestations (duodenal adenoma and fundic gland polyps) in a FAP patient.[18] Application of NGS in genetic testing for patients with polyposis may increase the frequency of *APC* mosaicism in cases without familial history and those with mild phenotype.

After the surveillance of large intestine for eight years, the patient underwent subtotal colectomy in combination with ileorectal anastomosis because two of four biopsies of the colonic polyps histologically showed severe atypia. As we were unable to examine the DNA of his parents, we could not confirm *de novo APC* mutation in the patient. In addition, although his children have not been investigated, we should consider the possibility that germline mosaicism may lead to more severe phenotypes in the next generation.

On the basis of our result from deep sequencing, the frequency of the mutant allele was not constant among different types of tissues, and even in different sections isolated from the same tissue. The mutant allele was also found to a relatively higher extent in normal gastric mucosa (18.9–27.7%) compared with that in peripheral blood (12.2%), hair follicles (4.5%), buccal mucosa (3.0–7.3%) and normal colonic mucosa (3.4–12.3%). During embryogenesis, a zygote starts development and forms three germ layers; ectoderm, endoderm and mesoderm. Peripheral blood originates from mesoderm, hair follicles and buccal mucosa from ectoderm, and gastric and colonic mucosa from endoderm. Therefore the mutation should occur before the separation of these three layers. Although cells carrying the mutation were delivered into different tissues at different frequencies, we may assume from the frequencies that the mutation occurred at the four- or eight-cell stage. As the patient carried the mutation in non-tumorous gastric mucosa at a relatively high frequency, future surveillance paying special attention to his stomach is essential because the relative risk for gastric cancer in FAP patients is higher than the normal population in Asia.[19,20] Interestingly we also found that the mutation frequency is different at the location even in the same tissue, suggesting a possibility of intra-organic mosaicism.

Sequencing by the Sanger method is a gold standard of genetic testing for FAP. However, it is reported that the sensitivity of mutation by the direct sequence method is ~15%.[21] Consistent with this data, we failed to identify the mutation in our initial screening. Although PTT, DGGE, DHPLC and HRM are also applicable for the initial screening and may have higher sensitivities compared with Sanger's method, they are indirect detection systems and additional confirmatory sequencing is essential. In the near future, sequencing by NGS will replace the screening strategies for polyposis because the cost for NGS is dramatically decreasing. Previous reports also revealed the effectiveness of NGS for the detection of mosaic mutations.[21,22] Consistently, our data show that amplicon sequence with NGS is useful for the quantification of mosaic mutation (Figure 1 and Table 2). We additionally confirmed the mutation using a different set of primers, and the degree of mosaicism was comparable to that using the initial set of primers (data not shown), suggesting that the ratio is not affected by the set of primers used. Therefore, the amplicon-based NGS approach is reliable for confirming low-level mutation and quantifying mosaic mutations. It is of note that the sensitivity of detection of mosaic mutation by NGS totally depends on the number of reads. Although we identified the mosaic mutation in 6 out of 50 reads, we might miss the mutation if the depth of reads at the region was less than 10 or so. Although the sensitivity to identify low levels of mosaic mutation will be increased by the use a high depth sequence method such as amplicon sequence or whole-exome sequence, these methods may overlook structural changes such as translocation and deletion/amplification of large regions. Therefore, we should take advantage of the most appropriate method for the detection of different types of alterations. In addition, it may be possible to analyze multiple polyps, if available, in the patients, because the tumors are largely composed of relatively homogeneous cell population, and the mosaic mutation should be shared in the DNA from the polyps.

In conclusion, we successfully identified a mosaic mutation in a patient with a fraction of 12% mutated allele in peripheral blood by whole-genome sequencing covered at 26x average depth. As mutational mosaicism of the *APC* gene has relevance to cancer risk, genetic diagnosis is useful for the decision of surveillance and personalized treatment of the patients, and may be applied for the pre-symptomatic diagnosis of their children. These results will accelerate the application of NGS in clinical settings.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Chapter 7

The Role of DNA Methylation in the Genetics and Epigenetics of Multiple Myeloma

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Abstract

Multiple myeloma (MM) arises through an accumulation of multiple genetic and epigenetic changes, which play a significant role in tumorigenesis and tumor development. DNA methylation is often found in cancers including MM at the 5-carbon on cytosine residues within CpG islands of genes whose products are associated with the promoter regions of protein-coding genes. This methylation is an epigenetic alteration that leads to heritable changes in gene expression through the recruitment of histone deacetylases and histone methyltransferases. We and other researchers have reported the association of global and regional DNA methylation status with MM. Global DNA hypomethylation is the predominant early change during plasma cell oncogenesis from monoclonal gammopathy of undetermined significance to MM, while regional DNA hypermethylation occurs in tumor relapse and during disease progression. Thus, DNA methylation could be a useful biomarker of MM tumorigenesis and progression. In the current review, we discuss the role of DNA methylation changes: their potential application as epigenetic biomarkers to facilitate risk assessment, diagnosis, prediction of prognosis, and sensitivity to treatment; and epigenetic therapy in MM.

