れ以外の糖アルコールは口腔内細菌によって発酵されない。表4の糖アルコールはどれも非う蝕誘発性（あるいは極めて低う蝕誘発性）であることが動物実験で確かめられている[21]。

表2. フッ化物を使用している国のスクロース消費量とう蝕リスクの間の因果関係

歯列	研究デザイン	研究水準スコア	関係の強弱
永久歯	断面調査	60	中等度
永久歯	断面調査	72	弱い
永久歯	断面調査	62	弱い
永久歯	コホート調査	66	弱い
永久歯	断面調査	77	弱い
永久歯（根面う蝕）	症例	59	中等度
乳歯	断面調査	66	弱い
乳歯	コホート調査	59	中等度
永久歯	断面調査	74	中等度
乳歯	断面調査	63	弱い
乳歯	コホート調査	72	弱い
乳歯	コホート調査	70	強い
永久歯	断面調査	55	弱い
乳歯	断面調査	58	弱い
乳歯	断面調査	70	中等度
永久歯	断面調査	79	中等度
永久歯	断面調査	63	中等度
乳歯	断面調査	67	弱い
永久歯	コホート調査	67	中等度
永久歯	断面調査	68	弱い
永久歯	断面調査	55	弱い
永久歯	断面調査	72	弱い
永久歯	断面調査	64	弱い
乳歯	コホート調査	70	中等度
乳歯	コホート調査	60	中等度
乳歯	断面調査	58	中等度
永久歯（根面う蝕）	断面調査	61	中等度
永久歯	断面調査	65	強い
乳歯	コホート調査	57	中等度
永久歯	コホート調査	75	弱い
乳歯	断面調査	60	中等度
乳歯	断面調査	58	中等度
永久歯	断面調査	59	弱い
永久歯	コホート調査	66	弱い
乳歯	コホート調査	71	中等度
乳歯	コホート調査	59	弱い

【関係の強弱】強い：オッズ比（リスク比）＝2.5以上、中等度：オッズ比（リスク比）＝1.5～2.4、弱い：オッズ比（リスク比）＝1.4以下（文献18より改変）

表3. 各種オリゴ糖の性状

	酸産生基質	糖代謝阻害	グルカン生成基質	GTF阻害	う蝕誘発性	う蝕抑制	一過性下痢	エネルギー値（kcal/g）
スクロース	++	−	++	−	++	−	−	4
パラチノース	−	−	−	+	−	±	−	4
トレハルロース	−	−	−	+	−	±	−	
トレハロース	(±)	−	−	±	(−)		−	4
キシロシルフルクトシド	(±)	−	−	++	(−)	(+)	−	
カップリングシュガー	+	−	±	+	±	±	−	4
イソマルトオリゴ糖	+	−	−	+	±	±	−	3
パノースオリゴ糖	+	−	−	+	±	±	−	3

（ ）は検討中を、空欄は不明を意味する

単糖類のグルコース、フルクトースは発酵性の糖質であるため、一度プラークが形成されてしまうとプラーク内で資化されてpHをエナメル質脱灰に十分な値にまで低下させる。グルコースのう蝕誘発性はスクロースに比べれば弱いながら実験動物にう蝕を発生させる。その割合はスクロースの約25%程度である[20]。また、サルやヒトでの研究の中には、スクロースとフルクトースの間、スクロースと異性化糖（グルコースとフルクトースの等量混合物）との間ではう蝕誘発性に差がないという報告もある[22]。前述のTurku市でのヒトでの2年間の疫学研究でも1年目まではスクロースとフルクトースのう蝕誘発性に差はみられなかったが、2年目にはフルクトースのう蝕誘発性はスクロースのそれの約1／2であった。

前出のKoulouridesら[13]はICT法により9種類の糖質のう蝕誘発性を調べた結果、キシリトールとキシロースは非う蝕誘発性であり、マンニトール、ソルビトール、乳糖、メリビオースは低う蝕誘発性であり、グルコース、フルクトース、ラフィノースはう蝕誘発性だとした。

以上のように、スクロース以外の発酵性糖質、あるいは発酵性糖質を含む混合糖はスクロースほど強くないにしても明らかにう蝕誘発性であるといえる。一方、糖アルコールのう蝕誘発性は一般に極めて低いと考えて差し支えないと思われる。

表4. 各種糖アルコールの性状

	酸産生	糖代謝阻害	グルカン生成	GTF阻害	う蝕誘発性	再石灰化	一過性下痢	最大無作用量 (g/kg体重)	エネルギー量 (kcal/g)
エリスリトール	−	−	−	−	−	−	−	0.66〜0.80	0.3(0)
キシリトール	−	±	−	−	−	+	+	*	2.8〜3.6(3)
ソルビトール	±	±	−	−	−	+	+	0.15〜0.3	2.8〜3.0(3)
マンニトール	±	−	−	−	−	+	+		2.0〜2.1(2)
マルチトール	−	−	−	−	−	+	+	0.3	1.8〜2.9(2)
パラチニット	−	−	−	−	−		+	0.3	1.2〜1.6(2)

*適応が起こると1日あたり90g（約1.6g/kg体重）まで安全であるとされる

表5. 糖アルコールについての長期の疫学的臨床研究

試験地域	試験時期	期間(年)	キシリトール摂取量 (g/day)	う蝕発症の減少率 (対照群との比較)
フィンランド　トゥルク	1972〜1974	2	67	キシリトール入り食事:85% フルクトース入り食事:30% (スクロース入り食事)
フィンランド　トゥルク	1973〜1974	1	6.7	キシリトールガム:85% (スクロースガム)
旧ソ連　カザン共和国	1975〜1977	2	30	キシリトール菓子:73% (スクロース菓子)
タイ(WHO)	1970年代後半	3	5〜7	フッ素含有キシリトールガム :0.2% NaF洗口とほぼ同等の効果
フランス領ポリネシア(WHO)	1980年代初期	2.7	14〜20	キシリトールガム:37% (非摂取)
ハンガリー(WHO)	1980年代前半	2〜3	14〜20	キシリトールガム:35〜45% (フッ化物含有牛乳)
カナダ　モントリオール	1980年代前半	1	3.4	キシリトールガム:54〜59% (非摂取)
フィンランド　ユリビエスカ	1982〜1985	2〜3	10	キシリトールガム:30〜80% (非摂取)
ベリーズ共和国	1989〜1993	2〜3.3	4.3〜9	100%キシリトール粒状ガム:71% (非摂取)
エストニア	1994〜1997	2.3	5	キシリトール＋マルチトール キャンデー:33〜59% (非摂取)
		3	5	キシリトールガム:53.5% (非摂取)

4. ミュータンスレンサ球菌の糖代謝

スクロースのう蝕誘発性はミュータンスレンサ球菌のもつ2つの性質によって説明されている[23]。1つはミュータンスレンサ球菌がスクロースをGTF（グルコシルトランスフェラーゼ）の基質として利用し、粘着性の強い非水溶性グルカンを合成してバイオフィルム形成を促進することであり、もう1つはミュータンスレンサ球菌が細胞膜に存在する糖輸送系（ホスホエノールピルビン酸依存糖ホスホトランスフェラーゼ系：PTS）によってスクロースをリン酸化しながら細胞内に取り込み、一旦加水分解してからエムデン・マイヤーホフ経路を経て最終的には有機酸（乳酸、ギ酸、酢酸など）にまで代謝し、歯質の脱灰を起こすことである。

ミュータンスレンサ球菌は他の発酵性糖質（グルコース、フルクトース、マンノース、マルトースなど）もPTSによって細胞内に取り込み、スクロース同様、最終的には有機酸にまで代謝してプラークpHを低下させ、条件が整えば歯質の脱灰を惹起させる。しかし、これらの発酵性糖質はGTFの基質にはならない。その点でこれらの発酵性糖質のう蝕誘発性は一般にスクロースのもつう蝕誘発性よりも弱いと考えられている。

5. 糖質によるう蝕予防

スクロースの替わりにう蝕誘発性のない（またはごく弱い）代用甘味料を用いてう蝕を予防することが可能かどうかを調べた研究がある[24]。表5はキシリトールについての長期の疫学的臨床研究の結果を改変してまとめたものである。この中には前述のTurku市での調査も含まれている。表から分かるように、キシリトールに関しては1970年代前半から1990年代後半まで継続的に調査が行われてきた。食事の甘味料を全てキシリトールに置換した研究のほか、キシリトール含有チューインガムを摂取する研究、キシリトール菓子やキシリトールキャンデーを摂取する研究などである。1年～3.3年の長期間摂取し、う蝕発症の減少率を対照群と比較している。その結果、キシリトール含有食品の摂取によりう蝕発症が30～85%減少しており、う蝕予防におけるキシリトールの有効性が示されている。

キシリトールをラットに摂取させた研究でも対照群に対するう蝕減少率は25～100%であった[25]。

以上の結果は食品中にキシリトールを用いることによってう蝕の発生を予防できることを示唆している。

近年、日本において再石灰化能のある糖質食品素材が2種開発され、実際にそれらを含んだガムが創出され、厚生労働省許可の特定保健用食品として市販されている[26]。2種の食品素材とは、1つは紅藻フクロノリから抽出されたフノランという多糖類であり、もう1つは馬鈴薯でんぷんから調製されたリン酸化オリゴ糖である。エナメル質再石灰化能をもつキシリトールをベースとしてフノランとリン酸一水素カルシウムを含むチューインガムからの抽出液は、あらかじめ表層下脱灰させたエナメル質歯片の再石灰化を促進することが示された[27]。また、やはりキシリトールをベースとして馬鈴薯でんぷんの加水分解産物でリン酸化されたオリゴ糖のカルシウム塩を含むチューインガムを摂取し、そのときの唾液に表層下脱灰させたエナメル質歯片を浸漬するとエナメル質の再石灰化が促進されること[28]、また、同チューインガムを摂取するとヒト口腔内に装着した脱灰エナメル歯片の再石灰化が促進されることが示された[29]。これら2種の糖質の再石灰化作用のメカニズムはまだ十分に解明されていないが、いずれもカルシウムを可溶化する作用をもつので、これらの糖質はカルシウムイオンと結合することにより、カルシウムがリン酸と沈殿物を形成してしまうのを防ぎ、脱灰部位へカルシウムを運ぶと推察されている。

