

報 文

キラル高速液体クロマトグラフィー/大気圧化学イオン化質量分析法による グリシドール脂肪酸エステルの光学異性体分析

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キラル高速液体クロマトグラフィー/質量分析法 (HPLC/MS) を用いて, 食用油に微量存在し, 安全性が危惧されているグリシドール脂肪酸エステル (GE) の光学異性体を分離, 定量する方法を検討した. HPLC 分析には, アミロース誘導体を固定相とするカラムとアセトニトリルに少量のメタノールを添加した移動相を使用した. MS には四重極型質量分析計を用いて, 大気圧化学イオン化法で正イオンスペクトルを測定した. その結果, 炭素数と不飽和度の異なる GE の光学異性体が明瞭に分離され, それぞれから顕著なプロトン化分子 $[M+H]^+$ が検出された. このイオンを用いる選択イオン検出法によって, ジアシルグリセロールを約 80 % 含有する食用油 (DAG 油) を直接分析した結果, アシル基の異なる 5 種の GE (16:0-GE, 18:0-GE, 18:1-GE, 18:2-GE, 18:3-GE) が検出され, これらはいずれも (R)-異性体と (S)-異性体のほぼ等量混合物であることが明らかになった. 得られた結果から, DAG 油に含まれる GE の光学異性体は, ほぼラセミ体の 1-モノアシルグリセロールから生成するものと考えられた.

1 緒 言

食用油 (精製植物油) に微量含まれるグリシドール脂肪酸エステル (GE, 1-アシル-2,3-エポキシプロパン) の安全性が危惧されており, 世界的に大きな問題となっている¹⁾²⁾. GE は, 食用油の主成分であるトリアシルグリセロール (TAG) やジアシルグリセロール (DAG) が脱臭などの精製工程で高温, 高圧下に置かれることで生成すると考えられている²⁾³⁾.

食用油に含まれる微量の GE を精確に定量することは容易ではなく, 信頼のおける分析法の開発が緊急の課題となっている¹⁾. これまで, GE の分析法がいくつか報告されている. たとえば, Masukawa⁴⁾⁵⁾らは食用油中の GE を 2 回の固相抽出によって濃縮した後, 逆相高速液体クロマトグラフィー/質量分析法 (HPLC/MS) を用いてアシル基の異なる 5 種の GE を定量している. WeiBhaar³⁾らは食用油中の GE をゲル透過によって分離後, ガスクロマトグラ

フィー/質量分析法 (GC/MS) を用いて同定している. 一方, Deutsche Gesellschaft für Fettwissenschaft (DGF, ドイツ脂質学会)⁶⁾は, GE を加水分解して生成する 3-モノクロロ-1,2-プロパンジオールをフェニルホウ酸で誘導体化した後, GC/MS を用いて GE を間接的に定量する方法を推奨している. これらの方法は, 食用油に含まれる GE の検出と定量に有用であると思われるが, 存在可能な 1 対の光学異性体 (Fig. 4 参照) については何も考慮されていない. GE の光学異性体間の生物活性の差異は明らかではないが, 光学異性体は, 一方が有効な活性を示し, もう一方が毒性を示すなど生物に対する作用が異なる場合があることから, 食品中の GE をリスク評価する際には, 安全性が危惧されている GE の光学異性体分析は不可欠であると考えられる.

本研究では逆相キラル HPLC を用いて GE の光学異性体を分離し, MS を併用して前処理なしで実試料中の GE 光学異性体を直接分析する方法を検討した. 得られた結果から GE 光学異性体の生成経路を推測した.

2 実 験

2.1 試 薬

以下の試薬を用いた. パルミチン酸 (16:0), ステアリン酸 (18:0), オレイン酸 (18:1), リノール酸 (18:2), リノレン酸 (18:3) (Sigma 製, 純度 99 % 以上); パルミチン酸グリシジル (16:0-GE, 純度 98 % 以上), オレイン酸グリシジル (18:1-GE, 純度 98 % 以上), リノール酸グ

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リシジル (18:2-GE, 純度 90 % 以上), リノレン酸グリシジル (18:3-GE, 純度 85 % 以上) (和光純薬工業製); 4-ジメチルアミノピリジン (DMAP), 無水硫酸ナトリウム, *N,N*-ジシクロヘキシルカルボジイミド (DCC) (ナカライテスク製); (*R*)-, (*S*)-及びラセミ体グリシドール (Aldrich 製, 純度 96~97 %); DAG 高含有 (約 80 %) 食用油⁷⁾ (DAG 油, 花王製, 2008 年に函館市内の食料品店にて購入).

