

男性より約3倍患者数が多く、地域別でみると東北や北関東では少なく、九州、四国、近畿（女性）に多く、西高東低の関係があることがわかった。

## (2) 脊椎椎体骨折

脊椎骨折は骨粗鬆症に関連する骨折の中ではもっとも頻度が高いが、軽症あるいは無症状で経過することが多いため、発生率を知るためには初回調査からある一定の期間をおいて再度同条件でX線調査を行う必要がある。このような煩雑さのため、わが国における脊椎骨折の疫学調査の報告はきわめて少ない。

脊椎椎体骨折の発生率については、広島、長崎での出生年別コホート研究が、一般住民を対象とした脊椎椎体骨折発生率の唯一の調査であった<sup>27)</sup>。Fujiwaraらは、2年ごとに撮影された胸部X線写真を用いて、胸椎骨折の有無を診断し、出生コホート別に長期トレンドを推計した。その結果、女性においては、60歳以降に胸椎骨折の発生率が急激に上昇していたこと、胸椎骨折の発生率は近年に生まれた人ほど低くなり、10年出生が遅いと発生率は約1/2に低下していたことを報告している。その後、Fujiwaraらは同コホートの参加者2,356人を4年間追跡し、脊椎椎体骨折の発生率も推計した<sup>28)</sup>。これから脊椎椎体骨折の発生率は女性に多く、加齢とともに著明な上昇を示すことが明らかになった。

これらの結果に加えて、著者らは和歌山県の山村において設定した40～79歳の男女400人を10年間追跡し、脊椎椎体骨折の10年間の累積発生率を報告した<sup>29)</sup>。ベースライン調査時に骨折が認められず、10年後に骨折が認められるものを脊椎椎体骨折発生と定義すると、10年間の脊椎椎体骨折の累積発生率は60歳代男性で5.1%、女性で14%、70歳代男性で10.8%、女性で22.2%であることがわかった(図2)。

## (3) 橈骨遠位端骨折および上腕骨近位端骨折の発生率

わが国における大腿骨頸部骨折、脊椎椎体骨折

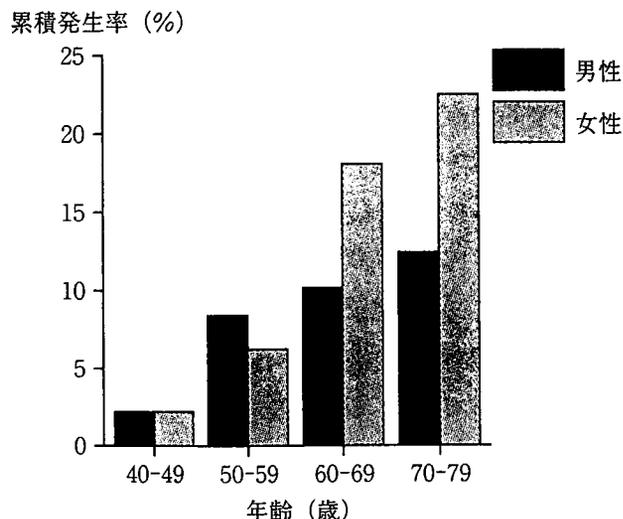


図2 脊椎椎体骨折の累積発生率 (10年間の追跡)  
(文献29)より作成)

以外の部位の骨粗鬆症関連骨折の頻度については、鳥取県の悉皆調査の結果<sup>30)</sup>が詳しい。Haginoらは、1986～1988年、1992年、1995年において鳥取県の橈骨遠位端骨折の発生数を調査し発生率を推計した。1986年と1995年の性・年齢別橈骨遠位端骨折の発生率をみると、橈骨遠位端骨折は女性において50歳代以降に多く発症し年齢とともに上昇傾向にある。男女別にみると女性に多く、経年的には9年前の調査時よりも女性において上昇傾向にあることがわかる(図3)。

上腕骨近位端骨折の発生率についてもHaginoらは橈骨遠位端骨折と同様、1986～1988年、1992年、1995年において鳥取県の上腕骨近位端骨折の発生数を調査し発生率を推計した<sup>30)</sup>。これをみると、上腕骨近位端骨折は男女とも60歳代以降に多く発症し、女性において年齢とともに上昇傾向にある。男女別には女性に多く、経年的には9年前の調査時よりも女性の65歳以降において上昇傾向にあることが明らかになった(図4)。

## 2) 骨粗鬆症に伴う骨折の予後

疫学において目的疾患の予後を知ることは、個々の疾病予防の面からもまた行政的な施策の面からもきわめて重要なことである。骨粗鬆症の予後とは、骨粗鬆症に伴う骨折の予後にほかならない。

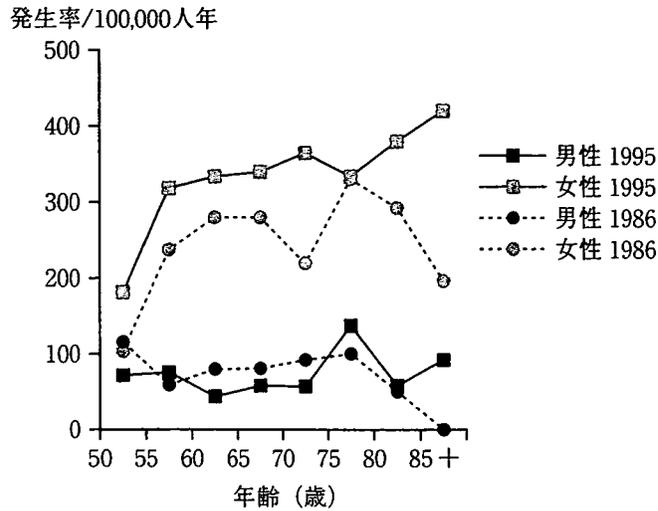


図3 橈骨遠位端骨折の発生率とその推移 (文献30) より引用)

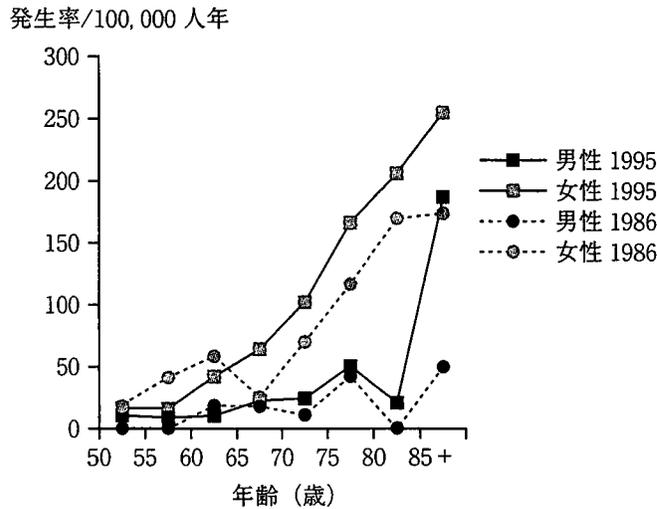


図4 上腕遠位端骨折の発生率とその推移 (文献30) より引用)

骨粗鬆症による骨折後の死亡率について、Johnell<sup>31)</sup>らは、Malmöの2847人の骨折患者を追跡し、骨折部位別に骨折後の死亡率を同定した(図5)。彼らは、脊椎椎体骨折、大腿骨頸部骨折、上腕骨折においては死亡率は骨折後すぐに著明に上昇するが、骨折後1年を経過すると死亡率の上昇には歯止めがかかる。その一方、脊椎椎体骨折、大腿骨頸部骨折においては、骨折後5年がたってもその死亡に対する相対危険度は有意に高いと報告した。

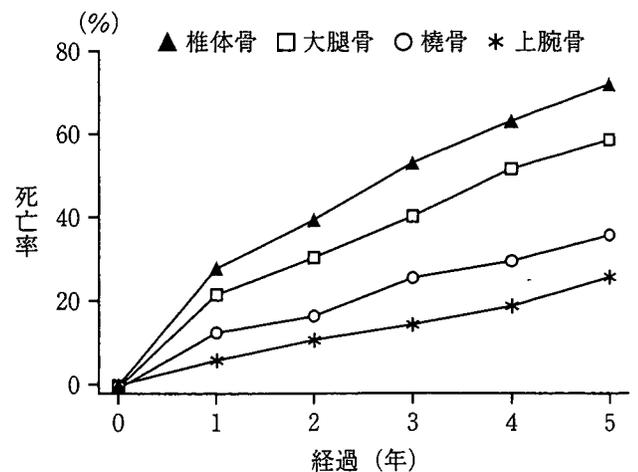


図5 骨粗鬆症性骨折の生命予後 (文献31) より引用)

わが国における骨粗鬆症による骨折の生命予後

表2 骨折の危険因子 (メタアナリシス, システマティック・レビューによる結果)

危険因子	文 献	成 績
低骨密度	77) 112)	BMD 1SD 低下で RR 1.5 腰椎 BMD: 椎体骨折 RR 2.3, 大腿骨頸部 BMD—大腿骨頸部骨折 RR 2.6 BMD 1SD 低下で 65 歳男大腿骨頸部骨折 RR 2.94, 65 歳女 RR 2.88
骨密度とは 独立した危険因子	既存骨折	75) 既存椎体骨折: 椎体骨折 RR 4, その他の組み合わせ RR 2 113) 既存骨折: すべての骨折 RR 1.86
	喫 煙	115) 喫煙 RR 1.25 116) 喫煙: すべての骨折 RR 1.26, 大腿骨頸部骨折 RR 1.39, 椎体骨折 RR 1.76
	飲 酒	118) 1 日 2 単位以上: 骨折 RR 1.23, 骨粗鬆症性骨折 1.38, 大腿骨頸部骨折 1.68
	ステロイド 使用	120) 骨粗鬆症性骨折 RR 2.63~1.71, 大腿骨頸部骨折 RR 4.42~2.48 119) GPRD: 骨折 RR 1.33, 大腿骨頸部 1.61, 椎体 2.6, 手首 1.09 その他: 骨折 1.91, 大腿骨頸部 2.01, 椎体 2.86, 手首 1.13
	骨折家族歴	121) 親の大腿骨頸部骨折: 大腿骨頸部骨折 RR 2.3 親の骨折: 骨折 RR 1.17, 骨粗鬆症性骨折 1.18, 大腿骨頸部 1.49
	運 動	122) 大腿骨頸部骨折リスク 20~40%低い 123) 最大で 50%の予防効果
骨密度を介した 危険因子	体重, BMI	124) 骨密度を調整しない場合, BMI (1 kg/m <sup>2</sup> ) 高いと骨粗鬆症性骨折 RR 0.93
	カルシウム摂取	125) カルシウム補助薬: 椎体骨折 RR 0.77(0.54~1.09), 非椎体骨折 RR 0.86(0.43~1.72): 有意ではない

BMD: 骨密度, RR: 相対リスク, GPRD: general practice research database

(文献 42) より引用)

に関する研究において, Muraki らは, 65 歳以上の大腿骨頸部骨折患者 480 人を追跡し, 1 年生存率は男性 83.1%, 女性 89.1%であることを報告している<sup>32)</sup>. 一方, 大腿骨頸部骨折以外の骨折の生命予後に関する研究はわが国では十分であるとはいえない.