上記2種のチューインガムは機能性糖質を含む食品として2001年と2003年にそれぞれ特定保健用食品に許可されたが、これらとは別に再石灰化促進作用をもつペプチドを含むチューインガムも2000年に特定保健用食品として許可されている。このチューインガムは牛乳に含まれるカゼイン由来のカゼインホスフォペプチド（CPP）と非結晶性リン酸カルシウムの複合体[30]を再石灰化促進因子として含んでいる。現在までに再石灰化を促す食品として特定保健用食品に許可されているのは3種の食品であるが、異なる構造をもつ糖質とペプチドが同じ機能を発揮しており、今後さらに別の再石灰化促進食材が開発されてくる可能性は高い。

しかし、これら再石灰化促進作用をもつ食品がう蝕予防にどれだけ貢献できるかはまだ調査されていない。今後その点の検討が必要である。

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【食品による齲蝕予防】

Prevention of Dental Caries by Functional Foods

今井　奨

Imai　Susumu

Key words
Dental caries, Functional food, Sucrose, Sugar substitute, Xylitol

Abstract

齲蝕は多因子性疾患であり，その発症には宿主因子，食餌因子，細菌因子，時間因子が関わっている。食餌因子のなかでも特にスクロースの役割は大きい。スクロース含有食品をコントロールすれば完全とはいかないまでも齲蝕を制御できる可能性はある。スクロースの齲蝕誘発性，代用甘味料の齲蝕誘発性について考え，食品による齲蝕予防という概念が実際に有効であるかを考察した結果，キシリトールをはじめとする糖アルコールを中心とした非齲蝕誘発性甘味料配合の食品を上手に摂取することにより齲蝕を幾分かは予防できる可能性が示された。我が国では特定保健用食品の制度が導入され，食品によって疾病を予防しようとする動きが活発になってきており，再石灰化を促進するような機能性食品も創出されて特定保健用食品として許可されている。今後は新機能の再石灰化促進作用をもつ食品群が齲蝕予防にどれだけ貢献できるか，臨床試験等での調査が期待される。

はじめに

キシリトールが日本で食品添加物として厚生省（当時）に認可されたのが1997年4月である。それを4半世紀ほどさかのぼる1970年代初頭にはすでにスウェーデンのTurku市で大掛かりなキシリトールについての疫学研究が始まっていた。食品中のスクロースをすべてキシリトールに置き換えて，2年間にわたり齲蝕歯面数を追跡調査したものである。結果は明らかで，スクロース群の齲蝕歯面数が増加の一途を辿るのに対してキシリトール群では新たな齲蝕歯面はほとんど観察されなかった。この結果は，スクロースが齲蝕発生と密接に関連していること，スクロースを非発酵性の代用甘味料キシリトールに置き換えると齲蝕はほとんど発生しないことを如実に示しており，その後の代用甘味料研究の端緒となったともいえる。

齲蝕は多因子性疾患であり，その発症には宿主因子，食餌因子，細菌因子，時間因子が関わっている[1]。特に食餌因子のなかでもスクロースの役割は大きい。したがって，食品をコントロールすれば完全とはいかないまでも齲蝕を制御できる可能性はある。本稿では，はじめにスクロースの齲蝕誘発性，代用甘味料の齲蝕誘発性に触れ，食品による齲蝕予防という概念が実際に有効であるのかについて考えてみたい。

1．スクロースの齲蝕誘発性

スクロースの齲蝕誘発性は多くの証拠によってゆるぎないものになっている。たとえば証拠の一つに疫学調査がある[2]。我国の一人当たりの年間スクロース消費量が第2次世界大戦時に激減するのに呼応して子供の齲蝕罹患率が激減し，戦後スクロース消費量が増加するとそれに伴って齲蝕罹患率も増加している。これは間接的な証拠で，これをもって直ちにスクロースと齲蝕の関係を結論づけることはできないが，齲蝕がスクロース消費量という環境要因によっても影響されることを示唆するものである。スクロースと齲蝕の関係をより直接的に明らかにした調査にオーストラリアの孤児院Hopewood Houseでの研究，スウェーデンのVipeholm精神病院での研究，前出のスウェーデンのTurku市での研究などがある。これらはいずれもヒト被験者による複数年にわたる特定の食品の摂取により齲歯数，あるいは齲蝕歯面数がどのように変化していくかを調べたものである。スクロースを多く含んだ食品を摂取したグ

国立保健医療科学院口腔保健部：Department of Oral Health, National Institute of Public Health
〒351-0197　埼玉県和光市南2-3-6　Fax: 048-458-6288　E-mail: simai@niph.go.jp

ループでは齲蝕の増加が顕著であるが，スクロースを含まない食品を摂取したグループでの齲蝕発生は非常に低いことが示された。また，Vipeholm研究で示唆されたように，同じスクロース含有おやつを摂取するにしても3度の食事時に食べた方が食間の自由な時間に食べるよりも齲蝕の発生が少ないこともわかった。齲蝕予防には何を食べるかも重要であるが，どのように食べるか，摂取方法も重要であることが示唆された。事実，Weissら[3]は，就学前の5～6歳児について調べ，スナックなどを間食する回数が増えるほど，齲歯数が多くなることを示している（図）。動物実験においても齲蝕誘発食をラットに規則的に与えた方が自由に食べさせた場合より齲蝕スコアが有意に低いことが報告されている[1]。

スクロースの齲蝕誘発性は実験動物による検討[5]やヒト被験者によるIntraoral Cariogenicity Test[6]でも確かめられている。

スクロースが齲蝕誘発性を発揮するメカニズムは次のように理解されている[7]。スクロースは齲蝕原性細菌のミュータンスレンサ球菌をはじめとする口腔内細菌表層に存在する糖輸送系によって菌体内に取り込まれ，EM経路を経由して最終的にはエナメル質脱灰性の乳酸，ギ酸，酢酸などの有機酸に代謝されると同時に，ミュータンスレンサ球菌のグルコシルトランスフェラーゼ(GTF)の基質となって粘着性の強い非水溶性グルカンへと変換され，バイオフィルム形成を促進する。スクロースはこの2つの性状により齲蝕誘発性を発揮すると考えられている。

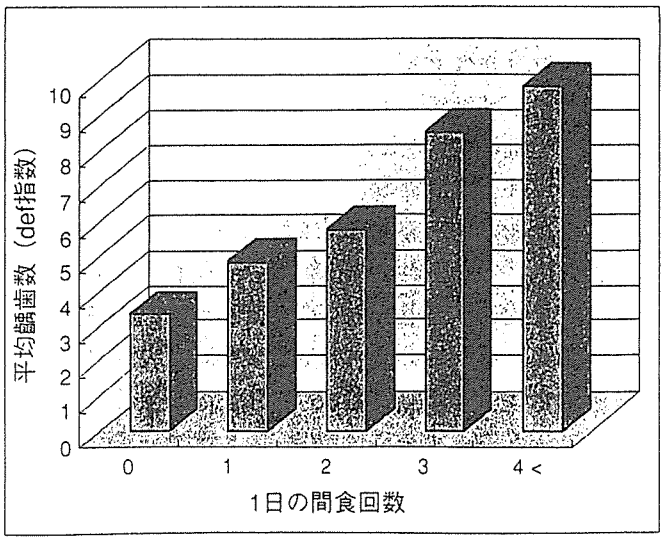

図　1日の間食回数と平均齲歯数との関係

2．代用甘味料の齲蝕誘発性

代用甘味料には合成品，単糖，オリゴ糖，糖アルコール，その他と多数知られているが，特にオリゴ糖，糖アルコールに重要なものが多い。オリゴ糖のパラチノース，トレハルロースは酸産生基質にもグルカン生成基質にもならず，GTF阻害活性をもち，齲蝕誘発性をもたない。カップリングシュガー，イソマルトオリゴ糖，パノースオリゴ糖はいくつかの糖質の混合物でGTF阻害活性をもつが発酵性糖質も含んでいるのでスクロースより弱いが酸産生基質になり，弱い齲蝕誘発性をもつ。一方，糖アルコールのエリスリトール，キシリトール，ソルビトール，マンニトール，マルチトール，パラチニットはいずれもグルカン生成基質にならず，齲蝕誘発性をもたない。ソルビトール，マンニトールを除いて酸産生基質にもならない。ソルビトール，マンニトールはミュータンスレンサ球菌に利用されて酸を産生するがその速度は非常に遅い。キシリトール，ソルビトール，マンニトール，マルチトールには再石灰化作用が報告されている。

3．キシリトール含有食品による齲蝕予防

ラットに*Streptococcus mutans*，または*S. mutans*と*Actinomyces viscosus*を植菌して主食にスクロース含有食を与え，間食にスターチ，またはスクロース含有食を与えた群を対照とし，間食にキシリトール含有食を与えた群と比較した多くの研究では，キシリトール群に25～95%の齲蝕抑制が観察されている[8]。

表1はキシリトールの長期疫学臨床研究の結果を示している。1970年代前半から1990年代後半まで継続して調査が行われてきた。前述のTurku市での調査のように食事に含まれる甘味料をすべてキシリトールに置き換えた研究，あるいはキシリトール菓子を摂取する研究，キシリトール含有チューインガムを摂取する研究，キシリトール入り粒状ガムを摂取する研究などが北欧，東南アジア，旧ソ連，北米などで1～3.3年の長期にわたり行われた。その結果，キシリトール入り食事摂取群では対照のスクロース入り食事摂取群に比べ2年間に約85%の齲蝕発症の減少が観察された。キシリトール入りガム摂取の場合には対照を非摂取群とすると，30～80%の

表1　キシリトールに関する長期の疫学的臨床研究

試験地域	試験時期	期間（年）	齲蝕発症の減少率 （対照群との比較）
フィンランド　トゥルク	1972-1974	2	キシリトール入り食事：85% フルクトース入り食事：30%（スクロース入り食事）
フィンランド　トゥルク	1973-1974	1	キシリトールガム：85%（スクロースガム）
旧ソ連　カザン共和国	1975-1977	2	キシリトール菓子：73%（スクロース菓子）
タイ(WHO)	1970年代後半	3	フッ素含有キシリトールガム：0.2% NaF 洗口とほぼ同等の効果
フランス領ポリネシア(WHO)	1980年代初期	2.7	キシリトールガム：37%（非摂取）
ハンガリー(WHO)	1980年代前半	2-3	キシリトールガム：35-45%（フッ化物含有牛乳）
カナダ　モントリオール	1980年代前半	1	キシリトールガム：54-59%（非摂取）
フィンランド　ユリビエスカ	1982-1985	2-3	キシリトールガム：30-80%（非摂取）
ベリーズ共和国	1989-1993	2-3.3	100%キシリトール粒状ガム：71%（非摂取）
エストニア	1994-1997	2.3	キシリトール＋マルチトールキャンデー：33-59%（非摂取）
		3	キシリトールガム：53.5%（非摂取）