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Introduction

Cancers. including multiple myeloma (MM). arise because of an accumulation of multiple genetic changes. which play a significant role in tumorigenesis and tumor development. In addition to genetics. recent studies revealed the role of epigenetics—heritable information that does not affect DNA sequence—in the pathogenesis of cancers. including MM [1-4]. Among epigenetic changes. DNA methylation and histone modification have been well-studied.

DNA methylation occurs at the 5-carbon on cytosine residues in cytosine-guanine pairs known as CpG dinucleotides. DNA methylation is catalyzed by three DNA methyltransferases. including DNMT1. DNMT3A. and DNMT3B. and is a crucial regulator in different biological processes. such as embryonic development. transcription. chromatin structure. X chromosome inactivation. genomic imprinting. genomic instability. and tumorigenesis [5]. Since transcriptionally active regions of the genome are usually CpG rich. methylation of CpG sites is a critical factor affecting gene transcription. DNA hypermethylation of the large clusters of CpG dinucleotides. referred to as CpG islands. at gene promoters and transcription start sites is an epigenetic alteration that can suppress gene expression through the recruitment of methyl-CpG binding domain proteins. histone deacetylases. and histone methyltransferases. thus causing chromatin condensation [6]. Genome-wide hypomethylation and regional hypermethylation are common events in tumors. including hematological malignancies. In MM. DNA hypomethylation was reported as the predominant early change during tumorigenesis that gradually transforms to regional DNA hypermethylation during disease progression [7-9].

In the current review. we discuss the role of alterations in DNA methylation. potential application of epigenetic biomarkers. and target therapeutics in MM.

2. Molecular Mechanism Involved in Tumorigenesis of MM

MM is a neoplastic plasma cell disorder that is characterized by the clonal proliferation of malignant plasma cells in the bone marrow. the presence of monoclonal immunoglobulin in the serum and/or urine in most cases. and associated organ dysfunction. including lytic bone lesions. compromised immunity. anemia. renal failure. and hypercalcemia [10-12]. Recent studies have shown that MM is consistently preceded by a premalignant stage of clonal plasma cell proliferation. termed monoclonal gammopathy of undetermined significance (MGUS) [13.14]. Approximately 1% of MGUS cases evolve to MM per year [15].

MM advances through a multistep transformation process of specific events. including somatic mutations. chromosomal copy-number changes. and non-random chromosomal translocations such as immunoglobulin gene rearrangements involved in cyclin D; furthermore. epigenetic changes drive progression from MGUS. to symptomatic MM. and ultimately to recurrent myeloma. including extramedullary disease and. in some cases. plasma cell leukemia [4.12.16.17] (Figure 1).

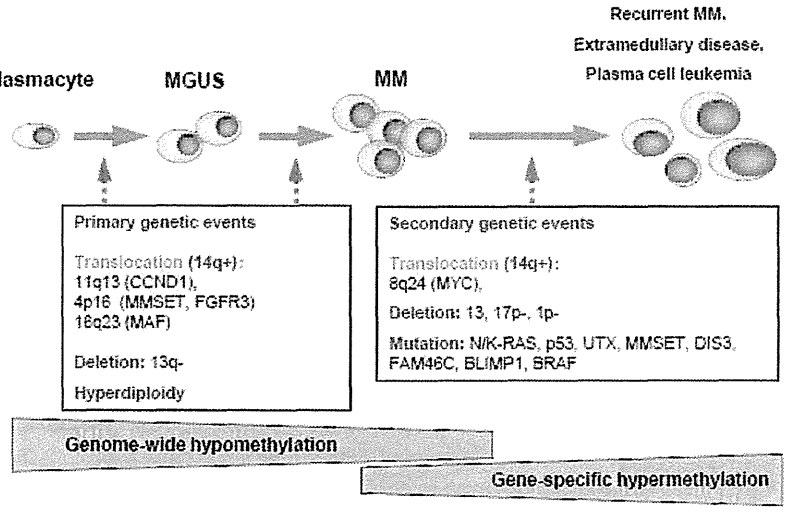

Figure 1. Multiple myeloma (MM) advances through a multistep transformation process due to specific events. These events include somatic mutations, chromosomal copy-number changes, and non-random chromosomal translocations, such as immunoglobulin gene rearrangements involved in cyclin D. Further, epigenetic changes drive progression from monoclonal gammopathy of undetermined significance (MGUS), to symptomatic MM, and ultimately to recurrent myeloma, including extramedullary disease and plasma cell leukemia. Genome-wide hypomethylation occurs during the events indicated by a blue wedge, and gene-specific hypermethylation occurs during the events indicated by a blue wedge.