### 3) 骨粗鬆症に伴う骨折の危険因子

骨粗鬆症による骨折の危険因子について, WHO collaborating centre for metabolic bone diseases では John Kanis 教授を中心として, 世界で代表的な 9 つのコホートの結果を集め, 46,000 人以上の個別データをそろえて meta-analysis することにより, 骨粗鬆症による骨折の統合された危険因子を明らかにしてきた. この研究には, わが国からも広島 Adult Health Study コホート (主任: 藤原佐枝子)<sup>33, 34)</sup> が参加し, 危険因子を定めその評価を行うことに大きな貢献をしている. この meta-analysis の結果, 骨粗鬆症による骨折の

危険因子は, 高齢, 性別 (女), 低 BMI<sup>35, 36)</sup>, 50 歳以降の骨折の既往<sup>36, 37)</sup>, 大腿骨頸部骨折の家族歴<sup>36, 38)</sup>, 喫煙<sup>36, 39)</sup>, ステロイド使用<sup>36)</sup>, 関節リウマチ<sup>36)</sup>, 一日 2 ユニット以上のアルコール摂取<sup>36, 40)</sup>, 大腿骨頸部あるいは total hip の骨密度<sup>36, 41)</sup> であることが明らかになった.

骨粗鬆症の予防と治療ガイドライン 2006 年度版<sup>42)</sup> では, 骨粗鬆症に伴う骨折の危険因子として, WHO の結果を踏襲し, 低骨密度の他に骨密度と独立した危険因子として年齢 (高齢), 性 (女性), 骨折の既往, 喫煙, 飲酒, ステロイド使用, 骨折家族歴, 運動 (予防), やせがあげられている (表 2).

## おわりに

本稿では, 骨粗鬆症とそれに関連する骨折の疫学について述べた. 稿を終えるにあたり, 今後骨粗鬆症の分野において解明していくべき課題につ

いて述べたいと思う。

骨粗鬆症の頻度については前述のごとく、骨粗鬆症の発生率、および脊椎椎体骨折の発生率については報告が極めて少ない。さらに骨粗鬆症に伴う骨折のうち上腕骨近位端、大腿骨頸部以外の大腿骨、肋骨、骨盤、鎖骨・肩甲骨・胸骨、下腿骨（脛骨、腓骨）の骨折の頻度も明らかになっていない。これらの頻度については今後わが国の研究結果から新しい報告が待たれるところである。

骨質の概念の指標化についても今後の課題である。骨粗鬆症において骨質を客観的に無侵襲にそして効率的に判定するための多くの指標が考案され、それにより骨折予測が可能かどうかについて検討がなされてきている。それらの指標はいずれも貴重な成果をあげているが、今はまだいずれも gold standard とはなりえていないようである。

危険因子については、基礎研究や診断方法の著しい進歩による効率的な危険因子の検出が期待される。たとえば、骨代謝マーカーの測定は骨折の高危険群の絞り込みに有益な指標としてすでに用いられているが今後もさらに新しいマーカーの提案が期待される。ゲノム疫学の進歩により新たな遺伝子多型や変異が疾病の早期発見につながるものが期待されている。

## 文 献

- 1) 内閣府：平成 18 年度版高齢社会白書。  
<http://www8.cao.go.jp/kourei/whitepaper/w-2006/zenbun/18index.html>
- 2) Kanis, J. A., et al. : J. Bone Miner. Res., 9 : 1137, 1994.
- 3) NIH Consensus Development Panel : JAMA, 285 : 785, 2001.
- 4) 厚生労働省ホームページ統計情報：平成 16 年国民生活基礎調査の概況。  
<http://www.mhlw.go.jp/toukei/saikin/hw/k-tyosa/k-tyosa04/index.html>
- 5) 折茂 肇, 坂田清美 : 日本医事新報, 4180 : 25, 2004.
- 6) 骨粗鬆症の予防と治療ガイドライン作成委員会 (代表 折茂肇) : ライフサイエンス出版, 東京, 2006.
- 7) 日本骨代謝学会骨粗鬆症診断基準検討委員会 : Osteoporosis Japan, 4 : 643, 1996.
- 8) 折茂 肇 : Osteoporosis Jpn, 9 : 9, 2001.
- 9) 藤原佐枝子, 他 : Osteoporosis Jpn, 5 : 223, 1997.
- 10) 山本逸雄 : Osteoporosis Jpn, 7 : 10, 1999.
- 11) 吉村典子, et al. : Osteoporosis Jpn, 14, suppl : 149, 2006.
- 12) 伊木雅之, 他 : 日衛誌, 58 : 311, 2003.
- 13) 伊木雅之 : 財団法人日本公衆衛生協会, 東京, 2004.
- 14) 骨粗鬆症の予防と治療ガイドライン作成委員会 (代表 折茂肇) : p38, ライフサイエンス出版, 東京, 2006.
- 15) 骨粗鬆症の予防と治療ガイドライン作成委員会 (代表 折茂肇) : p41, ライフサイエンス出版, 東京, 2006.
- 16) 骨粗鬆症の予防と治療ガイドライン作成委員会 (代表 折茂肇) : p64, ライフサイエンス出版, 東京, 2006.
- 17) 骨粗鬆症の予防と治療ガイドライン作成委員会 (代表 折茂肇) : p67, ライフサイエンス出版, 東京, 2006.
- 18) Yoshimura, N. : J. Bone Miner. Metab., 14 : 171, 1996.
- 19) Yoshimura, N., et al. : Osteoporos. Int., 8 : 604, 1998.
- 20) 折茂 肇, 他 : 日本医事新報, 3420, p43, 1989.
- 21) Orimo, H., et al. : J of Bone and Miner. Metab., 9 (suppl) : 15, 1991.
- 22) 折茂 肇, 他 : 日本医事新報, 3707 : 27, 1995.
- 23) Orimo, H., et al. : J. Bone Miner. Metab., 15 : 100, 1996.
- 24) 厚生省長寿科学総合研究事業 骨粗鬆症予防のための危険因子に関する研究班 (班長 折茂肇) : 日本医事新報, 3916 : 46, 1999.
- 25) Orimo, H., et al. : in press, J. Bone Miner. Metab., 1999.
- 26) Yoshimura, N., et al. : J. Bone Miner. Metab., 23 suppl : 78, 2005.
- 27) Fujiwara, S., et al. : J. Clin. Epidemiol., 44 : 1007, 1991.
- 28) Fujiwara, S., et al. : J. Bone Miner. Res., 18 : 1547, 2003.
- 29) Yoshimura, N., et al. : Archives Osteoporos, DOI 10.1007/s11657-006-0007-0, 2006.

- 30) Hanigo, H., et al. : Bone, 24 : 265, 1999.
- 31) Johnell, O., et al. : Osteoporos. Int., 15 : 38, 2004.
- 32) Muraki, S., et al. : J. Bone Miner. Metab., 24 : 101, 2006.
- 33) Fujiwara, S., et al. : J. Bone Min. Res., 18 : 1547, 2003.
- 34) Fujiwara, S., et al. : J. Bone Min. Res., 12 : 998, 1997.
- 35) De Laet, C., et al. : Osteoporos. Int., 16 : 1330, 2005.
- 36) Kanis, J. A., et al. : Osteoporos. Int., 16 : 581, 2005.
- 37) Kanis, J. A., et al. : Bone, 35 : 375, 2004.
- 38) Kanis, J. A., et al. : Bone, 35 : 1029, 2004.
- 39) Kanis, J. A., et al. : Osteoporos. Int., 16 : 222, 2005.
- 40) Kanis, J. A., et al. : Osteoporos. Int., 16 : 737, 2005.
- 41) Johnell, O., et al. : J. Bone Miner. Res., 20 : 1185, 2005.
- 42) 骨粗鬆症の予防と治療ガイドライン作成委員会 (代表 折茂肇) : p34, ライフサイエンス出版, 東京, 2006.
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# 骨粗鬆症発生頻度の動向

吉村 典子

## ポイント

- ① 骨粗鬆症の発生率には50歳以上で年間男性2%、女性6%との報告があるが、まだ一定の見解は得られていない。
- ② 2002年の全国調査の結果、大腿骨頸部骨折の発生数は12万人と増加しており、年齢別発生率も依然として増加傾向にある。
- ③ 脊椎椎体骨折の10年間の発生率は、70歳台で男性の約1割、女性の約2割であると報告されている。

平成18年度版高齢社会白書<sup>1)</sup>によると、2005年10月1日現在のわが国の人口は、1億2,776万人と戦後初めて減少に転じたのに対して、65歳以上の高齢者人口は過去最高の2,560万人となった。また高齢化率(総人口に占める65歳以上の高齢者の割合)が20.04%となり、初めて20%を超えたと報告されている。今後も高齢者人口は増加が見込まれ、その一方で総人口の減少は続くと考えられることから、高齢化率は2015年に26.0%、2050年には35.7%となると予測されており、わが国の高齢化はまさに世界に類のない速さで進行し続けている。

このような状況のなかで、高齢者の生活の質(quality of life: QOL)を著しく阻害する骨粗鬆症とそれによる骨折の予防は、超高齢社会が目前に迫るわが国にとって焦眉の急であるといえる。しかしながら、骨粗鬆症やそれによる骨折の発生頻度については、まだ明らかになっていない点も多く、骨粗鬆症予防対策遅延の原因となっている。

そこで本稿においては、わが国において、現状で把握できる限りの骨粗鬆症および骨折の頻度の動向について述べることにする。

## 骨粗鬆症の発生率の動向

原発性骨粗鬆症とは、「骨量の低下、骨組織の微細構造の変化を特徴とし、骨の脆弱化とその結果骨折の危険の増大をきたした疾患<sup>2)</sup>と定義されてきた疾患であるが、最近ではこれに骨質の概念を加え<sup>3)</sup>、「骨強度が低下することにより骨折のリスクが高くなる骨の障害」とする定義が一般的となりつつある。