減少率が，また，対照をスクロース入りガム摂取群とすると，85%の減少率が観察されている。キシリトール菓子摂取群はスクロース菓子摂取群に比べ73%の齲蝕の減少率を示した。また，フッ素含有キシリトールガム摂取は0.2% NaF洗口とほぼ同等の齲蝕抑制効果を示すことが示された。このように，スクロース含有食品を対照とすると大きな齲蝕発症減少率(73～83%)が得られており，非摂取群を対照とした場合でも研究によりその減少率には幅(30～80%)があるが，いずれの研究でもキシリトール含有食品の摂取により対照よりも顕著に齲蝕発症を抑制することが示唆されている。

van Loveren[9]は糖アルコールの齲蝕予防効果，齲蝕治療効果についての総説の中で，糖アルコールの種類を問わず1日3回以上シュガーレスガムを噛むことにより齲蝕罹患率が低減すること，ポリオール（糖アルコールはこの仲間にはいる）含有ガムまたはポリオール含有キャンデーの齲蝕予防効果はポリオール自身の効果よりも噛むプロセスによって唾液分泌を促すことが効果につながると述べている。ただし，糖アルコールの再石灰化作用は示唆されているが臨床試験では明確ではなく，糖アルコールの齲蝕治療効果については証拠なしとしている。

4．機能性食品による齲蝕予防

近年，厚生労働省許可の特定保健用食品として再石灰化作用をもつチューインガムが3種市販されている[10]。日本で開発された機能性食品素材であり，紅藻フノリから抽出されたフノランまたは馬鈴薯デンプンから調製されたリン酸化オリゴ糖を含むガム，およびオーストラリアで牛乳から調製された食品素材のカゼインホスフォペプチド(CPP)を含むガムである（表2）。エナメル質再石灰化能をもつキシリトールをベースとしてフノランとリン酸一水素カルシウムを含むチューインガムからの抽出液は，あらかじめ表層下脱灰させたエナメル質歯片の再石灰化を促進することが示されている。また，リン酸化オリゴ糖カルシウム塩を含むチューインガムもキシリトールをベースとしており，これを摂取したときに得られる唾液に表層下脱灰させたエナメル質歯片を浸漬するとエナメル質の再石灰化が促進されること，また，ヒト口腔内に装着した脱灰エナメル歯片の再石灰化も同チューインガムを摂取することにより促進されることが示されている。また，CPPは非結晶性リン酸カルシウムの共存下で複合体を形成するが，キシリトールをベースとしてこの複合体を含むガムも脱灰エナメル歯片の再石灰化を促進することが観察されている。

これら3種のガムの再石灰化作用のメカニズムはまだ十分に解明されていないが，いずれもカルシウムを可溶化する作用をもつので，それぞれの機能性素材はカルシウムイオンと結合することにより，カルシウムがリン酸と沈殿物を形成してしまうのを防ぎ，脱灰部位へカルシウムを運ぶ役割をもつと推定されている。上記ガムを含め歯科関連の特定保健用食品は現在31品目で，まだ特定保健用食品の総数の6.5%にすぎない。

表2　再石灰化促進物質

	再石灰化物質（由来）	化学物質名	補助物質	構　造	作　用
1	糖アルコール	キシリトール ソルビトール マンニトール		OH基を複数個もつ 糖アルコール	カルシウム可溶化 エナメル質再石灰化
2	ガゼインホスフォペプチド（牛乳）	ガゼインホスフォペプチド	非結晶性リン酸カルシウム	アミノ酸残基21～25個にリン酸基4～5個結合	カルシウム吸収促進 カルシウム可溶化 エナメル質再石灰化 齲蝕抑制
3	フノリ抽出物（紅藻類フノリ）	フノラン	第2リン酸カルシウム	多糖類	カルシウム可溶化 エナメル質再石灰化
4	リン酸化オリゴ糖（馬鈴薯デンプン）	リン酸化オリゴ糖カルシウム		グルコース残基3～7個のマルトオリゴ糖にリン酸基1～2個結合	pH緩衝作用 カルシウム可溶化 エナメル質再石灰化

おわりに

スクロースの齲蝕誘発性，代用甘味料の齲蝕誘発性について考察し，食品による齲蝕予防という概念が実際に有効であるのかについて考えてきたが，糖アルコールを中心とした非齲蝕誘発性甘味料配合の食品を上手に摂取することにより齲蝕を幾分かは予防できる可能性が示されたと思われる。今後は新機能の再石灰化促進作用をもつ食品が齲蝕予防にどれだけ貢献できるか，臨床試験等での調査が期待される。

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p53 gene status and expression of p53, MDM2, and $p14^{ARF}$ proteins in ameloblastomas

Hiroyuki Kumamoto[1], Takashi Izutsu[1], Kousuke Ohki[1], Nobuhiro Takahashi[2], Kiyoshi Ooya[1]

[1]Division of Oral Pathology, Department of Oral Medicine and Surgery, and [2]Division of Oral Ecology and Biochemistry, Department of Oral Biology, Tohoku University Graduate School of Dentistry, Sendai, Japan

BACKGROUND: To clarify the roles of the p53–MDM2–$p14^{ARF}$ cell cycle regulation system in oncogenesis and cytodifferentiation of odontogenic tumors, *p53* gene status and expression of p53, MDM2, and $p14^{ARF}$ proteins was analyzed in ameloblastomas as well as tooth germs.

METHODS: Paraffin sections of 16 tooth germs and 46 benign and 5 malignant ameloblastomas were examined immunohistochemically for the expression of p53, MDM2, and $p14^{ARF}$ proteins. Frozen tissue samples of 10 benign ameloblastomas and 1 malignant (metastasizing) ameloblastoma were analyzed by direct DNA sequencing to detect *p53* gene alteration.

RESULTS: Immunohistochemical reactivity for p53 was detected in 2 of 13 tooth germs, 13 of 29 ameloblastomas, and 5 of 5 malignant ameloblastomas, and the expression ratio of p53 in tooth germs was significantly lower than those in benign and malignant ameloblastomas. Direct DNA sequencing showed no alteration of *p53* gene exons 5–8 in any sample of 10 benign ameloblastomas and 1 metastasizing ameloblastoma. Expression of MDM2 and $p14^{ARF}$ was detected in all samples of normal and neoplastic odontogenic epithelium, and the expression ratios in tooth germs tended to be lower than those in benign and malignant ameloblastomas. In ameloblastomas, expression of p53, MDM2, and $p14^{ARF}$ was significantly higher in plexiform cases than in follicular cases. Markedly decreased reactivity for p53, MDM2, and $p14^{ARF}$ was detected in keratinizing and granular cells in ameloblastoma subtypes. Basal cell ameloblastoma showed slightly higher reactivity for p53, MDM2, and $p14^{ARF}$ as compared with other subtypes.

CONCLUSION: Elevated expression of p53, MDM2, and $p14^{ARF}$ in benign and malignant ameloblastomas suggests that alteration of the p53–MDM2–$p14^{ARF}$ cascade is involved in oncogenesis and/of malignant transformation of odontogenic epithelium. *p53* gene status implied that *p53* mutation might play a minor role in neoplastic changes of odontogenic epithelium. Immunoreactivity for p53, MDM2, and $p14^{ARF}$ in ameloblastoma variants suggests that these factors might be associated with tissue structuring and cytodifferentiation of ameloblastomas.

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Keywords: ameloblastoma; MDM2; $p14^{ARF}$; p53

Correspondence: Hiroyuki Kumamoto, Division of Oral Pathology, Department of Oral Medicine and Surgery, Tohoku University Graduate School of Dentistry, 4-1 Seiryo-machi, Aoba-ku, Sendai 980-8575, Japan. Tel.: +81 22 717 8303. Fax: +81 22 717 8304. E-mail: kumamoto@mail.tains.tohoku.ac.jp
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Introduction

Tumors arising from epithelium of the odontogenic apparatus or from its derivatives or remnants exhibit considerable histologic variation and are classified into several benign and malignant entities (1–4). Ameloblastoma is the most frequently encountered tumor arising from odontogenic epithelium and is characterized by a benign but locally invasive behavior with a high risk of recurrence (1, 2, 4). Histologically, ameloblastoma shows considerable variation, including follicular, plexiform, acanthomatous, granular cell, basal cell, and desmoplastic types (1). Malignant ameloblastoma is defined as a neoplasm in which the pattern of an ameloblastoma and cytologic features of malignancy are shown by the primary growth in the jaws and/or by any metastatic growth (1). Recently, malignant ameloblastoma has been subclassified into metastasizing ameloblastoma and ameloblastic carcinoma on the basis of metastatic spread and cytologic malignant features (3). Several recent studies have detected genetic and cytogenetic alterations in these epithelial odontogenic tumors (5–8); however, the detailed mechanisms of oncogenesis, cytodifferentiation, and tumor progression remain unknown.

A series of genetic alterations appears to promote the development of tumors via multiple steps (9, 10). *p53* gene is well recognized as a tumor suppressor gene situated on chromosome 17p13 and is one of the most frequently altered genes in tumors (11–13). Its gene product is a transcriptional factor that plays an important role in response to cellular DNA damage by inducing either G1/S cell cycle arrest to allow DNA repair or apoptosis if DNA has suffered irreversible damage (13, 14). Mutation and loss of heterozygosity

(LOH) of *p53* gene and/or accumulation of p53 product protein have been associated with increased cellular proliferation and malignant transformation (10, 12, 15–21). *MDM2* gene, mapped to chromosome 12q13-14, was originally identified as a highly amplified gene in a transformed tumorigenic fibroblast cell line (22). Its product protein forms a tight complex with both wild- and mutant-type p53 protein and inactivates wild-type p53 function (23). Amplification of *MDM2* gene and overexpression of its product protein have been reported to be involved in tumorigenesis or tumor development in several human malignancies (7, 17, 19, 24). *p14ARF* gene is located at the INK4a/ARF locus on chromosome 9p21, a region with a high rate of LOH in human tumors, and shared exons with *p16INK4a* gene, which encodes a cyclin-dependent kinase inhibitor, in an alternative reading frame (25, 26). p14ARF product directly interacts with MDM2 and neutralizes MDM2-mediated inhibition of p53 (27). Certain tumors have shown *p14ARF* gene alterations, including LOH, mutation, and hypermethylation (21, 28, 29).