2.2 GE 標準品の合成

(*R*)-グリシドール, (*S*)-グリシドール及びラセミ体グリシドール (各 0.9 mmol), 脂肪酸 (1.0 mmol), DCC (1.2 mmol), DMAP (0.2 mmol) をクロロホルム 5 mL に溶解し, 攪拌しながら 20 °C で 16 時間反応させた. 反応後, クロロホルムを窒素気流下で除去して得られた残渣約 20 mg を 200 μ L のクロロホルムに再度溶解させた. このクロロホルム可溶物からシリカゲル薄層クロマトグラフィー {Merck 製シリカゲル 60 F₂₅₄ (厚さ 0.25 mm, 20 cm \times 20 cm)} を用いて GE を単離した. 展開溶媒にはヘキサン-ジエチルエーテル (60:40, v/v) を使用した. 展開後, 0.2 % 2',7'-ジクロロフルオレセイン試薬を噴霧して, 紫外線照射下で市販標準品と同一の R_f 値を示すバンドを確認し, ジエチルエーテルを用いて抽出した. 得られた GE 画分を大気圧化学イオン化質量分析法 (APCI-MS) で確認し, その純度を光散乱検出逆相 HPLC (HPLC/ELSD) で求めた. MS には四重極型質量分析計 LC/MS-2010EV (島津製作所製) を用い, インフュージョン法で GE のマススペクトルを m/z 200 ~ m/z 1000 の範囲で測定した. ネブライザーガス 2.5 L min⁻¹, インターフェース温度 400 °C, 加熱ブロック温度 200 °C に設定し, 正イオンスペクトル測定した. HPLC 分析には Gemini 5u C18 110A カラム (Penomenex 製, 内径 4.6 mm, 長さ 25 cm, 粒径 5 μ m), LC-20AD ポンプ (島津製作所製), カラム恒温槽 Cool Pocket (Thermo Fisher Scientific 製), 蒸発光散乱検出器 (SEDEX55, SEDERE 製) 及びクロマトパック C-R6A (島津製作所製) を使用した. 試料は 2-プロパノールに 1 mg mL⁻¹ の濃度になるように溶解し, その 5 μ L を Rheodyne 製インジェクター (Model 7725) を用いてカラムに注入した. 移動相にはアセトニトリル/メタノール (10:90, v/v) (関東化学製 HPLC 用) を用いて 0.5 mL min⁻¹ の流量で送液した. カラム温度は 20 °C とし, 検出器の温度を 50 °C (空気圧力 2.3 bar, Gain 8) に設定して溶出成分を検出した.

2.3 キラル HPLC/ELSD 分析

キラル HPLC 分析には, 固定相に Amylose tris(3,5-dimethylphenylcarbamate) を含むダイセル製の CHIRALPAK IA カラム (内径 4.6 mm, 長さ 25 cm, 粒径 5 μ m) と 2.2 に記載した機器類を使用した. カラム温度は 5 °C または

25 °C に設定し, GE の光学異性体分離を検討した. 合成した GE 標準品を 2-プロパノールに 200 μ g mL⁻¹ の濃度になるように溶解し, その 5 μ L を Rheodyne 製インジェクター (Model 7725) を用いてカラムに注入した. 移動相には, アセトニトリルに少量のメタノール, 2-プロパノール, エタノールまたは水 (いずれも関東化学製 HPLC 用) を加えたものを使用し (組成については 3.1 参照), 0.5 mL min⁻¹ の流量で送液した.