骨粗鬆症の診断に必須となる低骨量を有する者は、ほとんどの場合が無症状であるため、医療機関での早期診断は困難であり、住民を対象とした集団検診などで発見することが必要となってくる。骨粗鬆症の発生率を求めるためには、地域代表性をもつ住民の骨量評価を少なくとも2回行う必要があり、この困難さのために、わ

よしむらのりこ：東京大学大学院医学系研究科関節疾患総合研究講座 ㊚ 113-8655 東京都文京区本郷 7-3-1

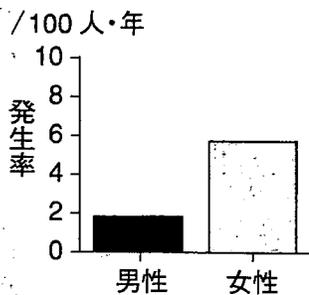


図1 骨粗鬆症の発生率 (50歳以上) (文献4より改変して引用)

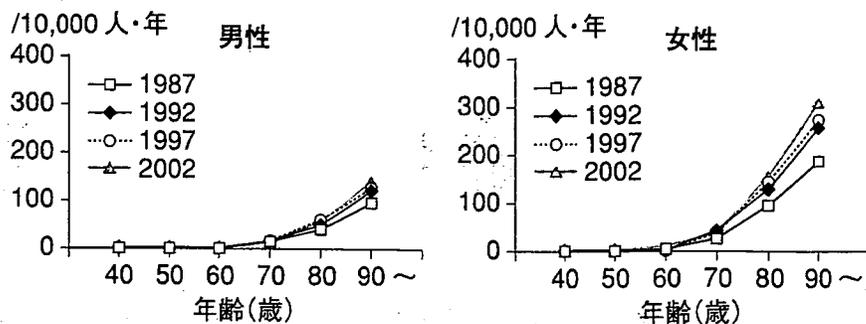


図2 年代別にみた大腿骨頸部骨折の発生率 (1987～2002年) (文献11より改変して引用)

が国における骨粗鬆症の発生率の報告はきわめて少ない。筆者らは、和歌山県の山村での10年間における継続的な調査から、腰椎骨密度で診断した骨粗鬆症の発生率を推計した(図1)。この結果、50歳以上の男性においては1年間に約2%、女性では約6%に新規骨粗鬆症が発生することが明らかになった<sup>4)</sup>。しかし、この推計は全体数が少ない一地域での結果であり、今後、ほかのコホートでの検証が必要であると考える。

## 骨粗鬆症による骨折の発生率の動向

骨粗鬆症そのものがほとんど症状を伴わないとすれば、なぜ治療の必要性があるのか？ それは、合併症である骨折が重大な結果をきたす場合が多いからである。そのため、骨粗鬆症の治療は、骨粗鬆症による骨折の発生予防のために行っていると考えてよい。これは脳卒中や心筋梗塞発症予防のために高血圧を治療する構図と類似している。

骨粗鬆症によって骨折を起こす部位は、大腿骨頸部、脊椎椎体のほか、橈骨遠位端、上腕骨近位端、大腿骨頸部以外の大腿骨、肋骨、骨盤、鎖骨・肩甲骨・胸骨、下腿骨(脛骨、腓骨)などが挙げられる。これらの部位のなかで、発生率についての疫学的研究結果の蓄積が最も進

んでいるのが大腿骨頸部骨折であり、次いで脊椎椎体骨折である。

## ■ 大腿骨頸部骨折

大腿骨頸部骨折は、寝たきりの原因となり、高齢者のQOLを著しく阻害する疾患である。わが国における大腿骨頸部骨折の発生率については、厚生省(現 厚生労働省)シルバーヘルスサイエンス研究老人性骨粗鬆症の予防および治療法に関する総合的研究班(1987年, 班長: 折茂 肇)<sup>5,6)</sup>, 厚生省骨粗鬆症の予防に関する総合的研究班(1992年, 班長: 折茂 肇)<sup>7,8)</sup>, 厚生省骨粗鬆症予防のための危険因子に関する研究班(1997年, 班長: 折茂 肇)<sup>9,10)</sup>, 厚生労働科学研究長寿科学総合研究事業骨粗鬆症におけるテーラーメイド医療の確立に関する研究班(2002年, 班長: 折茂 肇)<sup>11,12)</sup>により、過去5年ごとに4回の全国規模の調査が行われてきた。最新の調査である2002年の結果からは、大腿骨頸部骨折発生数は117,900人(男性25,300人, 女性92,600人)となり、これは初回(53,000人: 男性13,500人, 女性39,600人), 2回目(76,600人: 男性18,700人, 女性57,900人), 3回目(92,400人: 男性20,800人, 女性71,600人)の調査時の患者数を大きく上回っていることが報告された。年代別でも、大腿骨頸部骨折の発生率は年齢とともに上昇していた(図2)。さらに、女性は男性より約3倍患者

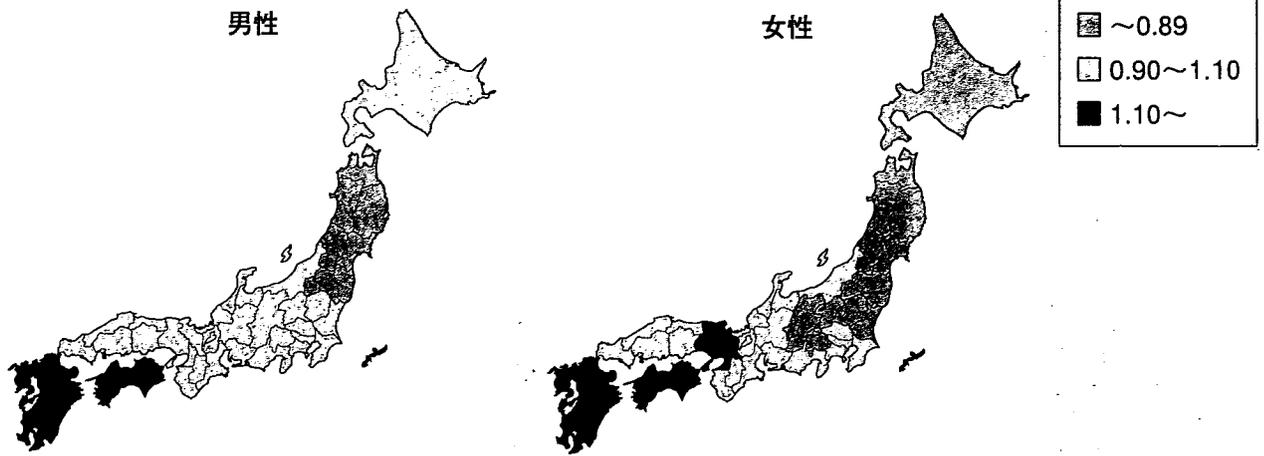


図3 大腿骨頸部骨折の標準化発生比 (文献11より改変して引用)

数が多く、地域別でみると東北や北関東では少なく、九州、四国、近畿(女性)に多く、西高東低の傾向があることがわかった(図3)。

2007年には第5次大腿骨頸部骨折発生率全国調査が計画されている。わが国の大腿骨頸部骨折の発生率が上げ止まりになるか、あるいはさらに上昇するかは、今後の骨粗鬆症予防対策のみならず、介護予防の面からも大きな意味をもっていることは疑いない。

### ■ 脊椎椎体骨折

脊椎椎体骨折は骨粗鬆症に関連する骨折のなかでは最も頻度が高いが、軽症あるいは無症状で経過することが多い。したがって、発生率を知るためには初回調査からある一定の期間において再度、同条件でX線調査を行う必要がある。このような煩雑さのため、わが国における脊椎椎体骨折の疫学調査の報告は今のところ乏しいといわざるを得ない。

脊椎椎体骨折の発生率については、広島、長崎での出生年別コホート研究が、一般住民を対象とした脊椎椎体骨折発生率の唯一の調査であった<sup>13)</sup>。Fujiwaraらは、2年ごとに撮影された胸部X線写真を用いて、胸椎骨折の有無を診断し、出生コホート別に長期トレンドを推計

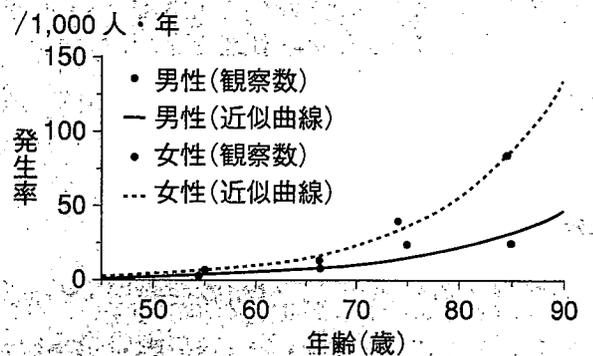
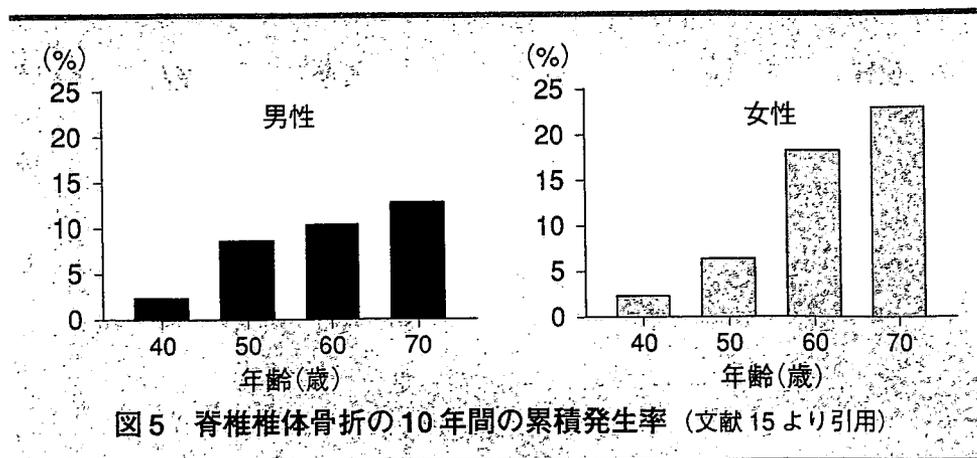