Our previous studies confirmed cellular kinetics, including proliferation and cell death modulators, in tooth germs and ameloblastomas, suggesting that these factors are associated with oncogenesis or cytodifferentiation of odontogenic epithelium (26, 30–34). Several studies have examined alteration of *p53* gene and expression of p53 and MDM2 proteins in specific odontogenic tumors (7, 8, 18, 35). In the present study, the immunohistochemical expression of p53, MDM2, and p14ARF proteins and mutation of *p53* gene was examined in ameloblastomas as well as in tooth germs to clarify the possible role of p53 and its upstream regulators in epithelial odontogenic tumors.

Materials and methods

The study protocol was reviewed and approved by the Research Ethics Committee of Tohoku University Graduate School of Dentistry.

Tissue preparation

Specimens were surgically removed from 51 patients with epithelial odontogenic tumor at the Department of Oral and Maxillofacial Surgery, Tohoku University Dental Hospital, and affiliated hospitals. The specimens were fixed in 10% buffered formalin for one to several days and were embedded in paraffin. The tissue blocks were sliced into 3-μm thick sections for routine histologic and subsequent immunohistochemical examinations. Tissue sections were stained with hematoxylin and eosin for histologic diagnosis according to the WHO histologic typing of odontogenic tumors (1). The tumors comprised 46 ameloblastomas and 5 malignant ameloblastomas. Ameloblastomas were divided into 29 follicular and 17 plexiform types, including 17 acanthomatous, 5 granular cell, 3 basal cell, and 4 desmoplastic subtypes. Malignant ameloblastomas were classified into two metastasizing ameloblastomas and three ameloblastic carcinomas according to the criteria provided by Eversole (3). For direct DNA sequencing, tumor tissues were immediately frozen on dry ice and stored at −80°C. Specimens of 16 tooth germs of the mandibular third molars, enucleated for orthodontic reasons at the stage of crown mineralization, were similarly prepared and compared with the epithelial odontogenic tumors.

Immunohistochemistry for p53, MDM2, and p14ARF expression

The tissue sections were deparaffinized and immersed in methanol with 0.3% hydrogen peroxide. For antigen retrieval, the sections were heated in 0.01 M citrate buffer (pH 6.0) for 10 min by autoclave (121°C, 2 atm). After treatment with normal serum for 30 min, the sections were incubated with primary antibodies at 4°C overnight. The applied antibodies were mouse anti-p53 monoclonal antibody (Dako, Glostrup, Denmark; subclass IgG2b; diluted at 1 : 50), mouse anti-MDM2 monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA; subclass IgG1; diluted at 1 : 200), and rabbit anti-p14ARF polyclonal antibody (Santa Cruz Biotechnology; diluted at 1 : 200). The standard streptavidin–biotin–peroxidase complex method was performed to bind the primary antibodies with the use of Histofine SAB-PO Kits (Nichirei, Tokyo, Japan). Reaction products were visualized by immersing the sections in 0.03% diaminobenzidine solution containing 2 mM hydrogen peroxide for 1–3 min. Nuclei were lightly counterstained with methylgreen. For control studies of the antibodies, the serial sections were treated with phosphate-buffered saline, mouse anti-chromogranin A monoclonal antibody (Dako; subclass IgG2b), mouse anti-desmin monoclonal antibody (Nichirei; subclass IgG1), and normal rabbit IgG instead of the primary antibodies and were confirmed to be unstained.

Immunohistochemical reactivity for p53, MDM2, and p14ARF was evaluated and classified into four groups: (−) negative, (±) weakly positive (less than 5% of epithelial or neoplastic cells), (+) moderately positive (5–25% of epithelial or neoplastic cells), and (++) strongly positive (more than 25% of epithelial or neoplastic cells) positive. The statistical significance of differences in the percentages of cases with different reactivity levels was analyzed by the Mann–Whitney *U*-test for differences between two groups or the Kruskal–Wallis test for differences among three or more groups. *P*-values less than 0.05 were considered to indicate statistical significance.

Direct DNA sequencing for p53 gene mutation

Genomic DNA was extracted from frozen tissue samples of 10 benign ameloblastomas and 1 malignant ameloblastoma, which immunohistochemically showed moderately positive (+) reactions for p53 protein, using a QIAamp DNA Mini

Table 1 Primers for *p53* sequencing

Exon	*Codon*	*Sequence (5′–3′)*	*Product (bp)*
5	146–186	Forward: GCTGTGGGTTGATTCCACAC	167
		Reverse: AACCAGCCCTGTCGTCTCTC	
6	187–224	Forward: GCCTCTGTTCCTCACTGATT	175
		Reverse: TCCTCCCAGAGACCCCAGTT	
7	225–261	Forward: CCTCATCTTGGGCCTGTGTT	171
		Reverse: CAGTGTGCAGGGTGGCAAGT	
8	262–306	Forward: TTCCTTACTGCCTCTTGCTT	206
		Reverse: CACCGCTTCTTGTCCTGCTT	

Kit (Qiagen, Hilden, Germany). *p53* exons 5–8, where most mutations of *p53* gene occur in human tumors, were separately amplified using a HotstarTaq Master Mix Kit (Qiagen) with specific primers (Table 1) in a DNA thermal cycler (Eppendorf, Hamburg, Germany). Polymerase chain reaction (PCR) was performed in a total volume of 50 µl, containing 0.5 µg of template DNA and 0.5 mM of each specific primer set. The procedure for amplification included 35 cycles of denaturation at 94°C for 45 s, annealing at 55°C for 45 s and elongation at 72°C for 60 s with heat starting at 95°C for 15 min and final elongation at 72°C for 10 min.

Sequencing reactions of each *p53* exon were carried out with the PCR products purified using a GFR PCR DNA and Gel Band Purification Kit (Amersham Biosciences, Little Chalfont, UK), the above-mentioned PCR primers and a Thermo Sequenase Cy5 Dye Terminator Sequencing Kit (Amersham Biosciences). The sequencing products were separated on denaturing 8% polyacrylamide gel on an automated laser fluorescence sequencer (ALFexpress II DNA Sequencer; Amersham Biosciences), and the sequencing data were analyzed with the use of an ALFwin Sequence Analyser (Amersham Biosciences).

Results

Immunohistochemical reactivity for p53, MDM2, and p14ARF

The results of immunohistochemical studies of p53, MDM2, and p14ARF are summarized in Table 2. Immunohistochemical reactivity for p53 was detected in the nuclei of normal and neoplastic odontogenic epithelial cells (Fig. 1). In tooth germs, p53 expression was found in limited epithelial cells in 2 of 13 dental laminae. Ameloblastomas showed p53 reactivity scatteredly in peripheral columnar or cuboidal cells in 13 of 29 follicular cases and 16 of 17 plexiform cases (Fig. 1A,B). p53 expression in ameloblastomas was significantly higher than that in enamel organs ($P < 0.05$) and dental laminae ($P < 0.01$) of tooth germs. Plexiform ameloblastomas exhibited statistically higher p53 expression than follicular ameloblastomas ($P < 0.001$). Keratinizing cells in acanthomatous ameloblastomas and granular cells in granular cell ameloblastomas were not reactive with anti-p53 antibody. Basal cell ameloblastomas showed p53 reactivity in scattered neoplastic cells, whereas p53 expression in desmoplastic ameloblastomas was found in a few neoplastic cells. Expression of p53 in malignant ameloblastomas was detected in all five cases and was significantly higher than that in enamel organs and dental laminae ($P < 0.01$). Metastasizing ameloblastomas showed a p53 expression pattern similar to that of follicular ameloblastomas, while ameloblastic carcinomas demonstrated increased p53 expression in neoplastic cells (Fig. 1C).

Immunohistochemical reactivity for MDM2 and p14ARF was detected in the nuclei of normal and neoplastic odontogenic epithelial cells: mesenchymal cells in tooth germs and stromal cells in benign and malignant ameloblastomas were faintly reactive with anti-MDM2 and anti-p14ARF antibodies (Figs. 2 and 3). In all tooth germs, MDM2 expression was found in scattered epithelial cells of outer enamel epithelium and dental laminae. Ameloblastomas showed MDM2 reactivity in many peripheral columnar or

Table 2 Immunohistochemical reactivity for p53, MDM2, and p14ARF proteins in tooth germs and ameloblastomas

	p53				*MDM2*				*p14ARF*			
	(−)	(±)	(+)	(++)	(−)	(±)	(+)	(++)	(−)	(±)	(+)	(++)
Tooth germ ($n = 16$)												
Enamel organ ($n = 8$)	8 (100)	0 (0)	0 (0)	0 (0)	0 (0)	2 (25)	6 (75)	0 (0)	2 (25)	5 (62)	1 (13)	0 (0)
Dental lamina ($n = 13$)	11 (85)	2 (15)	0 (0)	0 (0)	0 (0)	2 (15)	6 (47)	5 (38)	0 (0)	5 (38)	4 (31)	4 (31)
Ameloblastoma ($n = 46$)	17 (37)	9 (20)	20 (43)	0 (0)	0 (0)	3 (7)	17 (37)	26 (56)	0 (0)	5 (11)	30 (65)	11 (24)
Follicular type ($n = 29$)	16 (55)	6 (21)	7 (24)	0 (0)	0 (0)	3 (10)	14 (48)	12 (42)	0 (0)	4 (14)	23 (79)	2 (7)
Plexiform type ($n = 17$)	1 (6)	3 (18)	13 (76)	0 (0)	0 (0)	0 (0)	3 (18)	14 (82)	0 (0)	1 (6)	7 (41)	9 (53)
Acanthomatous subtype ($n = 17$)	6 (35)	2 (12)	9 (53)	0 (0)	0 (0)	2 (12)	7 (41)	8 (47)	0 (0)	2 (12)	13 (76)	2 (12)
Granular subtype ($n = 5$)	2 (40)	3 (60)	0 (0)	0 (0)	0 (0)	1 (20)	3 (60)	1 (20)	0 (0)	1 (20)	4 (80)	0 (0)
Basal cell subtype ($n = 3$)	0 (0)	0 (0)	3 (100)	0 (0)	0 (0)	0 (0)	1 (33)	2 (67)	0 (0)	0 (0)	0 (0)	3 (100)
Desmoplastic subtype ($n = 4$)	2 (50)	2 (50)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	4 (100)	0 (0)	0 (0)	4 (100)	0 (0)
Malignant ameloblastoma ($n = 5$)	0 (0)	2 (40)	2 (40)	1 (20)	0 (0)	1 (20)	0 (0)	4 (80)	0 (0)	0 (0)	2 (40)	3 (60)
Metastasizing ameloblastoma ($n = 2$)	0 (0)	1 (50)	1 (50)	0 (0)	0 (0)	1 (50)	0 (0)	1 (50)	0 (0)	0 (0)	2 (100)	0 (0)
Ameloblastoma carcinoma ($n = 3$)	0 (0)	1 (33)	1 (33)	1 (33)	0 (0)	0 (0)	0 (0)	3 (100)	0 (0)	0 (0)	0 (0)	3 (0)