2.4 キラル HPLC/APCI-MS

実試料 (DAG 油) に含まれる GE の光学異性体の分離と定量にはキラル HPLC/APCI-MS を使用した. キラル HPLC は 2.3 と同一の条件で行った. APCI-MS は 2.2 と同一条件で行い, プロトン化分子を用いる選択イオン検出 (SIM) により各光学異性体を定量した. 合成した GE 標準品 (ラセミ体) を 2-プロパノールに 0.5, 1, 5, 10, 20, 50 μ g mL⁻¹ の濃度になるように溶解し, その 5 μ L をオートインジェクター用いてカラムに注入した. 溶出した各光学異性体のピーク面積を測定して検量線を作成した. 検出限界と定量限界は, それぞれ $S/N=3$, $S/N=10$ で求めた. DAG 油は 2-プロパノールに 30 mg mL⁻¹ の濃度になるように溶解し, その 20 μ L をオートインジェクターを用いてカラムに注入した. 移動相を 150 分間送液して, GE の後に溶出する多量の DAG などの夾雑物をカラムから排出した.

3 結果と考察

3.1 GE 標準品の純度検定

インフュージョン法による MS 分析の結果, 本研究で合成したすべての GE からベースピークとして $[M+H]^+$ が検出された. また, C18 カラムを用いた逆相 HPLC/ELSD 分析では, いずれの試料からも単一のピークが得られ, 合成した GE 標準品はすべて高純度であることが確認された.

3.2 キラル HPLC による GE の光学異性体分離

キラル HPLC 分析に使用した CHIRALPAK IA カラムは従来型の CHIRALPAK AD カラムと同様, 固定相として Amylose tris(3,5-dimethylphenylcarbamate) を含むが, 耐溶剤性に優れ, 順相と逆相の両モードで使用できる特徴を有する⁸⁾. 本研究では, HPLC/MS でのイオン化を促進するために, またアシル基の異なる種々の GE の光学異性体を一斉に分離するために逆相モードを選択した. 移動相にアセトニトリル/メタノール, アセトニトリル/2-プロパノール, アセトニトリル/エタノール (組成はいずれも 98.5:1.5, v/v), 及びアセトニトリル/水 (90:10, v/v) を用いて GE の光学異性体分離を検討した結果, アセトニトリル/メタノールで最も良い分離が得られた. たとえば,