図4 脊椎椎体骨折の発生率(文献14より引用)

した。その結果、女性においては、60歳以降に胸椎骨折の発生率が急激に上昇していたこと、胸椎骨折の発生率は近年に生まれた人ほど低くなり、10年出生が遅いと発生率は約1/2に低下していたことを報告している。その後、Fujiwaraらは同コホートの参加者2,356人を4年間追跡し、脊椎椎体骨折の発生率も推計した<sup>14)</sup>。それによると、脊椎椎体骨折の発生率は女性に多く、加齢とともに著明な上昇を示すことが明らかになった(図4)。

これらの結果に加えて、筆者らは和歌山県の山村において設定した40~79歳の男女400人を10年間追跡し、脊椎椎体骨折の10年間の累積発生率を報告した<sup>15)</sup>。ベースライン調査時に骨折が認められず、10年後に骨折が認められ





る者を脊椎椎体骨折発生と定義すると、10年間の脊椎椎体骨折の累積発生率は60歳台男性で5.1%、女性で14%、70歳台男性で10.8%、女性で22.2%であることがわかった(図5)。これは、現在脊椎椎体骨折をもたない70歳台の男性で1割、女性では2割以上がその後の10年間に骨折をきたすことを意味している。

## おわりに

稿を終えるにあたり、骨粗鬆症発生率の動向というタイトルに関連して、わが国の将来の骨粗鬆症発生率に考えを巡らすことにしよう。はたして超高齢社会を迎えた将来のわが国において骨粗鬆症の発生は増えていくのだろうか？それとも減少に転じるのであろうか？

筆者らは、和歌山県に設定した一般住民コホートの10年間の観察から、一般住民の骨密度

の推移を年代別に比較し、男性では60歳台、女性では50歳台において10年前の世代よりも明らかに骨密度が上昇していることを明らかにした<sup>16)</sup>。この結果は、今後男性の60歳台、女性の50歳台以下の若い世代が骨粗鬆症の好発年齢になってくる10~20年後には低下に転じる可能性があると思われる。

しかし、骨粗鬆症は、骨密度のみにて定義されるものではなく、骨質を考慮に入れた骨強度が低下することにより、骨折のリスクが高くなる骨の障害であることを考えると、この予測もいささか心もとないものになってくる。現在のところ、骨強度を評価する簡便な指標はまだ存在しないので、今後の骨粗鬆症の発症頻度の評価は、その確立を待ち、診断基準が対応してからということになるだろう。

## 文 献

- 1)内閣府：平成18年度版高齢社会白書。http://www8.cao.go.jp/kourei/whitepaper/w-2006/zenbun/18index.html
- 2)Kaniz JA, et al : The dignosis of osteoporosis. J Bone Miner Res 9 : 1137-1141, 1994
- 3)NIH Consensus Development Panel : Osteoporosis prevention, diagnosis, and therapy. JAMA 285 : 785-795, 2001
- 4)吉村典子, 他 : 骨粗鬆症の有病率の推移と発生率の推定. Osteoporos Jpn 14 : 149-149, 2006
- 5)折茂 肇, 他 : 大腿骨頸部骨折全国頻度調査報告(昭和62年). 日本医事新報 No. 3420 : 43-45, 1989
- 6)Orimo H, et al : Hip fracture incidence in Japan. J Bone Miner Metab 9(Suppl) : 15-19, 1991
- 7)折茂 肇, 他 : 大腿骨頸部骨折全国頻度調査—1992年における新発症患者数の推定と5年間の推移. 日本医事新報 No. 3707 : 27-30, 1995
- 8)Orimo H, et al : Nation-wide incidence survey of femoral neck fracture in Japan, 1992. J Bone Miner Metab 15 : 100-106, 1996
- 9)厚生省長寿科学総合研究事業 骨粗鬆症予防のための危険因子に関する研究班(班長：折茂 肇) : 第3回大腿骨頸部骨折全国頻度調査成績—1997年における新発症患者数の推定と10年間の推移. 日本医事新報 No. 3916 : 46-49, 1999
- 10)Orimo H, et al : Trend of incidence of hip fracture in Japan, 1987-1997 ; The third nation-wide survey. J Bone Miner Metab 18 : 126-131, 2000
- 11)折茂 肇, 坂田清美 : 第4回大腿骨頸部骨折全国頻度調査成績—2002年における新発症患者数の推定と15年間の推移. 日本医事新報 No. 4180 : 25-30, 2004
- 12)Yosihmura N, et al : Epidemiology of hip fracture in Japan ; Incidence and risk factors. J Bone Miner Metab

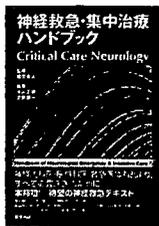
- 23(Suppl) : 78-80, 2005
- 13) Fujiwara S, et al : The incidence of thoracic vertebral fractures in a Japanese population, Hiroshima and Nagasaki, 1958-1986. J Clin Epidemiol 44 : 1007-1014, 1991
- 14) Fujiwara S, et al : Fracture prediction from bone mineral density in Japanese men and women. J Bone Miner Res 18 : 1547-1553, 2003
- 15) Yoshimura N, et al : Cumulative incidence and changes in prevalence of vertebral fractures in a rural Japanese community ; A 10-year follow-up of the Miyama Cohort. Archives Osteoporos, DOI 10.1007/s11657-006-0007-0, 2006
- 16) Yoshimura N, et al : Bone loss at the lumbar spine and the proximal femur in a rural Japanese community, 1990-2000 ; The Miyama Study. Osteoporos Int 13 : 803-808, 2002

## 書評

●金澤 一郎(国立精神・神経センター総長)

## 神経救急・集中治療ハンドブック

Critical Care Neurology



監修：篠原 幸人  
編集：永山 正雄，濱田 潤一

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われわれが医師になったのはちょうどインターン闘争から大学紛争に拡大していく時期であった。そこで「患者のためになる良い医療を」という旗印の下で、青年医師連合を結成した。医師とは関係のないところで政治的に活動していた仲間もいたが、われわれノンポリは医師国家試験をボイコットした非合法的なインターンとして医療の世界に飛び込んでいった。すでに医師として社会で働いていた先輩たちの技術や知恵を授けてもらいながら医療に邁進した。実に充実していた。中でも、いわゆる救急には心をときめかした。自分こそがこの患者を救うのだ！ という心意気に燃えていた。ただ心意気だけでは救えないので、いざというときはすぐに先輩に聞けるように「連絡網」を作成していた。救急医療は、医師になりたての身には格別の魅力があった。さしもの大学紛争も少し落ち着いた頃、大学に戻り、患者の受け持ちになってみると、病室で急変した患者に対応する先輩たちの動作の鈍いのにあきれた。しかし、今思えばその頃がわれわれの一般医療の腕前としてはピークだったように思う。頭で反応するよりも体が反応していたような気がする。

ところで、神経疾患の医療については、診断が

難しいだけでなく治療も明確なものがなくて医師としてやりがいがない、などと心ないことをいう人がいまだにいるので困る。神経学領域にも、脳卒中、頭部外傷、てんかん性痙攣、重症筋無力症のクリーゼ、などの本格的な救急症例はあるし、急性脳脊髄膜炎やギラン・バレー症候群なども急いで対応する必要がある。脳卒中以外の疾患は幸いにもあまり多いものではないが、決して侮れるものではない。命にかかわる。自分の経験でも、幸いにも助けることができた場合もあるし、残念ながら力が及ばなかったこともある。

このたび、私の尊敬する篠原幸人先生監修による神経救急と集中治療に関する本格的な教科書が上梓された。この本が、わが国における神経救急の初めての専門書である、と聞いてその事実にビックリした。以前から神経救急の必要性を説かれていた篠原先生が、米国で神経救急を本格的に学んでこられた永山・濱田両先生を編者に据えてまとめ上げられたものである。神経内科医は無論のこと、脳外科医、救急医などあらゆる領域の臨床医の必携の書である。かゆいところに手が届く、とはまさにこの本の読後感であった。悪性症候群、spinal emergency、などを一つの章として扱っているのはユニークでありプラクティカルでもある。内容も極めて充実している。本書のもう一つの特筆すべき点は、終章に「精神疾患におけるDo-Not-Resuscitate(DNR)と脳死」と題して、社会的にも注目すべき課題について、そのエッセンスを学べるように編纂されていることである。DNRはいわゆる尊厳死と共通部分をもった考え方であり、わが国でも十分議論する必要がある。いろいろ学べる書であり、考えさせる書でもあり、臨床医にとって必読の書である。

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変形性関節症の治療・予防の標的分子の  
同定とその臨床応用

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主任研究者 中村 耕三

平成 20 年 3 月

### Ⅲ. 研究成果の刊行物・別刷②

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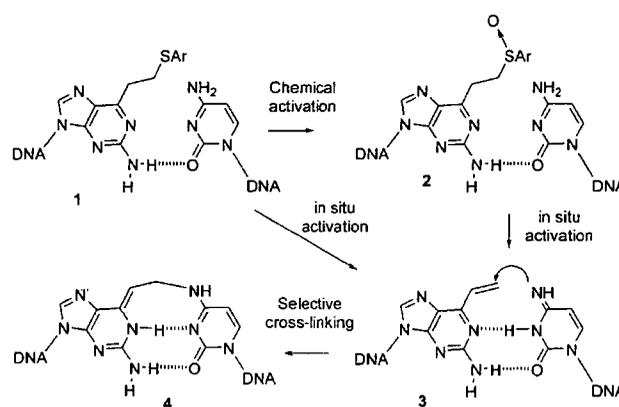
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**Intracellular Inducible Alkylation System That Exhibits Antisense Effects with Greater Potency and Selectivity than the Natural Oligonucleotide\*\***

Md. Monsur Ali, Motoi Oishi, Fumi Nagatsugi, Kenya Mori, Yukio Nagasaki, Kazunori Kataoka, and Shigeki Sasaki\*