Immunohistochemical reactivity: (−) negative; (±) weakly (less than 5% of epithelial or neoplastic cells) positive; (+) moderately (5–25% of epithelial or neoplastic cells) positive; (++) strongly (more than 25% of epithelial or neoplastic cells) positive.
Values in parentheses denote percentage values.
Statistical significance: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

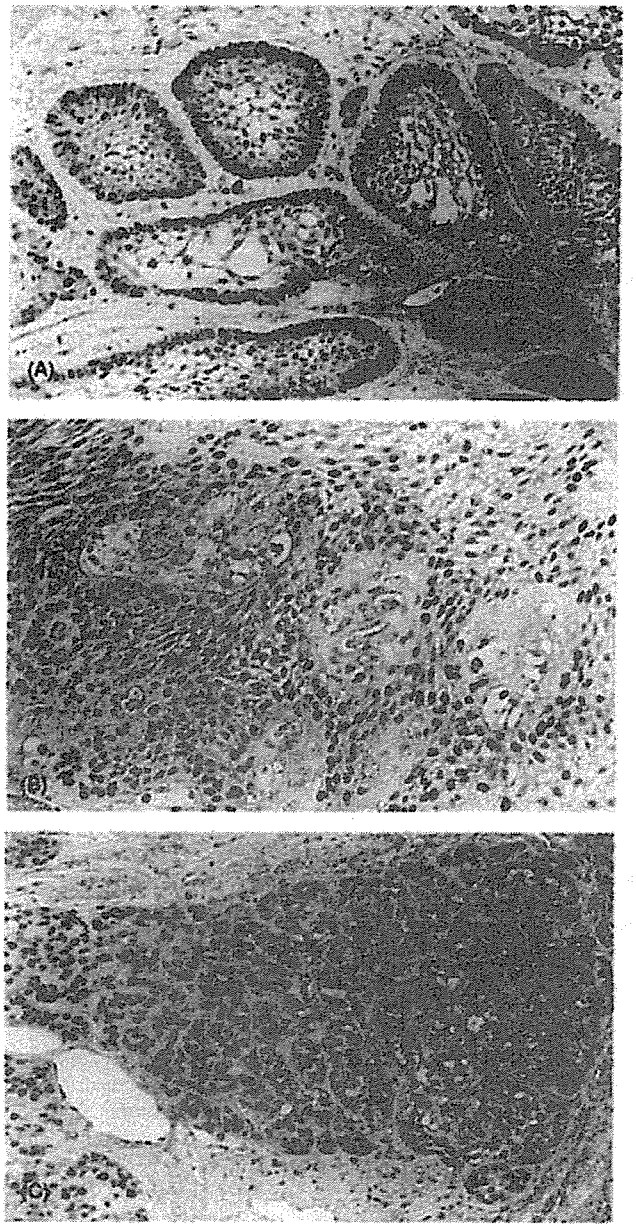

Figure 1 Immunohistochemical reactivity for p53 in ameloblastoma (A,B) and malignant ameloblastoma (C). Follicular ameloblastoma (A) and plexiform ameloblastoma (B) showing reactivity scatteredly in peripheral columnar or cuboidal cells (A: ×160; B: ×160). Ameloblastic carcinoma (C) showing reactivity in most neoplastic cells (×160).

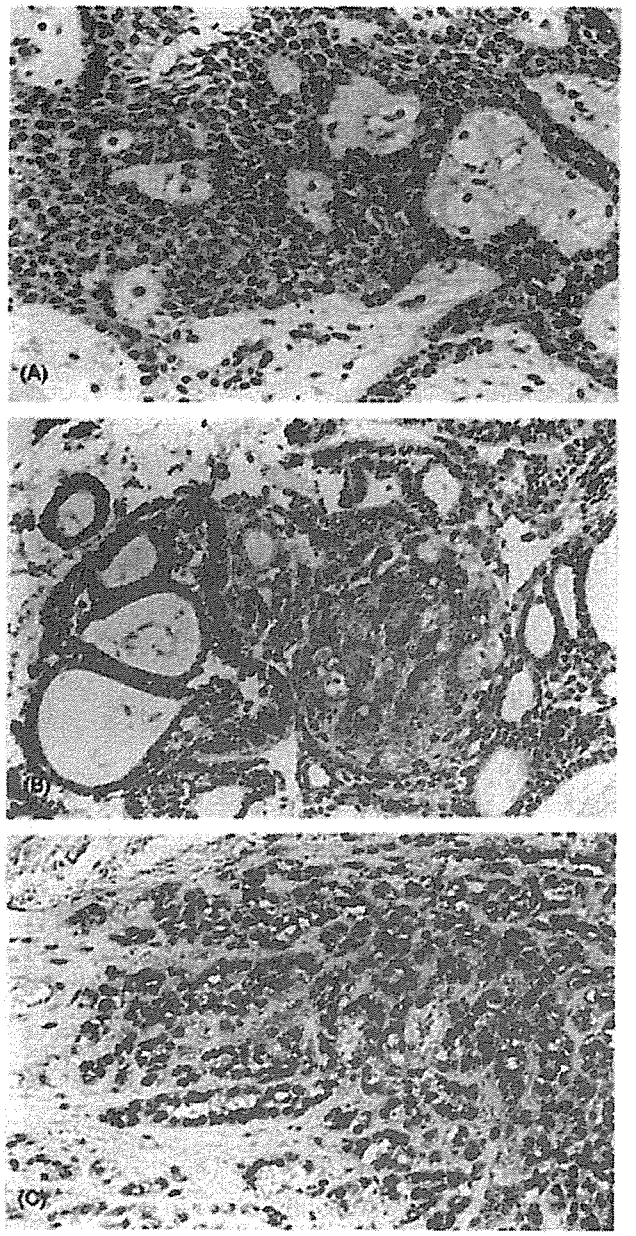

Figure 2 Immunohistochemical reactivity for MDM2 in ameloblastoma (A,B) and malignant ameloblastoma (C). (A) Plexiform ameloblastoma showing reactivity in many peripheral cuboidal cells and some central polyhedral cells (×175). (B) Granular cell ameloblastoma showing a little reactivity in granular cells (×140). (C) Ameloblastic carcinoma showing reactivity in most neoplastic cells (×180).

cuboidal cells and some central polyhedral cells in all cases (Fig. 2A). MDM2 expression in ameloblastomas was significantly higher than that in enamel organs of tooth germs ($P < 0.05$). Plexiform ameloblastomas exhibited statistically higher MDM2 expression than follicular ameloblastomas ($P < 0.01$). MDM2 reactivity was markedly decreased in keratinizing cells in acanthomatous ameloblastomas and granular cells in granular cell ameloblastomas (Fig. 2B). Basal cell ameloblastomas and desmoplastic ameloblastomas showed diffuse MDM2 expression in neoplastic cells, and staining intensity in desmoplastic ameloblastomas was low. Expression of MDM2 in malignant ameloblastomas was detected in all cases. Metastasizing ameloblastomas showed a MDM2 expression pattern similar to that of follicular ameloblastomas, while ameloblastic carcinomas were diffusely positive for MDM2 in most neoplastic cells (Fig. 2C).

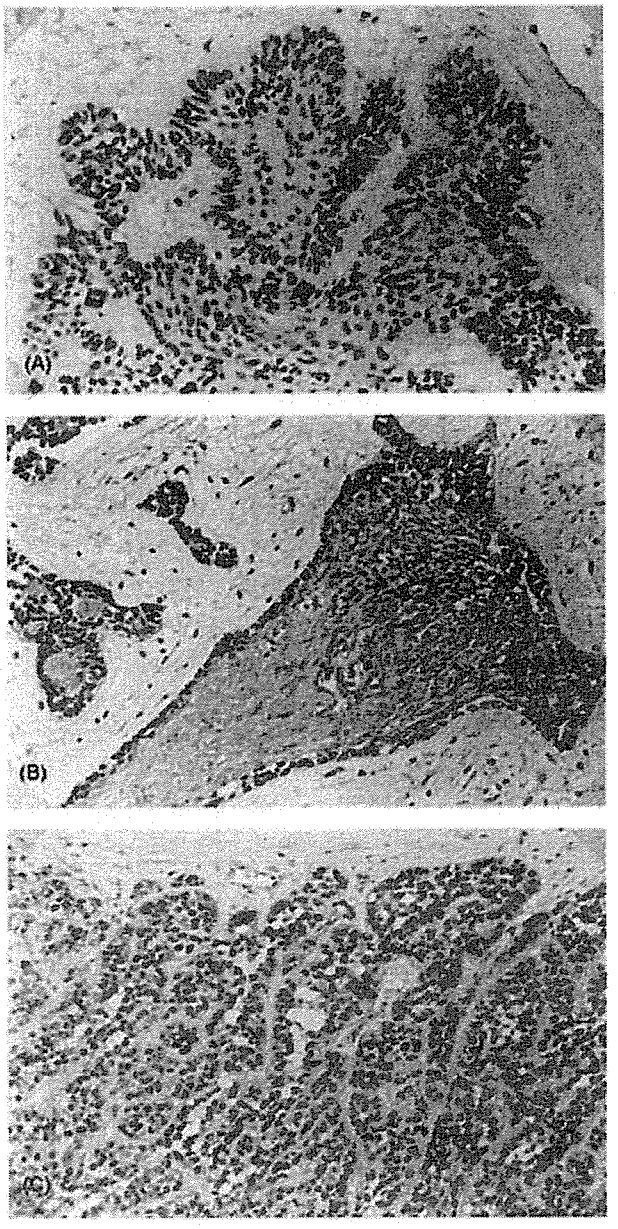

Figure 3 Immunohistochemical reactivity for $p14^{ARF}$ in ameloblastoma (A,B) and malignant ameloblastoma (C). (A) Follicular ameloblastoma showing reactivity in many peripheral columnar cells and some central polyhedral cells (×160). (B) Desmoplastic ameloblastoma showing reactivity in neoplastic cells neighboring the basement membrane (×130). (C) Ameloblastic carcinoma showing reactivity in most neoplastic cells (×140).