Recently, several studies using high-throughput sequencing technologies demonstrated heterogeneity of MM genomic evolution and subclonal structure [18-22]. Chapman et al., reported that the analysis of somatic mutations by tumor-genome sequencing in MM cases revealed that, of the numerous genes mutated, identified genes are specifically involved in NFκB activation, protein homeostasis, and histone methylation, which are processes consistent with MM biology [18]. However, the key steps in MM oncogenesis remain unclear [3,4,23,24]. Recent findings also revealed that epigenetics, including DNA methylation and histone modification, is also important in MM pathogenesis. Global methylation analyses in MM have revealed the role of DNA methylation in MM pathogenesis and progress.

2. DNA Hypomethylation in MM

Genome-wide DNA hypomethylation is a common epigenetic alteration in cancer cells. Low levels of DNA methylation in cancer cells is substantially due to the loss of methylation at repetitive sequences such as long interspersed nuclear element-1 (LINE-1, a kind of a retrotransposon), which accounts for 17% of the human genome [19,25]. The following mechanisms have been suggested for DNA hypomethylation in tumorigenesis and tumor development: increased instability of the genome and reactivation of transposable elements (transposons) that can move in DNA [26-28]. Importantly, we and others reported that global methylation levels of DNA repetitive sequences, including LINE-1, progressively decline during the development of MM from MGUS to aggressive myeloma such as plasma cell

leukemia [8,9]. We also reported that there is a significant inverse correlation between the degree of genomic loss and LINE-1 methylation levels, and MM cases with LINE-1 hypomethylation had a significantly poor prognosis [9]. Regarding the pathogenesis of MM plasma cells, microarray data examining genome-wide differences in CpG methylation patterns revealed that genome-wide hypomethylation occurs at the transition from MGUS to MM [7].

3. DNA Hypermethylation in MM

DNA hypermethylation at gene promoters and transcription start sites is an epigenetic alteration that suppresses gene expression. Global DNA hypomethylation is the predominant early change during plasma cell oncogenesis from MGUS to MM, while regional DNA hypermethylation occurs in tumor relapse and during disease progression [7]. We and others studied regional DNA hypermethylation in MM and identified certain key genes as targets for epigenetic inactivation (Table 1).

Table 1. Epigenetically silenced genes in multiple myeloma (MM)

Gene	Chromosome	Function	Frequency of DNA hypermethylation of patient MM samples (n = 50)
CDKN2A (p16)	9p21.3	Cell cycle	34% [29]
DAPK1	9q21.33	Apoptosis	52.7% [35]
BNIP3	10q26.3	Apoptosis	5% [36]
RASD1	17p11.2	Cell growth	6–8% [33,40]
SPARC	5q33.1	Cell-extracellular matrix interaction	8–18.2% [36,40]
CD38	4p15	Ectoenzyme	45.9% [40]
GPX3	5q23	Glutathione peroxidase	7.5% [40]
NCAM1 (CD56)	11q23.1	Cell adhesion	5% [40]
PDK4	7q21.3	Regulation of metabolism	15.1% [40]
RBP1	3q23	carrier protein involved in the transport of retinol	16.3% [40]
TGFBI	5q31	Inhibition of cell adhesion	18.2% [40]

These genes include the following: cell-cycle regulators, such as cyclin-dependent kinase inhibitor 2A (*CDKN2A*) [29] and 2B (*CDKN2B*) [30] and checkpoint with fork head and ring finger domains (*CHFR*) [31]; genes involved in cell signaling, such as Ras association

(RalGDS/AF-6) domain family member 1 (*RASSF1*) [32], RAS, dexamethasone-induced 1 (*RASD1*) [33], and transforming growth factor, beta receptor II (*TGFBR2*) [34]; genes involved in apoptosis, such as death-associated protein kinase 1 (*DAPK1*) [35] and BCL2/adenovirus E1B 19kDa interacting protein 3 (*BNIP3*) [36,37]; genes involved in antigen presentation, such as class II, major histocompatibility complex, transactivator (*CIITA*) [38]; genes involved in cell-extracellular matrix interaction, such as secreted protein, acidic, cysteine-rich (*SPARC*) [36]; and genes involved in polycomb repressive complexes, such as enhancer of zeste, drosophila, homolog 2 (*EZH2*) [39].