Synthetic oligonucleotides (ODNs) that incorporate a chemically reactive appendage have been widely investigated in the endeavor to create new functions through specific reactivity toward a target sequence. A number of biotechnological approaches have been invented based on sequence-selective alkylation,<sup>[1]</sup> cross-linking,<sup>[2,3]</sup> strand cleavage,<sup>[4-7]</sup> chemical

ligation,<sup>[8]</sup> signal amplification,<sup>[9]</sup> and other techniques. Cross-linking oligonucleotides have been used to stabilize complexes with the cellular target mRNA by covalent-bond formation, and have been shown to be useful in enhancing antisense effects. Emerging knowledge about the fundamental role of micro-RNAs (miRNAs) in gene regulation has led to great interest in efficient antisense oligonucleotides against miRNAs as a tool for specifically knocking down particular miRNAs.<sup>[10,11]</sup> Psoralen derivatives are activated by UV irradiation and are the only reagents applicable to *in vitro* and *in vivo* studies. They not only enhance antisense effects<sup>[12,13]</sup> but also induce specific mutations at the sites of the reaction within the triplex DNA.<sup>[14,15]</sup> However, until now the use of alkylating oligonucleotides in biological studies has been hampered by the lack of an efficient cross-linking agent. As alkylating or cross-linking agents tend to be unstable under physiological conditions, the use of stable precursors with inducible reactivity was expected to provide a solution to this problem.<sup>[2,16]</sup> We recently proposed a new concept of inducible reactivity, in which a phenylsulfide derivative used as a stable precursor to a 2-amino-6-vinylpurine nucleoside analogue is automatically activated in the proximity of the target cytidine residue to form a covalent bond selectively (Scheme 1).<sup>[17-20]</sup> The selectivity of this inducible alkylation



**Scheme 1.** In situ activation of the sulfide precursor 1 to the 2-amino-6-vinylpurine 3 for selective cross-linking with cytidine to give 4.<sup>[17]</sup> The vinyl compound 3 can be generated selectively in the hybrid with the complementary sequence from the sulfoxide 2 or the sulfide precursor 1. As intermediary formation of 2 has not been detected, it is anticipated that activation from 1 to 3 takes place directly.

system for cytidine is so high that a single nucleotide difference can be discriminated in the alkylation. Herein we describe the capacity of the inducible alkylation system to exhibit antisense effects in an intracellular environment with greater potency and selectivity than the corresponding unmodified oligonucleotide.

In a previous study, the phenylsulfide derivative 1 was oxidized chemically to produce the sulfoxide derivative 2, which was activated selectively to the vinyl compound 3 by hybridization with the complementary sequence. It has been shown that the cytidine residue at the target site enhances the elimination step from 2 to 3, which is followed by efficient

[\*] Dr. M. M. Ali, Prof. Dr. F. Nagatsugi, Prof. Dr. S. Sasaki  
Graduate School of Pharmaceutical Sciences  
Kyushu University  
3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582 (Japan)  
Fax: (+81) 92-642-6615  
E-mail: sasaki@phar.kyushu-u.ac.jp  
Dr. M. Oishi, Prof. Dr. Y. Nagasaki  
Institute of Materials Science  
Tsukuba University  
1-1-1 Ten-nodai, Tsukuba, Ibaraki 305-8573 (Japan)

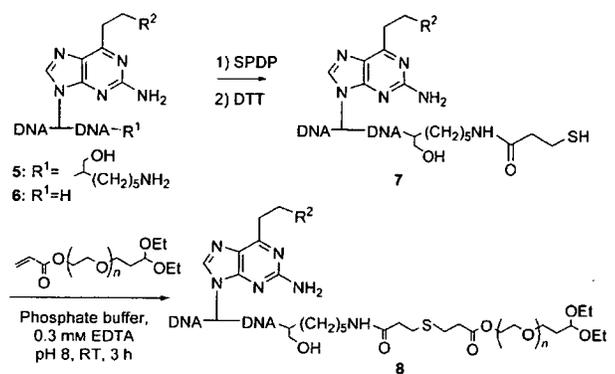
Dr. K. Mori  
GeneAct Ltd.  
2432-3 Kawaimachi, Kurume, Fukuoka 839-0861 (Japan)  
Prof. Dr. K. Kataoka  
Department of Materials Science and Engineering  
Graduate School of Engineering  
The University of Tokyo  
7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656 (Japan)

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Supporting information for this article is available on the WWW under <http://www.angewandte.org> or from the author.

alkylation to produce **4**.<sup>[17]</sup> The vinyl compound **3** can also be generated selectively from the sulfide precursor **1** in the hybrid with the complementary sequence. In this case it is anticipated that activation from **1** to **3** takes place directly. The elimination of the sulfide group is also accelerated by the cytidine residue at the target site. As the sulfide derivative **1** is chemically stable, we viewed it as a potential candidate for an alkylating agent with inducible reactivity for use in living systems.

Herein, we have applied polyion-complex (PIC) micelles of PEG conjugates of oligonucleotides for both the protection of ODNs from enzymatic digestion and their efficient delivery into the cellular interior.<sup>[21–23]</sup> Scheme 2 summarizes the



Substituent R<sup>2</sup>

- a: SMe  
 b: SPh  
 c: SC<sub>6</sub>H<sub>4</sub>-2-COOH  
 d: SC<sub>6</sub>H<sub>4</sub>-4-COOH  
 e: NHPh

Target sequence of firefly luciferase mRNA:  
 - 3' (446) TACGGGTATGA-C-AACTCGTTA (426) -

Synthesized ODN sequences:

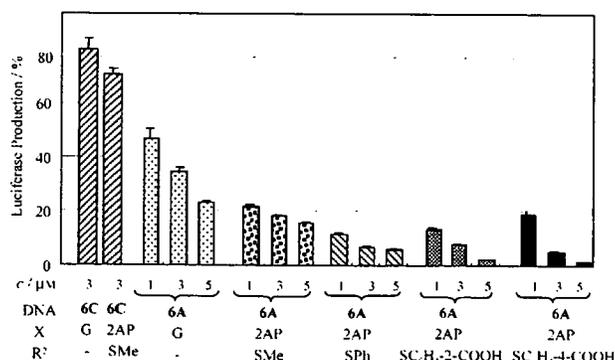
- A: 5' ATGCCCATACT-X-TTGAGCAAT  
 B: 5' ATGCCCATACT-G-XTGAGCAAT  
 C: 5' GCATAGCGTACT-X-TCTAACTA  
 X=2-amino 6-alkyl purine (2AP) or G

**Scheme 2.** Synthesis of the antisense PEG conjugates in which 2-amino 6-alkyl purine is incorporated. The sequence **A** has X at the complementary site to the cytidine residue of the target mRNA sequence, **B** contains X at the adjacent site, and **C** is a random sequence. The description **6A** refers to species with the ODN sequence **A** that are not conjugated with PEG; **8B** (X = AP, R<sup>2</sup> = SMe) is the PEG conjugate with the ODN sequence **B** in which a 2-amino-6-(2-methylthioethyl)purine derivative is incorporated adjacent to the complementary site to the cytidine residue of the target sequence. EDTA = ethylenediaminetetraacetic acid.

synthesis of PEG conjugates of the functional oligonucleotides. The antisense sequence to firefly-luciferase mRNA (sequence **A** in Scheme 2) was designed according to a reported technique.<sup>[24]</sup> The methylsulfide derivative of a 2-amino 6-alkyl purine was incorporated into the natural-type ODN (**5a** and **6a**),<sup>[17]</sup> and was then transformed into the phenylsulfide (**5b**, **6b**), 2-carboxyphenylsulfide (**5c**, **6c**), 4-carboxyphenylsulfide (**5d**, **6d**), and phenylamino derivatives (**5e**) by sequential reactions with magnesium monopropylphthalate (MMPP) and aqueous NaOH, following the addition of the corresponding thiol or amine. The amino group of the 3' terminus of **5** was modified with *N*-succinimidyl-3-(2-pyridyldithio)propionate (SPDP), followed by reduction with dithiothreitol (DTT) to form the thiol-containing ODN **7**. Finally,

the  $\beta$ -thiopropionate linkage of the corresponding ODN-PEG conjugates **8** was formed by a Michael reaction with a PEG polymer bearing a diethylacetal group at one terminus and an acrylate group at the other ( $M_n = 4460$ ). The conjugates **8** were isolated in good yields by purification by reversed-phase column chromatography. Sequence **A** has X at the complementary site to the cytidine residue of the target mRNA sequence, **B** contains X at the adjacent site, and **C** is a random sequence. The structures of the ODNs and the PEG-ODN conjugates were confirmed by MALDI-TOF MS. The PEG-ODN conjugates obtained were mixed with poly-L-lysine (PLL) in a 1:1 molar ratio with respect to the phosphate group in the PEG-ODN conjugate and the amino group in PLL to form the PIC micelles (N/P = 1).

Antisense effects of the reactive ODNs (**6A**, R<sup>1</sup> = H) were first evaluated in a transcription and translation assay without cells. The following order was found for the cross-linking rates of the derivatives of 2-aminopurine in the nonbiological system: phenyl sulfoxide > 2-carboxyphenylsulfide > phenylsulfide > methylsulfide.<sup>[17]</sup> However, as the phenyl sulfoxide derivatives were found to be unstable in the biological assay, the sulfide-functionalized ODNs were used in this study. Transcription, followed by translation, of firefly luciferase was performed by using ODN **6** (X = G or a sulfide derivative of 2-aminopurine) and firefly-luciferase DNA in a wheat-germ extract. Production of luciferase was monitored by measuring luminescence (Figure 1). All ODNs **6A** showed dose-dependent antisense inhibition. It should be noted that all sulfide-functionalized ODNs showed higher antisense effects than the corresponding natural antisense ODN.

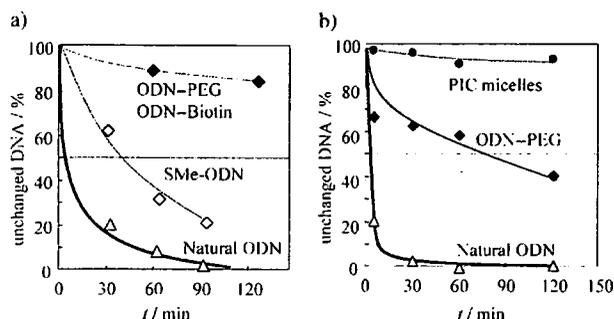


**Figure 1.** Antisense effects on luciferase production in an assay without cells. Transcription and translation were performed in vitro for 90 min at 30°C by using DNA (0.3 μg) in the absence or presence of the ODN in TNT coupled wheat germ extract system (Promega). The extent of luciferase production relative to that in the control is shown in the ordinate.