$p14^{ARF}$ expression was found in scattered epithelial cells of outer enamel epithelium and dental laminae in all but two tooth germs, and dental laminae showed significantly higher $p14^{ARF}$ expression than enamel organs did ($P < 0.05$). Ameloblastomas showed $p14^{ARF}$ reactivity in many peripheral columnar or cuboidal cells and some central polyhedral cells in all cases (Fig. 3A). $p14^{ARF}$ expression in ameloblastomas was significantly higher than that in enamel organs of tooth germs ($P < 0.01$). Plexiform ameloblastomas exhibited statistically higher $p14^{ARF}$ expression than follicular ameloblastomas ($P < 0.01$). Keratinizing cells in acanthomatous ameloblastomas and granular cells in granular cell ameloblastomas demonstrated little or no reactivity for $p14^{ARF}$. Basal cell ameloblastomas showed diffused $p14^{ARF}$ expression in neoplastic cells, whereas $p14^{ARF}$ reactivity in desmoplastic ameloblastomas was localized in neoplastic cells neighboring the basement membrane (Fig. 3B). $p14^{ARF}$ expression in basal cell ameloblastomas was significantly higher than that in acanthomatous ameloblastomas ($P < 0.05$) and granular cell ameloblastomas ($P < 0.01$). Expression of $p14^{ARF}$ in malignant ameloblastomas was detected in all cases and was significantly higher than that in enamel organs of tooth germs ($P < 0.01$). Metastasizing ameloblastomas showed a $p14^{ARF}$ expression pattern similar to that of follicular ameloblastomas, while ameloblastic carcinomas were diffusely positive for $p14^{ARF}$ in most neoplastic cells (Fig. 3C). Ameloblastic carcinomas exhibited statistically higher $p14^{ARF}$ expression than metastasizing ameloblastomas ($P < 0.05$).

Mutation analysis of p53 gene

Direct DNA sequencing for *p53* gene mutation was carried out in 10 ameloblastomas (five follicular and five plexiform cases) and 1 malignant ameloblastoma (one metastasizing ameloblastoma), which were moderately positive for p53 protein. Mutational alteration was not detected in *p53* gene exons 5–8, including hotspot codons 175, 245, 248, 249, 273, and 282, in any of the 11 cases (Fig. 4).

Discussion

Mutation of *p53* gene results in accumulation of a conformationally altered and functionally defective protein, and overexpression of p53 protein has been detected in various types of tumors (17–21). The present study was performed, employing a monoclonal antibody reactive with wild- and mutant-type p53 protein. Tooth germ tissue showed no or little p53 expression, whereas nuclear accumulation was recognized in benign and malignant ameloblastomas. These features suggest that p53 expression is associated with oncogenesis of odontogenic epithelium. In ameloblastomas, reactivity for p53 was significantly higher in plexiform-type than in follicular-type, suggesting that tissue structuring of ameloblastomas might be affected by p53 expression. In our previous study, keratinizing cells in acanthomatous ameloblastomas and granular cells in granular cell ameloblastomas showed increased apoptotic cell death as compared with other neoplastic cells (30, 32). The present study found no p53 expression in keratinizing or granular cells in ameloblastomas. Apoptosis of these cells was thus apparently not induced by a p53-dependent pathway.

In a wide variety of human tumors, *p53* gene mutations have been detected mainly in exons 5–8, including several hotspot codons (10, 12, 13, 15, 16, 19, 21). Ameloblastomas have shown infrequent *p53* mutations in limited number of neoplastic cells on ELISA and yeast functional assay (7, 8). In the present study using direct *p53* sequencing, alteration of *p53* exons 5–8 was not detected in 10 benign ameloblastomas or 1 metastasizing ameloblastoma, although these

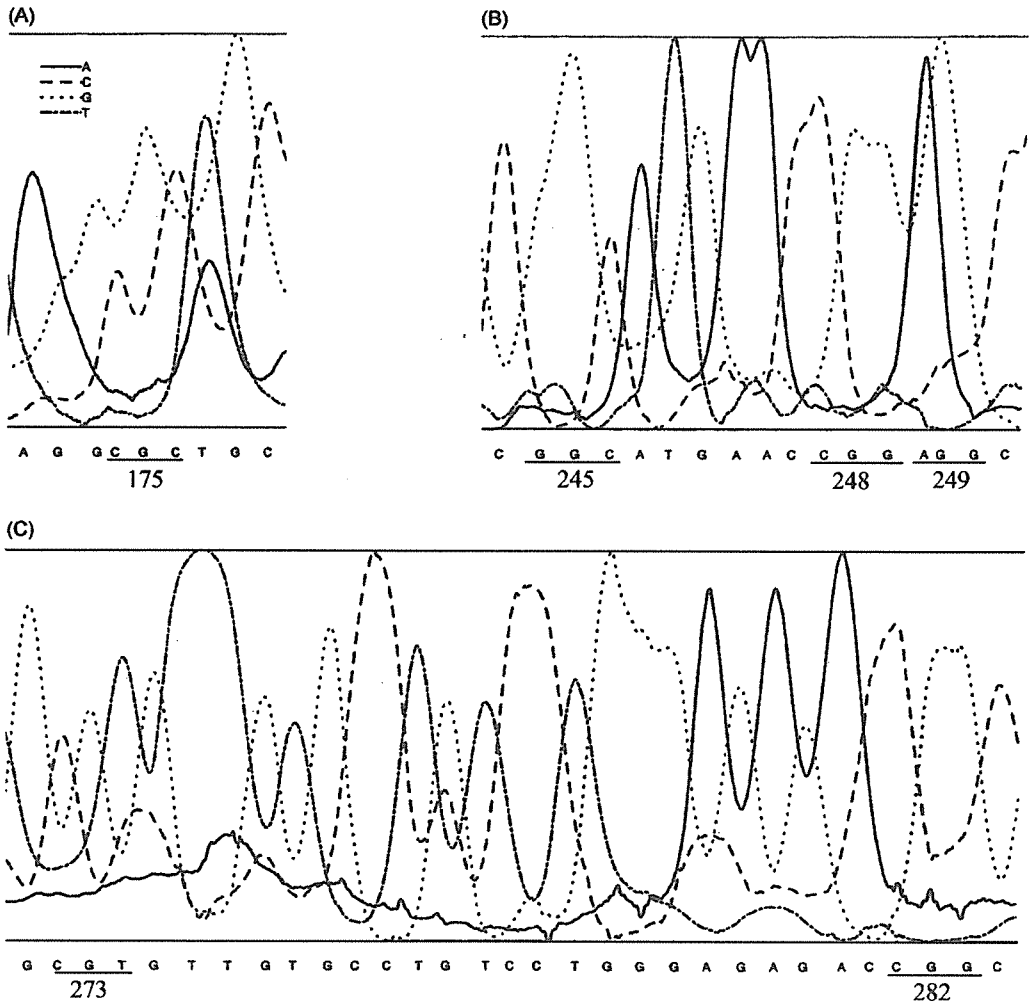

Figure 4 Direct DNA sequencing of *p53* gene in ameloblastoma. No mutation is detected at hotspot codons: codon 175 in exon 5 (A), codons 245, 248, and 249 in exon 7 (B) and codons 273 and 282 in exon 8 (C).

cases were immunohistochemically positive for p53. These findings suggest that mutation of *p53* gene might play a minor role in, and not be essential for, neoplastic changes of odontogenic epithelium. Wild-type p53 protein transcriptionally activates genes involved in cell cycle arrest, such as $p21^{WAF1/Cip1}$, or genes modulating apoptosis, such as bax (13, 36). Our previous study demonstrated low reactivity for bax protein and obvious $p21^{WAF1/Cip1}$ expression in ameloblastomas (26, 31). These features indicate that wild-type p53 protein regulates the cell cycle via $p21^{WAF1/Cip1}$ protein in ameloblastomas. In the present study, several ameloblastic carcinomas immunohistochemically showed increased p53 reactivity, and p53 was possibly associated with malignant transformation of odontogenic epithelium. Mutation analysis of *p53* gene in ameloblastic carcinomas could not be investigated because of the rarity of the malignancy, and further studies should be carried out to determine the association between p53 and malignant changes of odontogenic epithelium. Kropveld et al. (37) has revealed that 33% of all mutations are located outside the core domain of *p53* gene in head and neck squamous cell carcinomas. In our study, mutation analysis was performed only in the core domain of *p53* gene, and sequencing analysis of all 11 exons might be needed to guarantee absence of *p53* mutations.

The $p14^{ARF}$–MDM2–p53 cascade, called the p53 pathway, is an important cell cycle regulatory system in G1 arrest (25, 38), and aberration of this system strongly correlates with neoplastic transformation (7, 17, 19, 21, 24, 28, 29). The ability to generate mice lacking *p53* implies that *p53* is dispensable for embryonic development, while expression of MDM2 and $p14^{ARF}$ during development suggests that these molecules have a primary role in developmental processes (13,39). In the present study, tooth germs showed higher immunohistochemical reactivity for MDM2 and $p14^{ARF}$ as compared with that for p53, indicating that MDM2 and $p14^{ARF}$ play certain roles in tooth development. MDM2 expression in ameloblastomas has been reported to be associated with proliferative activity (7). Our previous study revealed that expression of $p16^{INK4a}$ protein did not

differ distinctly between tooth germs and ameloblastomas (26). In the present study, expression of MDM2 and p14^{ARF} was higher in ameloblastomas and malignant ameloblastomas than in tooth germs, suggesting that these upstream regulators of p53 are involved in oncogenesis and/or malignant transformation of odontogenic epithelium. Plexiform ameloblastomas showed higher expression of MDM2 and p14^{ARF} than follicular ameloblastomas, and markedly decreased reactivity for MDM2 and p14^{ARF} was found in keratinizing and granular cells in ameloblastomas, similar to p53 expression in ameloblastomas. In addition, basal cell ameloblastomas demonstrated high reactivity for p53, MDM2, and p14^{ARF} as compared with other subtypes of ameloblastomas, and p14^{ARF} expression in desmoplastic ameloblastomas was localized in neoplastic cells of basal areas. These features suggest that the p53–MDM2–p14^{ARF} cell cycle regulation system might be related to tissue structuring and cytodifferentiation of ameloblastomas.