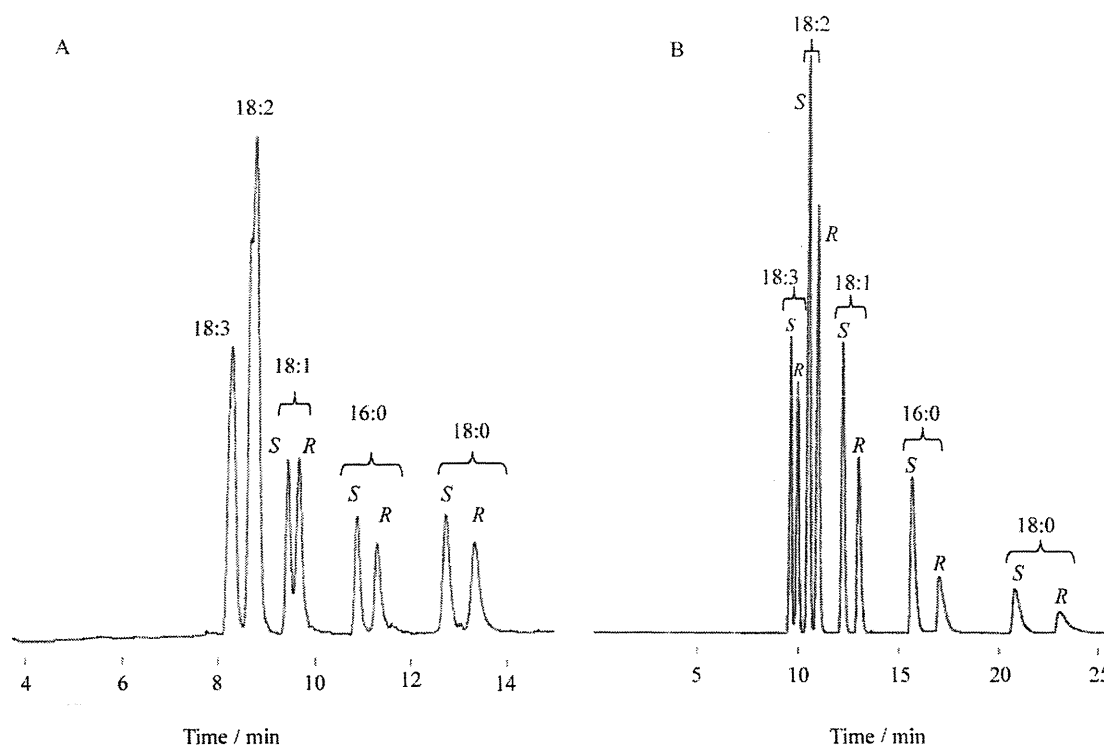


Fig. 1 Chiral-phase HPLC/ELSD profiles of racemic glycidyl fatty acid esters

18 : 3, glycidyl linolenate ; 18 : 2, glycidyl linoleate ; 18 : 1, glycidyl oleate ; 16 : 0, glycidyl palmitate ; 18 : 0, glycidyl stearate.

Column, CHIRALPAK IA (250 × 4.6 mm i.d.); Mobile phase, Acetonitrile/methanol. A, 90 : 10 (v/v); B, 98.5 : 1.5 (v/v). Column temperature : A, 25 °C ; B, 5 °C. Flow rate, 0.5 mL min⁻¹.

Table 1 Chromatographic parameters of enantiomeric glycidyl fatty acid esters (GE) obtained by chiral-phase HPLC/ELSD^{a)}

GE enantiomer		Column temperature							
		25 °C				5 °C			
		V_R ^{b)}	k' ^{c)}	α ^{d)}	R_S ^{e)}	V_R	k'	α	R_S
16 : 0	S	0.31	0.62	1.10	1.70	0.69	1.39	1.16	2.98
	R	0.34	0.69			0.81	1.61		
18 : 0	S	0.45	0.90	1.10	1.82	1.09	2.18	1.17	3.27
	R	0.49	0.99			1.27	2.55		
18 : 1	S	0.20	0.41	1.09	1.09	0.42	0.85	1.15	2.78
	R	0.22	0.44			0.49	0.97		
18 : 2	S	—	—	—	—	0.29	0.59	1.12	1.85
	R	—	—			0.33	0.66		
18 : 3	S	—	—	—	—	0.23	0.45	1.12	1.60
	R	—	—			0.25	0.50		

a) Mobile phase : Acetonitrile/methanol (98.5 : 1.5, v/v); b) V_R = retention volume (mL) corrected by subtracting the column void volume (Chiralpak IA, 3.35 mL); c) k' = capacity ratio ; d) α = separation factor ; e) R_S = peak resolution. $R_S = 1.18 (R_{t2} - R_{t1}) / (w_2 + w_1)$, where w = peak half width (min).

25 °C で分析した場合、16 : 0-GE、18 : 0-GE 及び 18 : 1-GE の分離係数は、アセトニトリル/2-プロパノールではそれぞれ 1.38, 1.40, 0.78, アセトニトリル/エタノールでは 1.42, 1.58, 0.79, アセトニトリル/水では 1.37, 1.16, 1.05 で

あったのに対し、アセトニトリル/メタノールではいずれの GE についても 1.70, 1.82, 1.09 と高い値が得られた。Fig. 1 に、アセトニトリル/メタノール (98.5 : 1.5, v/v) を移動相に用いて、GE のラセミ体混合物 (16 : 0-GE,