To check whether or not in situ activation would take place in a cell lysate, we mixed the reactive ODN **6A** (X = AP, R<sup>2</sup> = SMe) and the ODN with the target sequence in a cell lysate, and the reaction mixture was analyzed by HPLC.<sup>[25]</sup> Although the cross-linked adduct was not detected clearly, the formation of 2-amino-6-vinylpurine was evidenced by its intrinsic fluorescence. This vinyl derivative of ODN **6** was

only clearly observed in the presence of the ODN with the target cytidine, which suggests that the activation is dependent on cytidine.

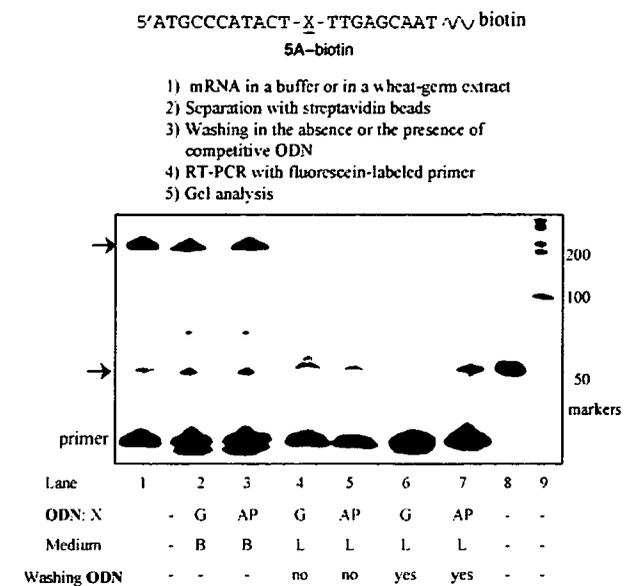
The biological stability of the ODNs was investigated in a cell lysate and in the presence of DNase I. It was shown that the modified ODN **6A** ( $X = \text{AP}$ ,  $R^2 = \text{SMe}$ ) was slightly more stable than the natural ODN **6A** ( $X = \text{G}$ ), and that the PEG conjugate **8A** ( $X = \text{G}$ ) exhibits high stability in a cell lysate (Figure 2 a). To rule out the possibility that the higher activity



**Figure 2.** Comparison of the stability of the ODN derivatives in a cell lysate (a) and in the presence of DNase I (b) at 30°C. Natural ODN: **6A** ( $X = \text{G}$ ), SMe-ODN: **6A** ( $X = \text{AP}$ ,  $R^2 = \text{SMe}$ ), ODN-PEG: **8A** ( $X = \text{G}$ ), ODN-biotin: biotin amide of **5A** ( $X = \text{G}$ ), PIC micelles: B-PEI + **8A** ( $X = \text{G}$ ) ( $N/P = 1$ ; B-PEI:  $M_w = 25\,000$ ,  $DP = 580$ ). B-PEI = branched polyethyleneimine,  $DP = \text{degree of polymerization}$ .

of the modified ODNs is merely an effect of higher biostability induced by the modification, we next investigated the antisense effect of the PEG conjugates. The ODN-PEG conjugates were incubated with mRNA of firefly luciferase for 30 min in a buffer, and translation was performed by treatment with an amino acid mixture in a cell lysate for 90 min at 30°C. It was clearly demonstrated that the PEG conjugate with the modified ODN, **8A** ( $X = 2\text{AP}$ ,  $R^2 = \text{SMe}$ ), exhibited a higher inhibitory effect than the conjugate with the natural ODN, **8A** ( $X = \text{G}$ ), and that the biostability of the modified ODN is not a major factor in the enhancement of the antisense effect.<sup>[25]</sup>

Hybridization of the ODN with the luciferase mRNA following a cross-linking reaction was next investigated by reverse-transcription polymerase chain reaction (RT-PCR). When primers were used for the upstream and downstream regions of the antisense target site, it was expected that the PCR with cross-linked mRNA would produce shorter PCR products than that with unmodified mRNA. First, ODN **6A** ( $X = \text{G}$  or  $2\text{AP}$ ,  $R^2 = \text{SPh}$ ) was mixed with mRNA in a buffer, the mixture was subjected to RT-PCR, and the products were analyzed by gel electrophoresis (Figure 3). The RT-PCR products corresponding to a 223-mer nucleotide of the full length were formed as the major product (Figure 3, lanes 2 and 3), thus indicating that neither covalent nor noncovalent hybridized complexes were formed effectively in a buffer. We next attempted to form complexes with mRNA in cell lysates, so that we could isolate the complexes by using streptavidin affinity beads. mRNA and biotin-conjugated ODN were incubated in a cell lysate, and the hybridized complexes were separated with streptavidin beads. (**5A**-biotin is as biostable

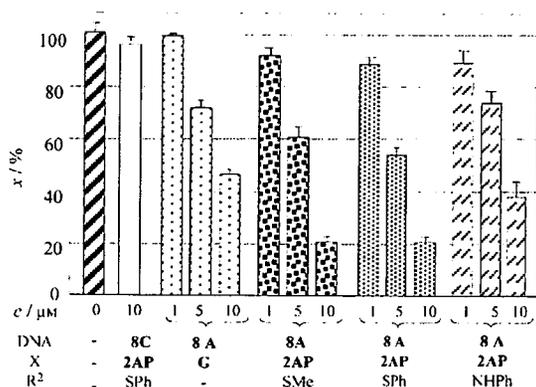


**Figure 3.** Analysis of RT-PCR products (denoted by arrows) of the reaction of the ODN and luciferase mRNA. Lane 1: control; lane 2: ODN **6A** ( $X = \text{G}$ ) and mRNA were subjected to RT-PCR; lane 3: ODN **6A** ( $X = 2\text{AP}$ ,  $R^2 = \text{SPh}$ ) was used in the same procedure as that for lane 2; lane 4: ODN **5A**-biotin ( $X = \text{G}$ ) and mRNA complexes were separated with streptavidin beads then washed several times with a buffer in the absence of the competitive ODN; lane 5: ODN **5A**-biotin ( $X = 2\text{AP}$ ,  $R^2 = \text{SPh}$ ) was used in the same procedure as that for lane 4; lane 6: washing was done in the presence of the competitive ODN in otherwise the same procedure as that used for lane 4; lane 7: washing was done in the presence of the competitive ODN in otherwise the same procedure as that used for lane 5; lanes 8 and 9: markers. G is guanosine and AP is 2-amino-6-(2-phenylthioethyl)purine ( $R^2 = \text{SPh}$ ; see Scheme 2). Medium B is a buffer and medium L is lysate. The ODN **5A**-biotin has a biotin unit at its 3' amino end. The sequence of the washing ODN (21 mer) is the sense sequence 3'-TACGGGTATGAC-AACTCGTTA-5': the target sequence of firefly-luciferase mRNA.

as the ODN-PEG conjugate, as shown in Figure 2 a.) The PCR in which separated streptavidin beads were used afforded shorter RT-PCR products of approximately 50-mer length (Figure 3, lanes 4 and 5), which showed that the RT reaction was inhibited before the site of cross-linking with both the natural ODN ( $X = \text{G}$ ) and the cross-linking ODN ( $X = 2\text{AP}$ ,  $R^2 = \text{SPh}$ ). These results also clearly indicate that tight ODN-mRNA complexes are formed in cell lysates, but not in a buffer, in the case of both the natural and the modified ODN. When the separated streptavidin beads were washed in the presence of the competitive ODN, the short RT-PCR products disappeared in lane 6 and were retained in lane 7, thus demonstrating that covalently bound ODN-mRNA complexes are formed with the cross-linking ODN ( $X = 2\text{AP}$ ,  $R^2 = \text{SPh}$ ). These results, together with the fact that the sulfide derivative is transformed into a 2-amino-6-vinyl-purine derivative in cell lysates,<sup>[25]</sup> strongly suggest that the ODN with the reactive 2-aminopurine residue forms the covalent bond with the target mRNA in the cell lysate to enhance the antisense effect.

We next evaluated the intracellular antisense activity of the reactive ODN-PEG conjugates by a dual-luciferase-

reporter assay. The PIC micelles of the reactive ODN-PEG conjugates exhibit high stability toward enzymatic digestion (Figure 2b) and were therefore used as the system for the delivery of the ODN into the cellular interior.<sup>[21,22,25]</sup> HuH-7 cells were cotransfected with plasmids of firefly and renilla luciferases in amounts at which both luciferases are expressed to a similar extent. The cells were incubated for 24 h in the presence of the reactive ODN-PEG as the PIC micelles and then for 24 h in a fresh medium. The PEG-ODN conjugates were mixed with PLL (poly-L-lysine, DP = 100,  $M_w = 20\,900$ ) with a 1:1 molar ratio of the phosphate group in the PEG-ODN conjugate and the amino group in PLL (N/P = 1) to form the PIC micelles. The luciferase expression was monitored by measuring luminescence with the dual luciferase assay kit. Antisense effects are summarized in Figure 4.

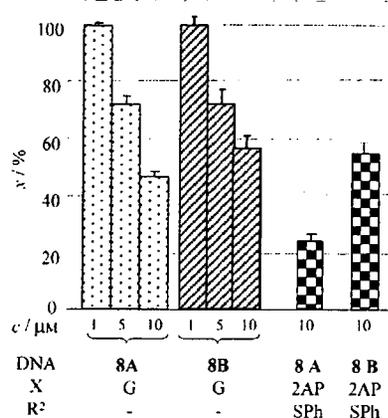


**Figure 4.** Antisense effects with PEG-ODN/PLL PIC micelles against gene expression of firefly luciferase in cultured HuH-7 cells. Normalized ratios  $x$  of firefly-luciferase activity to that of renilla luciferase are shown in the ordinate. G and 2AP represent guanosine and the 2-amino 6-alkyl purine nucleoside; substituents  $R^2$  are shown in Scheme 2.

Neither the antisense ODN **6A** nor the PEG-ODN **8A** alone showed antisense inhibition.<sup>[21,22]</sup> Under conditions under which the PIC micelles of the PEG-ODN **8C** with the random sequence did not show an antisense effect, the micelles of the natural PEG-ODN **8A** clearly showed potent antisense effects of about 50% inhibition at 10  $\mu\text{M}$ . Although higher concentrations of the PIC micelles were needed for potent inhibition than those required in the antisense experiments in cell lysates (compare Figures 1 and 4), the PIC micelles formed with the ODN-PEG conjugates and PLL did not cause cytotoxicity at concentrations of 10  $\mu\text{M}$ .<sup>[25]</sup> Thus, the inhibitory effects summarized in Figure 4 result from selective antisense effects of **8A**. The sulfide derivatives of 2-aminopurine (**8A**, X = AP,  $R^2 = \text{SMe}$  and SPh) showed greater antisense effects than the PEG conjugate with the natural antisense oligonucleotide (**8A**, X = G). At a concentration of 10  $\mu\text{M}$  in the sulfide derivative, luciferase expression was inhibited to 20% of that in the control. This degree of expression corresponds to a background level. The ODN containing the aniline derivative (**8A**, X = AP,  $R^2 = \text{NHPh}$ ) was used as an unreactive antisense control. In the presence of this ODN, less effective antisense inhibition was observed,

which again supports the hypothesis that the 2-aminopurine derivative in the reactive ODN enhances antisense inhibition.