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K-*Ras* gene status and expression of Ras/mitogen-activated protein kinase (MAPK) signaling molecules in ameloblastomas

Hiroyuki Kumamoto[1], Nobuhiro Takahashi[2], Kiyoshi Ooya[1]

[1]*Division of Oral Pathology, Department of Oral Medicine and Surgery, and* [2]*Division of Oral Ecology and Biochemistry, Department of Oral Biology, Tohoku University Graduate School of Dentistry, Sendai, Japan*

BACKGROUND: To clarify the roles of rat sarcoma (Ras)/mitogen-activated protein kinase (MAPK) signaling pathway in oncogenesis and cytodifferentiation of odontogenic tumors, K-*Ras* gene status and expression of Ras, Raf1, MAPK/extracellular signal-regulated kinase (ERK) kinase (MEK)1, and ERK1/2 proteins were analyzed in ameloblastomas as well as in tooth germs.

METHODS: Paraffin sections of 10 tooth germs and 46 benign and 6 malignant ameloblastomas were examined immunohistochemically for the expression of K-Ras, Raf1, MEK1, and ERK1/2. Frozen tissue samples of 22 benign ameloblastomas and 1 malignant (metastasizing) ameloblastoma were analyzed by direct DNA sequencing to detect K-*Ras* gene alteration.

RESULTS: Immunohistochemical reactivity for K-Ras, Raf1, MEK1, and ERK1/2 was detected in both normal and neoplastic odontogenic epithelium, and these molecules were reactive chiefly with odontogenic epithelial cells neighboring the basement membrane. Plexiform ameloblastomas showed slightly stronger expression of these Ras/MAPK signaling molecules than follicular ameloblastomas. Keratinizing cells and granular cells showed decreased reactivity for the signaling molecules. Basal cell ameloblastomas showed slightly stronger reactivity for the signaling molecules than did the other subtypes. K-Ras immunoreactivity in malignant ameloblastomas was lower than that in dental lamina of tooth germs. Direct DNA sequencing showed a GGT to GCT point mutation at codon 12 of K-*Ras* gene in one ameloblastoma.

CONCLUSION: Expression of K-Ras, Raf1, MEK1, and ERK1/2 in tooth germs and ameloblastomas suggests that Ras/MAPK signaling pathway functions to regulate cell proliferation and differentiation in both normal and neoplastic odontogenic epithelium. K-*Ras* gene status implied that K-*Ras* mutations might play a minor role in oncogenesis of odontogenic epithelium.

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Keywords: ameloblastoma; ERK; K-Ras; MEK; Raf

Correspondence: Hiroyuki Kumamoto, Division of Oral Pathology, Department of Oral Medicine and Surgery, Tohoku University Graduate School of Dentistry, 4-1 Seiryo-machi, Aoba-ku, Sendai 980-8575, Japan. Tel.: +81 227178303. Fax: +81 22 7178304. E-mail: kumamoto@mail.tains.tohoku.ac.jp
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Introduction

Tumors arising from epithelium of the odontogenic apparatus or from its derivatives or remnants exhibit considerable histologic variation and are classified into several benign and malignant entities (1–4). Ameloblastoma is the most frequently encountered tumor arising from odontogenic epithelium and is characterized by a benign but locally invasive behavior with a high risk of recurrence (1, 2, 4). Histologically, ameloblastoma shows considerable variation, including follicular, plexiform, acanthomatous, granular cell, basal cell, and desmoplastic types (1). Malignant ameloblastoma is defined as a neoplasm in which the pattern of an ameloblastoma and cytological features of malignancy are shown by the primary growth in the jaws and/or by any metastatic growth (1). Recently, malignant ameloblastoma has been subclassified into metastasizing ameloblastoma and ameloblastic carcinoma on the basis of metastatic spread and cytological malignant features (3). Several recent studies have detected genetic and cytogenetic alterations in these epithelial odontogenic tumors (5–7); however, the detailed mechanisms of oncogenesis, cytodifferentiation, and tumor progression remain unknown.

Ras proto-oncogenes were originally characterized on the basis of homology with the transforming genes of rat sarcoma viruses (v-*Ras*), and three *Ras* genes, H-*Ras*, K-*Ras*, and N-*Ras*, were identified in the mammalian genome (8–10). Activation of *Ras* genes by mutation contributes to malignant transformation, and K-*Ras* mutations have been detected in various human neoplasms (10–16). *Ras* genes encode highly similar

guanine nucleotide-binding proteins of approximately 21 kDa ($p21^{Ras}$), and $p21^{Ras}$ is involved in the transduction of external stimuli most likely induced by growth factors (10, 17, 18). These stimuli activate $p21^{Ras}$ by inducing the exchange of GDP to GTP, and GTP-bound $p21^{Ras}$ contributes the activation of three closely related Raf serine/threonine kinases: Raf1, B-Raf, and A-Raf (10, 16, 18). In downstream, activated Raf phosphorylates and activates mitogen-activated protein kinase (MAPK) kinases, MAPK/extracellular signal-regulated kinase (ERK) kinase (MEK)1 and MEK2 (16,18). Phosphorylated MEK functions as dual-specificity kinases and phosphorylates tandem threonine and tyrosine residues in MAPK, ERK1, and ERK12 to activate them (16, 18). Once activated, ERK translocates to the nucleus and activates a variety of substrates, including nuclear transcription factors (16–18). Thus, Ras/MAPK signaling pathway functions as a key regulator of cell proliferation and differentiation, and aberration of involved signaling components has been identified in a various of human tumors (16, 18–23).

Our previous studies confirmed cellular kinetics, including proliferation and cell death modulators, in tooth germs and ameloblastomas, suggesting that these factors are associated with oncogenesis or cytodifferentiation of odontogenic epithelium (24–29). Several studies have examined the expression of specific Ras/MAPK signaling molecules in tooth germs or odontogenic cysts and tumors (30–32). In the present study, the immunohistochemical expression of K-Ras, Raf1, MEK1, and ERK1/2 proteins and mutation of K-*Ras* gene were examined in ameloblastomas as well as in tooth germs to clarify the possible role of Ras/MAPK signaling pathway in epithelial odontogenic tumors.

Materials and methods

The study protocol was reviewed and approved by the Research Ethics Committee of Tohoku University Graduate School of Dentistry.

Tissue preparation

Specimens were surgically removed from 52 patients with epithelial odontogenic tumors at the Department of Oral and Maxillofacial Surgery, Tohoku University Dental Hospital, and affiliated hospitals. The specimens were fixed in 10% buffered formalin for one to several days and embedded in paraffin. The tissue blocks were sliced into 3-μm-thick sections for routine histologic and subsequent immunohistochemical examinations. Tissue sections were stained with hematoxylin and eosin for histologic diagnosis according to the WHO histologic typing of odontogenic tumors (1). The tumors comprised 46 ameloblastomas and 6 malignant ameloblastomas. Ameloblastomas were divided into 30 follicular and 16 plexiform types, including 15 acanthomatous, 5 granular cell, 3 basal cell and 4 desmoplastic subtypes. Malignant ameloblastomas were classified into two metastasizing ameloblastomas and four ameloblastic carcinomas according to the criteria of Eversole (3). For direct DNA sequencing, tumor tissues were immediately frozen on dry ice and stored at −80°C. Specimens of 10 tooth germs of the mandibular third molars, enucleated for orthodontic reasons at the initial stage of crown mineralization, were similarly prepared and compared with the epithelial odontogenic tumors.

Immunohistochemistry for K-Ras, Raf1, MEK1, and ERK1/2 expression

The tissue sections were deparaffinized, immersed in methanol with 0.3% hydrogen peroxide, and heated in 0.01 M citrate buffer (pH 6.0) for 10 min by autoclave (121°C, 2 atm). After treatment with normal serum for 30 min, the sections were incubated with primary antibodies at 4°C overnight. The applied antibodies were mouse anti-K-Ras monoclonal antibody (Oncogene, Boston, MA, USA; subclass IgG2a; diluted at 1 : 20), mouse anti-Raf1 monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA; subclass IgG1; diluted at 1 : 100), mouse anti-MEK1 monoclonal antibody (Santa Cruz Biotechnology; subclass IgG2b; diluted at 1 : 100), and rabbit anti-ERK1/2 polyclonal antibody (Cell Signaling Technology, Beverly, MA, USA; diluted at 1 : 20). The standard streptavidin-biotin-peroxidase complex method was performed to bind the primary antibodies with the use of Histofine SAB-PO Kits (Nichirei, Tokyo, Japan). Reaction products were visualized by immersing the sections in 0.03% diaminobenzidine solution containing 2 mM hydrogen peroxide for 1–3 min. Nuclei were lightly counterstained with methylgreen. For control studies of the antibodies, the serial sections were treated with phosphate-buffered saline, mouse anti-L26 (CD20) monoclonal antibody (Nichirei; subclass IgG2a), mouse anti-OPD4 (CD45RO) monoclonal antibody (Dako, Glostrup, Denmark; subclass IgG1), mouse antichromogranin A monoclonal antibody (Dako; subclass IgG2b), and normal rabbit IgG instead of the primary antibodies and were confirmed to be unstained.

Immunohistochemical reactivity for K-Ras, Raf1, MEK1, and ERK1/2 was evaluated and classified into three groups: (+) focally weak to moderate reactivity; (++) focally strong reactivity or diffusely weak to moderate reactivity; and (+++) diffusely strong reactivity. The statistical significance of differences in the percentages of cases with different reactivity levels was analyzed by the Mann–Whitney *U*-test for differences between two groups or the Kruskal–Wallis test for differences among three or more groups. *P*-values less than 0.05 were considered to indicate statistical significance.

Direct DNA sequencing for K-Ras gene mutations

Genomic DNA was extracted from frozen tissue samples of 22 benign ameloblastomas and 1 malignant ameloblastoma using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). K-*Ras* exons 1 and 2, including hotspot codons 12, 13, and 61, were separately amplified using a HotstarTaq Master Mix Kit (Qiagen) with specific primers in a DNA thermal cycler (Eppendorf, Hamburg, Germany). Primers used in this study were as follows: 5′-GACTGAATATAAACTTGTGG-3′

(forward) and 5′-CTATTGTTGGATCATATTCG-3′ (reverse) for exon 1, yielding a 107-bp product, and 5′-GATTCCTACAGGAAGCAAGT-3′ (forward) and 5′-CTATAATGGTGAATATCTTTC-3′ (reverse) for exon 2, yielding a 185-bp product. Polymerase chain reaction (PCR) was performed in a total volume of 50 μl, containing 0.5 μg of template DNA, 1.5 mM (for exon 1) or 3 mM (for exon 2) of $MgCl_2$, and 0.5 mM of each specific primer set. The procedure for amplification included 35 cycles of denaturation at 94°C for 45 s, annealing at 55°C for 45 s, and elongation at 72°C for 60 s, with heat starting at 95°C for 15 min, and final elongation at 72°C for 10 min.