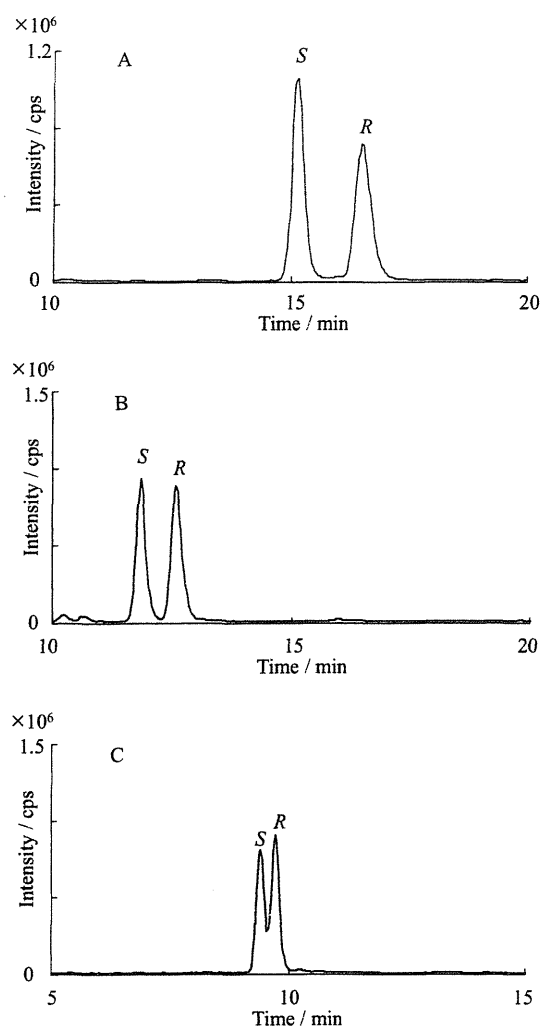


Fig. 2 Chiral-phase HPLC/APCI-MS profiles (selected ion monitoring of $[M+H]^+$) of racemic glycidyl fatty acid esters on CHIRALPAK IA

A, glycidyl palmitate (m/z 313); B, glycidyl oleate (m/z 339); C, glycidyl linolenate (m/z 335). Mobile phase and column temperature are the same as those in Fig. 1B. Other HPLC and MS conditions as given in text.

18:0-GE, 18:1-GE, 18:2-GE, 18:3-GE) を分析して得られたクロマトグラムを示す。25℃ のカラム温度で分析した結果、16:0-GE と 18:0-GE の光学異性体は完全に分離されたが、不飽和度の増加とともに GE の分離は低下し、18:3-GE の光学異性体分離は得られなかった (Fig. 1A)。各 GE の光学異性体を同時に分離するために 5℃ のカラム温度で分析した結果、すべての GE が明瞭に分離され、飽和及び不飽和 GE の光学異性体 10 成分の一斉分析が可能となった (Fig. 1B)。アシル基の鎖長と不飽和度は光学異性体の溶出順には影響を及ぼさず、いずれの GE も (S)-異性体が先に溶出した。Morante-Zarcero ら⁹⁾ は CHIRALPAK

AD-H カラムを用いてグリシドールの光学異性体分離を検討した結果、ヘキサン/エタノール (80:20, v/v) の移動相で良好な分離が得られ (分離度, $R_s = 1.8$)、S 配置が R 配置よりも強く保持されることを報告している。GE の光学異性体を順相移動相 (ヘキサン/2-プロパノール, 99:1, v/v) を用いて CHIRALPAK IA カラムで分析した場合、不飽和 GE (18:1-GE, 18:2-GE, 18:3-GE) の光学異性体は良好に分離され、逆相のときと溶出順は逆転して (R)-異性体が先に溶出した (クロマトグラムは省略)。多糖誘導体型固定相のキラル認識のメカニズムは十分解明されていないが¹⁰⁾、Amylose tris(3,5-dimethylphenylcarbamate) による GE のキラル認識には、水素結合形成、 π - π 相互作用、双極子-双極子相互作用などに加え、移動相溶剤の極性が大きく関与しているものと推察される。

GE 光学異性体の保持容量 (V_R)、保持係数 (k')、分離係数 (α) 及び分離度 (R_s) を Table 1 に示す。GE は二重結合数が増すにつれて極性が増大し、溶出時間が短くなって分離度が低下した。同様に、極性のより大きい 16:0-GE は 18:0-GE より早く溶出し、分離度が低下した。このようなアシル基の分離に与える影響はグリセロ脂質のキラル HPLC において広く認められている¹¹⁾¹²⁾。一般に光学異性体は低温で良い分離が得られるが¹¹⁾、本研究においてもカラム温度を 25℃ から 5℃ に下げることで、不飽和 GE (18:1-GE, 18:2-GE, 18:3-GE) についてはいずれも 1.6 以上の分離度が得られ、分離は著しく改善した (Table 1)。このことから、低温での分析は実試料中の GE 光学異性体の分析に特に有効であると考えられた。