The consequences of one mismatched site on the antisense effects are compared in Figure 5 for the natural antisense



**Figure 5.** Comparison of the consequences of one mismatched site on the antisense effects. Systems were assayed for their antisense effect in a similar way to that described in the footnote of Figure 4. G and 2AP represent guanosine and the 2-amino 6-alkyl purine nucleoside; substituents  $R^2$  are shown in Scheme 2.  $x$  = normalized ratios of firefly-luciferase activity to that of renilla luciferase. Target mRNA sequence: 3'-TACGGGTATGA-C-AACTCGTTA-; antisense sequences: **8A**: 5'-ATGCCCATACT-X-TTGAGCAAT-PEG, **8B**: 5'-ATGCCCATACT-G-XTGAGCAAT-PEG.

ODNs (**8A** and **8B**, X = G) and the reactive ODNs (**8A** and **8B**, X = 2AP,  $R^2 = \text{SPh}$ ). The natural ODN with one mismatched site (**8B**, X = G) exhibited similar inhibition to that of the fully matched ODN (**8A**, X = G), which demonstrates a disadvantage to the use of natural ODNs in terms of antisense inhibition. In marked contrast, the reactive ODN (**8B**, X = AP,  $R^2 = \text{SPh}$ ), in which the 2-aminopurine derivative is at the position adjacent to the target cytidine, was much less efficient than the fully matched ODN (**8A**, X = AP,  $R^2 = \text{SPh}$ ), thus demonstrating the superiority of the reactive ODN over the natural antisense ODN in discriminating a single nucleoside difference in cells. As the 2-amino-6-(2-phenylthioethyl)purine nucleoside is highly selective to cytidine at the complementary position,<sup>[17,18]</sup> the high sensitivity to a single mismatch site can also be reasonably interpreted as a result of selective cross-linking with the 2-amino-6-vinylpurine derivative.

In this study, we have applied the inducible alkylating system that has been developed by our research group to intracellular antisense oligonucleotides. It was found that the sulfide derivative of the 2-amino-6-vinylpurine nucleoside exhibited high reactivity in a cell lysate, in striking contrast to the chemical reactivity investigated in simple model systems. Detailed investigations of the reaction product of the reactive ODN with mRNA in a cell lysate strongly suggest that the reactive ODN that incorporates the 2-aminopurine nucleoside analogue forms a covalent bond with the target mRNA. The reactive ODN was subsequently applied in intracellular antisense inhibition of luciferase by using polyion-complex



(PIC) micelles of the PEG–ODN conjugate. In conclusion, we have successfully shown that the inducible alkylation system functions in an intracellular environment to promote efficient and selective antisense activity against luciferase production. A unique benefit of this alkylating agent is reflected in its high selectivity, which may permit the discrimination of a single nucleotide difference in cells. As the sulfide precursors to the 2-amino-6-vinylpurine derivative, unlike psoralen, do not need UV irradiation for activation, they are expected to be useful for further in vivo applications. This type of inducible alkylation system combined with the delivery system of PIC micelles for PEG conjugates opens new opportunities for the use of reactive oligonucleotides in vivo.

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- [1] K. B. Grant, P. B. Dervan, *Biochemistry* **1996**, *35*, 12313–12319.
- [2] For a recent review, see: S. Sasaki, *Eur. J. Pharm. Sci.* **2001**, *13*, 43–51; see also references therein.
- [3] Q. B. Zhou, S. E. Rokita, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 15452–15457.
- [4] Y. Yamamoto, W. Tsuboi, M. Komiyama, *Nucleic Acids Res.* **2003**, *31*, 4497–4502.
- [5] T. Fukuma, C. M. Walton, C. H. Wu, G. Y. Wu, *Bioconjugate Chem.* **2003**, *14*, 295–301.
- [6] R. N. Zuckermann, D. R. Corey, P. G. Schultz, *J. Am. Chem. Soc.* **1988**, *110*, 1614–1615.
- [7] K. Nagai, S. M. Hecht, *J. Biol. Chem.* **1991**, *266*, 23994–24002.
- [8] S. Sando, H. Abe, E. T. Kool, *J. Am. Chem. Soc.* **2004**, *126*, 1081–1087.
- [9] M. P. van de Corput, R. W. Dirks, R. P. M. van Gijlswijk, E. van Binnendijk, C. M. Hattinger, R. A. de Paus, J. E. Landegent, A. K. Raap, *J. Histochem. Cytochem.* **1998**, *46*, 1249–1259.
- [10] G. Meister, M. Landthaler, Y. Dorsett, T. Tuschl, *RNA* **2004**, *10*, 544–550.
- [11] G. Hutvagner, M. J. Simard, C. C. Mello, P. D. Zamore, *PLoS Biol.* **2004**, *2*, e98.
- [12] A. Murakami, A. Yamayoshi, R. Iwase, J. Nishida, T. Yamaoka, N. Wake, *Eur. J. Pharm. Sci.* **2001**, *13*, 25–34.
- [13] J. M. Kean, A. Murakami, K. R. Blake, D. D. Cushman, P. S. Miller, *Biochemistry* **1988**, *27*, 9113–9121.
- [14] N. Puri, A. Majumdar, B. Cuenoud, P. S. Miller, M. M. Seidman, *Biochemistry* **2004**, *43*, 1343–1351.
- [15] A. Majumdar, A. Khorlin, N. Dyatkina, F. L. Lin, J. Powell, J. Liu, Z. Fei, Y. Khripine, K. A. Watanabe, J. George, P. M. Glazer, M. M. Seidman, *Nat. Genet.* **1998**, *20*, 212–214.
- [16] Q. B. Zhou, S. E. Rokita, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 15452–15457.
- [17] T. Kawasaki, F. Nagatsugi, Md. M. Ali, M. Maeda, K. Sugiyama, K. Hori, S. Sasaki, *J. Org. Chem.* **2005**, *70*, 14–23.
- [18] F. Nagatsugi, T. Kawasaki, D. Usui, M. Maeda, S. Sasaki, *J. Am. Chem. Soc.* **1999**, *121*, 6753–6754.
- [19] F. Nagatsugi, K. Uemura, S. Nakashima, M. Maeda, S. Sasaki, *Tetrahedron* **1997**, *53*, 3035–3044.
- [20] F. Nagatsugi, K. Uemura, S. Nakashima, M. Maeda, S. Sasaki, *Tetrahedron Lett.* **1995**, *36*, 421–424.
- [21] M. Oishi, S. Sasaki, Y. Nagasaki, K. Kataoka, *Biomacromolecules* **2003**, *4*, 1426–1432.
- [22] M. Oishi, F. Nagatsugi, S. Sasaki, Y. Nagasaki, K. Kataoka, *ChemBioChem* **2005**, *6*, 1–6.
- [23] For a similar method, see: J. H. Jeong, S. W. Kim, T. G. Park, *Bioconjugate Chem.* **2003**, *14*, 473–479.
- [24] M. Mishra, J. R. Bennett, G. Chaudhuria, *Biochem. Pharmacol.* **2001**, *61*, 467–476.
- [25] See the Supporting Information.

# Cumulative incidence and changes in the prevalence of vertebral fractures in a rural Japanese community: a 10-year follow-up of the Miyama cohort

Noriko Yoshimura · Hirorufumi Kinoshita ·  
Hiroyuki Oka · Shigeyuki Muraki · Akihiko Mabuchi ·  
Hiroshi Kawaguchi · Kozo Nakamura

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## Abstract

**Introduction** The objective of this study was to clarify secular changes in the prevalence and cumulative incidence of vertebral fractures (VFX) among the general inhabitants of Miyama, a rural Japanese community.

**Methods** A cohort of 1,543 inhabitants aged 40–79 years was established using resident registration in 1989. Of these, 400 participants were selected and divided into four age strata, each containing 50 men and 50 women. Participants completed a self-administered questionnaire and anthropometric measurements were taken. In 1990, baseline radiographic examinations of the thoracolumbar spine in anteroposterior and lateral views were performed. Radiographic examinations of the same sites were performed on the same participants in 2000. All VFX were

diagnosed by the same experienced orthopedic surgeon. Evaluation of radiographic surveys after 10 years was completed for 287 of the 400 participants (132 men, 155 women; 71.8%).

**Results** The prevalences of VFX at follow-up in 2000 for subjects in their 50s, 60s, 70s, and 80s were 2.9%, 10.3%, 13.2%, and 25.0% for men, and 2.1%, 9.1%, 20.5%, and 54.2% for women, respectively. Prevalence in the present study was compared with that collected in 1990, showing improvements over time for men and women in their 50s, 60s, and 70s. Future problems with osteoporotic fractures in Japan might, thus, be less severe than previously predicted. Cumulative incidence over 10 years was detected by the number of incident cases divided by the number of all participants in the initial study. Incident cases were defined by two criteria. First, cases with prevalent VFX were excluded from both the numerators and the denominators. In this analysis, the cumulative incidence of the cases with first VFX was detected. Secondly, the cumulative incidence including the subjects who had suffered from the prevalent fractures and with an increased number of VFX in the following 10 years were calculated. This analysis clarified the cumulative incidence of cases with and without previous VFX. Incidence over 10 years of subjects in their 40s, 50s, 60s, and 70s with first VFX were 2.2%, 4.9%, 5.1%, and 10.8% for men, and 2.1%, 4.5%, 14.0%, and 22.2% for women, respectively. By contrast, if new cases were defined as including subjects who had suffered from previous fractures, but displayed an increased number of VFX, cumulative incidences for subjects in their 40s, 50s, 60s, and 70s were 2.1%, 8.3%, 10.0%, and 12.2% for men, and 2.1%, 6.1%, 18.0%, and 22.4% for women, respectively.