Sequencing reactions of each K-*Ras* exon were carried out with the PCR products purified using an GFR PCR DNA and Gel Band Purification Kit (Amersham Biosciences, Little Chalfont, UK), the above-mentioned PCR primers, and a Thermo Sequenase Cy5 Dye Terminator Sequencing Kit (Amersham Biosciences). The sequencing products were separated on denatured 8% polyacrylamide gel on an automated laser fluorescence sequencer (ALFexpress II DNA Sequencer; Amersham Biosciences), and the sequencing data were analyzed with the use of an ALFwin Sequence Analyzer (Amersham Biosciences).

Results

Immunohistochemical reactivity for K-Ras, Raf1, MEK1, and ERK1/2

Immunohistochemical reactivity for K-Ras was detected in the cytoplasm and cell membrane of normal and neoplastic odontogenic epithelial cells (Fig. 1). In tooth germs, K-Ras reactivity in inner and outer enamel epithelium and dental lamina was more evident than that in stratum intermedium and stellate reticulum (Fig. 1A). Ameloblastomas showed K-Ras reactivity in many peripheral columnar or cuboidal cells and some central polyhedral cells (Fig. 1B). K-Ras expression in keratinizing cells in acanthomatous ameloblastomas and granular cells in granular cell ameloblastomas was low. Basal cell ameloblastomas and desmoplastic ameloblastomas showed K-Ras reactivity in most neoplastic cells. Metastasizing ameloblastomas showed a K-Ras expression pattern similar to that of follicular ameloblastomas, while ameloblastic carcinomas demonstrated weak to moderate K-Ras reactivity in neoplastic cells (Fig. 1C). Reactivity for K-Ras in malignant ameloblastomas was significantly lower than that in dental lamina ($P < 0.05$) (Table 1).

Expression of Raf1 and MEK1 was found in the cytoplasm of normal and neoplastic odontogenic epithelial cells (Figs. 2 and 3). Tooth germs showed Raf reactivity in inner enamel epithelium and dental lamina (Fig. 2A). In ameloblastomas, Raf1 reactivity in peripheral columnar or cuboidal cells was more evident than that in central polyhedral cells. Plexiform ameloblastomas exhibited statistically higher Raf1 expression than follicular ameloblastomas ($P < 0.05$) (Table 1). Keratinizing cells in acanthomatous ameloblastomas and granular cells in granular cell ameloblastomas showed markedly decreased reactivity for Raf1. Basal cell ameloblastomas and desmoplastic ameloblastomas showed diffuse Raf1 expression in neoplastic cells, and staining intensity in basal cell ameloblastomas was

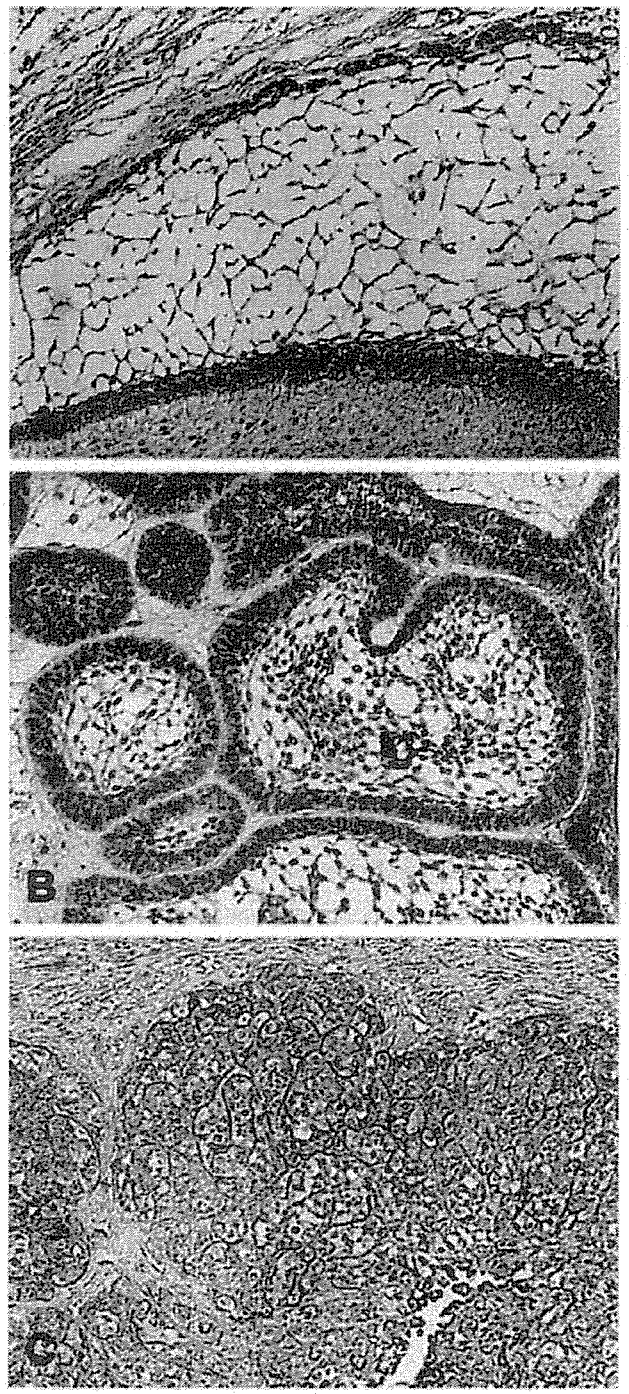

Figure 1 Immunohistochemical reactivity for K-Ras. (A) Tooth germ showing moderate to strong reactivity in inner and outer enamel epithelium and weak reactivity in stratum intermedium and stellate reticulum (×100). (B) Follicular ameloblastoma showing strong reactivity in peripheral columnar cells and weak to moderate reactivity in central polyhedral cells (×125). (C) Ameloblastic carcinoma showing weak reactivity in most neoplastic cells (×120).

Table 1 Immunohistochemical reactivity for K-Ras, Raf1, MEK1, and ERK1/2 in tooth germs and ameloblastomas

	K-Ras			*Raf1*			*MEK1*			*ERK1/2*		
	(+)	(++)	(+++)	(+)	(++)	(+++)	(+)	(++)	(+++)	(+)	(++)	(+++)
Tooth germ ($n = 10$)												
Enamel organ ($n = 10$)	0 (0%)	7 (70%)	3 (30%)	0 (0%)	10 (100%)	0 (0%)	0 (0%)	6 (60%)	4 (40%)	0 (0%)	5 (50%)	5 (50%)
Dental lamina ($n = 5$)	0 (0%)	3 (60%)	2 (40%)	0 (0%)	5 (100%)	0 (0%)	0 (0%)	2 (40%)	3 (60%)	0 (0%)	2 (40%)	3 (60%)
Ameloblastoma ($n = 46$)	4 (9%)	38 (82%)	4 (9%)	5 (11%)	30 (65%)	11 (24%)	1 (2%)	32 (70%)	13 (28%)	4 (9%)	29 (63%)	13 (28%)
Follicular type ($n = 30$)	4 (13%)	24 (80%)	2 (7%)	4 (13%)	22 (74%)	4 (13%)	1 (3%)	20 (67%)	9 (30%)	4 (13%)	19 (64%)	7 (23%)
Plexiform type ($n = 16$)	0 (0%)	14 (87%)	2 (13%)	1 (6%)	8 (50%)	7 (44%)	0 (0%)	12 (75%)	4 (25%)	0 (0%)	10 (62%)	6 (38%)
Acanthomatous subtype ($n = 15$)	2 (13%)	11 (74%)	2 (13%)	3 (20%)	10 (67%)	2 (13%)	0 (0%)	12 (80%)	3 (20%)	1 (9%)	10 (66%)	4 (27%)
Granular subtype ($n = 5$)	1 (20%)	4 (80%)	0 (0%)	1 (20%)	3 (60%)	1 (20%)	0 (0%)	4 (80%)	1 (20%)	1 (20%)	4 (80%)	0 (0%)
Basal cell subtype ($n = 3$)	0 (0%)	2 (67%)	1 (33%)	0 (0%)	1 (33%)	2 (67%)	0 (0%)	2 (67%)	1 (33%)	0 (0%)	1 (33%)	2 (67%)
Desmoplastic subtype ($n = 4$)	0 (0%)	4 (100%)	0 (0%)	0 (0 %)	4 (100%)	0 (0%)	0 (0%)	3 (75%)	1 (25%)	0 (0%)	1 (25%)	3 (75%)
Malignant ameloblastoma ($n = 6$)	1 (17%)	5 (83%)	0 (0%)	1 (17%)	4 (66%)	1 (17%)	0 (0%)	4 (67%)	2 (33%)	0 (0%)	4 (67%)	2 (33%)
Metastasizing ameloblastoma ($n = 2$)	0 (0%)	2 (100%)	0 (0%)	0 (0%)	2 (100%)	0 (0%)	0 (0%)	2 (100%)	0 (0%)	0 (0%)	2 (100%)	0 (0%)
Ameloblastic carcinoma ($n = 4$)	1 (25%)	3 (75%)	0 (0%)	1 (25%)	2 (50%)	1 (25%)	0 (0%)	2 (50%)	2 (50%)	0 (0%)	2 (50%)	2 (50%)

Immunohistochemical reactivity: (+) focally weak to moderate reactivity; (++) focally strong reactivity or diffusely weak to moderate reactivity; (+++) diffusely strong reactivity.
Statistical significance: $^*P < 0.05$.

Figure 2 Immunohistochemical reactivity for Raf1. (A) Tooth germ showing reactivity in inner enamel epithelium (×105). (B) Basal cell ameloblastoma showing reactivity in most neoplastic cells (×115). (C) Ameloblastic carcinoma showing reactivity in most neoplastic cells (×115).

remarkable (Fig. 2B). Metastasizing ameloblastomas showed a Raf1 expression pattern similar to that of follicular ameloblastomas, while ameloblastic carcinomas were positive for Raf1 in most neoplastic cells (Fig. 2C).