Recently, Kaiser et al., investigated the association between DNA methylation and MM prognosis using a genome-wide DNA methylation array of 159 patients treated in the Medical Research Council Myeloma IX trial [40]. They identified the following 8 epigenetically regulated genes with changes in DNA methylation status that were significantly associated with prognosis: *CD38*, *RASD1*, *SPARC*, glutathione peroxidase 3 (*GPX3*), neural cell adhesion molecule 1 (*NCAM1*), pyruvate dehydrogenase kinase 4 (*PDK4*), retinol-binding protein 1 (*RBP1*), and transforming growth factor, β induced (*TGFBI*). Importantly, multivariate analysis confirmed that *GPX3*, *RBP1*, *SPARC*, and *TGFBI* are associated with survival, and methylation of the genes is independent of established risk factors for MM. Methylation levels of these 4 genes is low in MGUS, and then increasing methylation is associated with more aggressive MM cellular phenotypes. Walker et al., investigated DNA methylation patterns associated with MM subtypes [7]. They found specific profiles with increased hypermethylation in clinically aggressive subtypes, such as plasma cell leukemia, and in the prognostically unfavorable t(4;14) cytogenetic subtype with overexpressed *MMSET*, which encodes a histone methyltransferase. These findings suggest that methylation changes affect disease biology.

Recent reports correlated hypermethylation of promoter-associated CpG islands with silencing of microRNAs (miRNAs), which are small 18–22 nucleotide RNAs that regulate many intracellular functions [41]. Dysregulation of miRNA genes has been implicated in MM. Moreover, several reports of MM described the role of hypermethylation of tumor-suppressor miRNA genes, including *miR-34b/c* [42], *miR-194-2-192* [43], and *miR-203* [44]. Combined genome-wide analysis of miRNA methylation and miRNA expression profiling is warranted to clarify the role of epigenetic regulation of miRNA in MM.

4. DNA Methylation As an Epigenetic Biomarker in MM

The current prognostic factors in MM include cytogenetic aberrations, such as the nonhyperdiploid, cytogenetically detected chromosomal 13q deletion, t(4;14), t(14;16), 1q gain, and del(17p) detected by fluorescence *in situ* hybridization [10]. Novel therapeutics, such as the proteasome inhibitor bortezomib, can partially overcome adverse outcomes conferred by these abnormalities [45]. However, there has been much less progress in the development of predictive biomarkers for specific treatments [46]. To identify predictive biomarkers for the effect of myeloma therapeutics, appropriate clinical trial designs are necessary. Since some novel MM therapeutics in development have specific molecular

targets, the identification of biomarkers that also characterize drug sensitivity is a promising therapeutic strategy [45].

As mentioned above, hypermethylation of *TGFB1*, *SPARC*, *RBP1*, and *GPX3* is associated with significantly shorter overall survival, independent of age, international staging system score, and adverse cytogenetics [40]. Future prospective studies will verify these genes as prognostic MM biomarkers.

We identified *RASD1* as a possible biomarker in MM [33]. *RASD1*, located on chromosome 17p11.2 with frequent loss of heterozygosity in various human tumors, encodes a Ras GTPase with tumor suppressor functions induced by dexamethasone [47,48]. Importantly, MM cells that show *RASD1* methylation are resistant to dexamethasone, and combined treatment with dexamethasone and the hypomethylating agent decitabine (5-aza-2′-deoxycytidine), which inhibits DNA methyltransferase, restores the cytotoxicity of dexamethasone to tumor cells. While the hypermethylation of *RASD1* was observed in approximately 10% of primary MM samples, the methylation levels of *RASD1* were elevated in all of the MM cases that had pair DNAs after repeated antimyeloma therapy, including dexamethasone.

Limited studies have addressed the antitumor effects of the hypomethylating agents decitabine and azacitidine (5-azacytidine) in MM, demonstrating significant *in vitro* antimyeloma activity. The mechanisms involve changes in gene expression and induction of DNA damage [49,50]. Recently, a gene expression-based DNA methylation score was reported, which relates the expression of methylation-regulated genes to predict the efficacy of hypomethylating agents—decitabine and azacitidine—in human MM cell lines and in patient MM cells *in vitro* [51,52]. Phase I/II clinical trials are ongoing to study the side effects and best dose of azacitidine in combination with lenalidomide and dexamethasone in MM; therefore, an investigation regarding association of the methylation score and the response of MM patients could provide promising information [53]. Taken together, these findings suggest the involvement of epigenetic gene silencing in MM progression and drug resistance and the usefulness of demethylation therapy for MM treatment.

Conclusion

In summary, DNA methylation functions in MM tumorigenesis and progression. Several reports have suggested that DNA methylation could be a useful biomarker to predict prognosis and sensitivity to treatment. A further comprehensive analysis using a genome-wide approach with high-throughput sequencing technologies will be necessary to clarify the molecular mechanisms of MM oncogenesis and progression. Epigenetics has become to an essential research area where important challenges should be resolved through further investigations of MM.

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