N. Yoshimura · H. Oka  
Department of Joint Disease Research, 22nd Century Medical  
and Research Center, Graduate School of Medicine,  
University of Tokyo,  
Tokyo, Japan

H. Kinoshita  
Department of Orthopaedic Surgery,  
Wakayama Medical University Kihoku Hospital,  
Wakayama, Japan

S. Muraki · A. Mabuchi  
Department of Clinical Motor System Medicine, 22nd Century  
Medical and Research Center, Graduate School of Medicine,  
University of Tokyo, Tokyo, Japan

H. Kawaguchi · K. Nakamura  
Department of Orthopaedic Surgery,  
Faculty of Medicine, University of Tokyo,  
Tokyo, Japan

N. Yoshimura (✉)  
7-3-1 Hongo, Bunkyo-ku,  
Tokyo 113-8655, Japan  
e-mail: yoshimuran-ort@h.u-tokyo.ac.jp

**Keywords** Cohort study · Cumulative incidence · Japanese · Prevalence · Vertebral fractures

## Introduction

Among fractures due to osteoporosis, vertebral fractures (VFX) are the most likely to lead to marked health problems. VFX are reportedly associated with functional impairment [1], back pain [2, 3], kyphosis [2, 3], esophageal reflux [4], depressive mood [5], respiratory dysfunctions [6], and decreased quality of life [7, 8]. In addition, VFX increase the risk of new VFX and hip fractures [9–13]. Furthermore, prevalent VFX reportedly increase mortality risk [14–16].

Although preventive strategies for VFX are urgently needed with the rapid aging of populations such as in Japan, little data is available concerning epidemiological characteristics in population samples, particularly in Japan [17–19]. A population-based cohort was established in 1990, comprising subjects aged 40- to 79-years-old from resident registrations for a village in a mountainous area, Miyama village, and follow-up studies were conducted in 1993, 1997, and 2000 [20–22]. In the previous report of the Miyama study, prevalences of VFX were ascertained for men and women [23].

In the present study, radiographic examination was performed on the same subjects after 10 years to assess secular trends of prevalence and to clarify cumulative incidences among different generations of VFX in a rural Japanese community.

## Participants and methods

Epidemiological research based on populations has been conducted in Miyama village, Wakayama prefecture, Japan. As a detailed profile of the Miyama cohort has already been described elsewhere [20–23], a brief summary is provided here. In this village, a list of inhabitants aged 40- to 79-years-old was compiled based on resident registrations as of the end of December 1988. As a result, a cohort of 1,543 inhabitants (716 men, 827 women) was identified. A self-administered questionnaire survey regarding 125 daily activities such as smoking habits, alcohol consumption, and physical exercise was performed (baseline study).

From the above-mentioned cohort, 50 men and 50 women in each decade of age between 40 and 79 years (for a total of 400 participants) was recruited based on resident registrations for the purpose of measuring bone mineral density (BMD) in 1990 (initial BMD survey). In addition to BMD measurements using dual energy X-ray absorptiometry (DXA; Lunar DPX, GE Medical Systems, Madison, WI, USA), an interviewer-administered questionnaire, medical examination, and physical measurements were also performed for these participants. The questionnaire comprised 70 items, such as past history, history of

medication, smoking habits, alcohol consumption, calcium intake, physical exercise, history of childbirth, history of lactation, and menstrual status. Radiographic examination of the spine was performed on all participants in 1990. Anteroposterior and lateral images of thoracolumbar vertebrae Th5-L5 were used for diagnosis (initial X-ray survey). Radiographic examination was again performed on subjects who provided consent after 10 years. Lateral images of thoracolumbar vertebrae Th5-L5 were again used for diagnosis (2nd X-ray survey).

Lateral spinal radiographs were examined for the presence of one or more VFX between Th5-L5, using the criteria defined by the Japan Bone and Mineral Society (Fig. 1) [24]. According to these criteria, the measurement of anterior, middle, and posterior heights on lateral radiography of the thoracic and lumbar spine is required, in order to determine ratios defining the anterior wedge, biconcave, and compound dimensions of the vertebral bodies. Diagnosis of VFX on all radiographs was performed by the same experienced orthopedic doctor (HK).

Prevalence and cumulative incidence were calculated by utilizing the results of diagnosis. Cumulative incidence is defined simply as the ratio of incident cases to the at-risk population at the beginning of the observation period. In the present study, cumulative incidence over 10 years was, thus, detected as the number of incident cases divided by the number of all participants in the initial study. Incident cases were defined using two criteria. First, cases with previous VFX were excluded from both numerators and denominators. In this analysis, the cumulative incidence of cases with first VFX was detected. Secondly, the cumulative incidence including subjects who had suffered from previous VFX but who displayed an increased number of VFX over the following 10 years was calculated. This analysis clarified the cumulative incidence of cases with and without previous VFX.

In the above-mentioned formula to obtain the cumulative incidence, the denominator was defined as the total number of participants in the initial X-ray survey. Using this definition, drop-outs during the observed 10 years were considered as non-VFX. To avoid underestimation caused

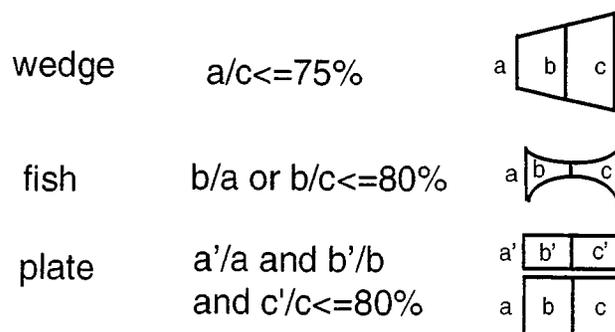


Fig. 1 Diagnostic criteria for vertebral fractures in Japan

by the inclusion of drop-outs, another cumulative incidence was calculated by excluding drop-outs during the study period from the denominator. This index was defined as the cumulative incidence among survivors.

All statistical analyses were performed using STATA statistical software (STATA Corp., College Station, TX, USA).

## Results

We note our findings as follows:

### 1. Eligible participants

Among 400 participants of the initial BMD examination, 390 provided consent to participate in the radiographic examination (194 men, 196 women; 97.5%) of the initial survey. Radiographic evaluation after 10 years was completed for 287 of the 400 participants (132 men, 155 women; 71.8%). The loss of 101 participants was explained as follows: 55 participants died (37 men, 18 women); 16 participants moved (8 men, 8 women); 13 participants were ill (4 men, 9 women); 8 participants

were busy (8 men); 5 participants refused to participate further (5 men); and 4 participants were away from the area at the time of follow-up (1 man, 3 women). Another 12 participants (5 men, 7 women) were excluded from analysis due to the poor quality of radiographic images. Table 1 shows the physical characteristics of the remaining participants at the time of the initial X-ray survey.

### 2. Prevalence of VFX at the survey in 2000 and secular trends 1990–2000

The prevalences of VFX at follow-up in 2000 were calculated. The prevalences of VFX for subjects in their 50s, 60s, 70s, and 80s were 2.9%, 10.3%, 13.2%, and 25.0% for men, and 2.1%, 9.1%, 20.5%, and 54.2% for women, respectively.

Figure 2 shows a comparison of VFX prevalences in the same age group according to the results of radiographic examination in 1990. The prevalences were significantly decreased between the 50s to 70s age groups in 2000 compared to 1990 ( $P < 0.05$ ) (Fig. 2).

### 3. Cumulative incidence of VFX

The cumulative incidences over 10 years for men and women sorted by the presence or absence of VFX at the

**Table 1** Characteristics of participants of the initial survey, classified by sex and age stratum

Birth cohort	Age strata of participants	No. of participants	No. of participants	Anthropometric measurements at the initial survey (mean (SD))				BMD measurements on initial survey (mean (SD))	
				Age (yrs)	Height (cm)	Weight (kg)	BMI (kg/m <sup>2</sup> )	BMD (L2-4)	BMD (femoral neck)
Men	Total	194	132	56.9 (10.3)	161.3 (6.7)	58.2 (9.2)	22.3 (2.5)	1.10 (0.19)	0.88 (0.14)
	1940–49	47	35	44.1 (3.2)	166.3 (5.8)	63.8 (8.4)	23.0 (2.3)	1.18 (0.17)	0.97 (0.15)
	1930–39	50	39	53.9 (2.6) <sup>a</sup>	162.0 (5.8) <sup>a</sup>	60.1 (8.2)	22.8 (2.4)	1.16 (0.18)	0.91 (0.12)
	1920–29	60–69	50	63.2 (2.8) <sup>ab</sup>	159.4 (5.4) <sup>a</sup>	56.1 (7.5) <sup>a</sup>	22.0 (2.4)	1.03 (0.19) <sup>ab</sup>	0.82 (0.12) <sup>ab</sup>
	1910–19	70–79	49	73.3 (2.8) <sup>abc</sup>	154.7 (4.8) <sup>abc</sup>	48.7 (6.8) <sup>abc</sup>	20.3 (2.5) <sup>ab</sup>	1.01 (0.16) <sup>ab</sup>	0.79 (0.10) <sup>ab</sup>
Women	Total	196	155	56.7 (10.1)	149.0 (6.0)	49.7 (8.1)	22.3 (2.8)	0.98 (0.23)	0.76 (0.14)
	1940–49	48	48	44.6 (3.0)	152.6 (4.7)	53.6 (8.3)	22.9 (2.8)	1.18 (0.16)	0.88 (0.12)
	1930–39	50	44	54.8 (2.7) <sup>a</sup>	149.9 (5.1)	50.1 (7.1)	22.3 (2.6)	0.99 (0.18) <sup>a</sup>	0.75 (0.12) <sup>a</sup>
	1920–29	60–69	50	64.3 (2.8) <sup>ab</sup>	147.4 (5.1) <sup>a</sup>	47.5 (6.9) <sup>a</sup>	21.9 (3.0)	0.85 (0.20) <sup>ab</sup>	0.69 (0.11) <sup>a</sup>
	1910–19	70–79	49	71.6 (1.8) <sup>abc</sup>	142.5 (5.6) <sup>abc</sup>	44.5 (7.6) <sup>ab</sup>	21.9 (3.0)	0.78 (0.16) <sup>ab</sup>	0.64 (0.09) <sup>ab</sup>

<sup>a</sup> Significantly different from values of the birth cohort group born in 1940–49

<sup>b</sup> Significantly different from values of the birth cohort group born in 1930–39

<sup>c</sup> Significantly different from values of the birth cohort group born in 1920–29