3.3 キラル HPLC-MS による GE 光学異性体の定量

移動相にアセトニトリル/メタノール (98.5:1.5, v/v) を用いて、カラム温度 5℃ で、各 GE 標準品をキラル HPLC/APCI-MS 分析した結果、すべての GE からプロトン化分子 $[M+H]^+$ がほぼ単一のピークとして検出された。このイオンを選択して得られた GE ラセミ体の SIM クロマトグラムを Fig. 2 に示す。いずれのラセミ体についても ELSD 検出の場合と同様に良好な分離が得られた。GE の MS において、 $[M+H]^+$ をモニターした場合、APCI-MS は ESI-MS の 10 倍高感度であることが報告されている⁴⁾。本研究でも ESI-MS を検討したが、適用した条件では $[M+H]^+$ は検出されず、 $[M+Na]^+$ が APCI-MS で得られた $[M+H]^+$ に匹敵する感度で検出された。したがって、ESI-MS でも GE の分析は十分可能であると考えられる。

Table 2 に検出限界、定量限界及び検量線について検討した結果を示す。各 GE の検量線の相関係数は 0.997 以上であり、良好な直線関係が得られた。検出限界 ($S/N = 3$) と定量限界 ($S/N = 10$) は、それぞれ 0.2, 0.67 $\mu\text{g mL}^{-1}$ であった。

Table 2 Limit of detection (LOD), limit of quantification (LOQ), and calibration lines obtained for enantiomeric glycidyl fatty acid esters (GE)

GE	LOD ^{a)} /μg mL ⁻¹		LOQ ^{a)} /μg mL ⁻¹		Calibration line ^{b)}			
	Enantiomer		Enantiomer		S-enantiomer		R-enantiomer	
	S	R	S	R	Equation	R ²	Equation	R ²
16 : 0	0.2	0.2	0.66	0.66	$y = 1061175x - 736213$	0.9967	$y = 953826x - 250272$	0.9997
18 : 0	0.2	0.2	0.66	0.66	$y = 743751x - 549055$	0.9978	$y = 699722x - 151986$	0.9985
18 : 1	0.2	0.2	0.66	0.66	$y = 802928x - 275120$	0.9993	$y = 801020x - 312693$	0.9988
18 : 2	0.2	0.2	0.66	0.66	$y = 954918x - 519142$	0.9980	$y = 984471x - 672675$	0.9973
18 : 3	0.2	0.2	0.66	0.66	$y = 580746x - 234941$	0.9984	$y = 588035x - 173547$	0.9990

a) Defined as $S/N = 3$ for LOD and 10 for LOQ based on 5 μL injection of the standard GE solution at a concentration of 0.2 μg mL⁻¹. b) Calculated from the equation $y = Ax + B$, where x is the injected concentration (μg mL⁻¹), y is the peak area, A is the slope, and B is the intercept in the range between 0.5 and 50 μg mL⁻¹ (0.5, 1, 5, 10, 20 and 50 μg mL⁻¹).

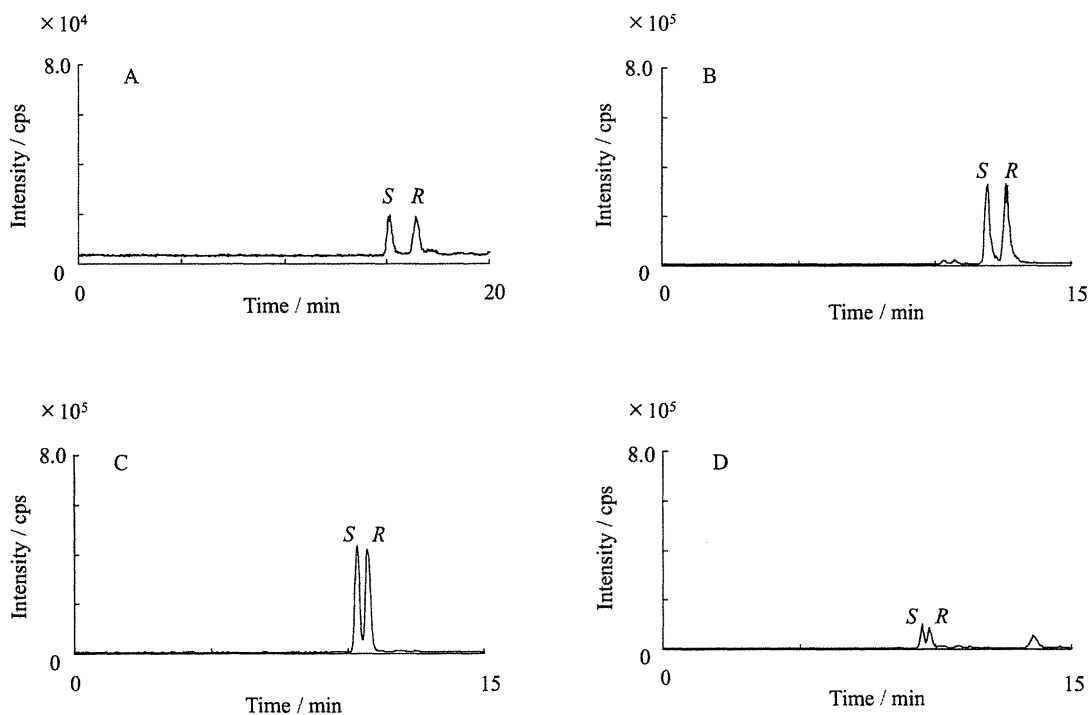


Fig. 3 Chiral-phase HPLC/APCI-MS profiles (selected ion monitoring of $[M+H]^+$) of the glycidyl fatty acid esters from a diacylglycerol-rich oil (DAG oil)

A, glycidyl palmitate (m/z 313); B, glycidyl oleate (m/z 339); C, glycidyl linoleate (m/z 337); D, glycidyl linolenate (m/z 335). Glycidyl stearate is not shown. Chiral-phase HPLC-MS conditions are the same as those in Fig. 2.

キラル HPLC/APCI-MS を用いて、DAG 油中の GE を分析して得られたクロマトグラムを Fig. 3 に示す。濃縮や単離などの前処理を行わずに GE を検出するために、600 μg の試料油をカラムに注入した。その結果、試料に含まれる 5 種の主要な GE のそれぞれの光学異性体は、25 分以内にはほぼ完全に分離して溶出し (18 : 0-GE のクロマトグラムは省略)、試料注入量の分離に及ぼす悪影響はほとんど認められなかった。得られた各 GE の光学異性体含量を Table 3 に示す。DAG 油の主成分である 1,3-DAG を構成する主な

脂肪酸は、16 : 0 (総脂肪酸中 3.4 %), 18 : 0 (同 1.1 %), 18 : 1 (同 29.9 %), 18 : 2 (同 59.1 %), 18 : 3 (同 5.2 %) であることが報告されている¹³⁾。この脂肪酸組成を反映して GE も 18 : 2-GE が最も高く (総 GE 中 44.9 %), 次いで 18 : 1-GE (同 40.5 %), 18 : 3-GE (同 10.4 %), 16 : 0-GE (同 2.1 %), 18 : 0-GE (同 2.1 %) であった。いずれの GE についても、(S)-異性体と (R)-異性体はほぼ等量存在した。試料油中の GE の総含量は 238 μg g⁻¹ であり、GE を固相抽出法で濃縮してから分析する方法で得られた値

Table 3 Levels of enantiomeric glycidyl fatty acid esters (GE) in a diacylglycerol-rich oil ($\mu\text{g g}^{-1}$)

GE	This study			Previous study ^{b)}
	S-enantiomer	R-enantiomer	S + R ^{a)}	
16 : 0	2.4	2.3	4.7	6
18 : 0	3.1	2.0	5.1	< 5
18 : 1	48.9	47.4	96.3	94
18 : 2	52.6	54.2	106.8	132
18 : 3	13.0	11.7	24.7	32
Total	120.0	117.6	237.6	269

a) Sum of S- and R-enantiomers. b) Data from Masukawa *et al.*⁴⁾

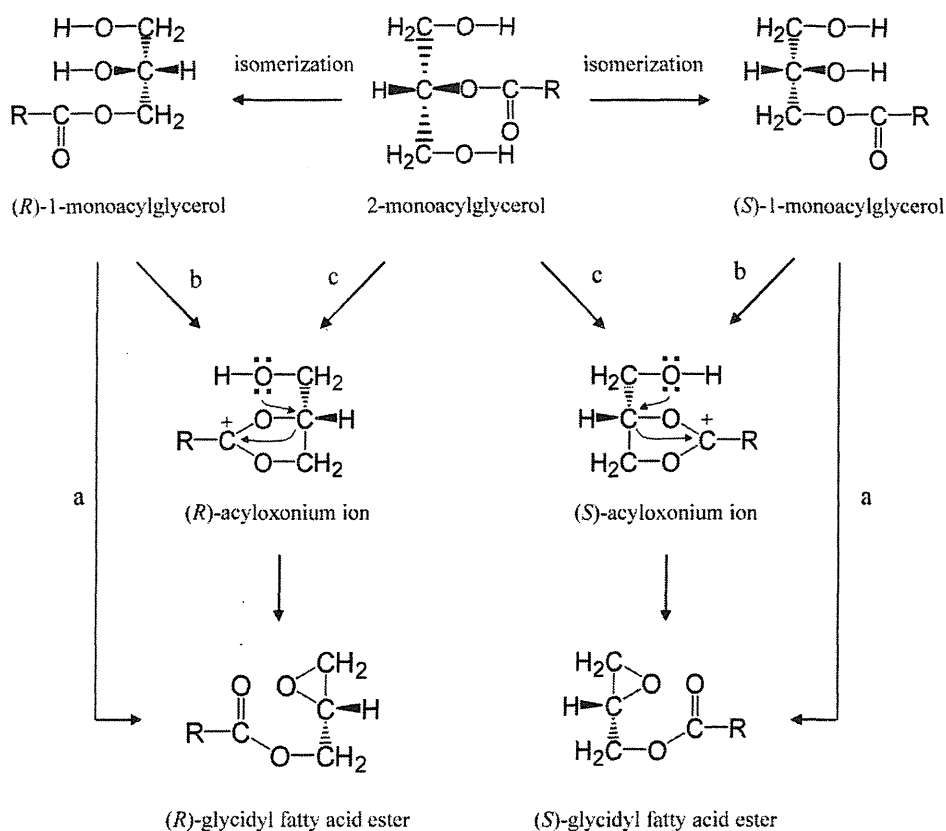


Fig. 4 Possible mechanism of formation of enantiomeric glycidyl fatty acid esters from refined edible oils

($264 \mu\text{g g}^{-1}$)⁴⁾と比較して幾分小さかった。この違いは不飽和度が高く、かつ主要成分である 18 : 2-GE と 18 : 3-GE において明瞭であった (Table 3)。今後、同一試料を両方法で分析して結果を比較することが必要であると考えられる。

DAG 油は他の食用油に比べて GE を数十倍多く含む⁴⁾ことから本法での検出が容易であったが、本法を極微量 ($0.1 \sim 0.5 \mu\text{g g}^{-1}$) の GE を含むとされる精製大豆油や精製ナタネ油など¹⁴⁾一般の食用油に広く適用するにはあらかじめ GE を濃縮したり、より高感度の MS 装置を使用することが有効であると考えられる。

3.4 GE 光学異性体の生成機構の推定

GE は食用油の脱臭工程において、TAG と DAG 由来のモノアシルグリセロール (MAG) から脱水またはアシルオキシニウムイオンの形成を経て生成すると考えられているが²⁾³⁾、光学異性体は考慮されていない。Fig. 4 に、本研究において推定した GE 光学異性体の生成機構を示す。DAG 油の主な原料である大豆油とナタネ油の TAG の *sn*-1 位と *sn*-3 位に結合する脂肪酸の組成はおおむね等しいことから¹⁵⁾、DAG 油から生成する 1-MAG も (*S*)-1-MAG (*sn*-1-MAG) と (*R*)-1-MAG (*sn*-3-MAG) のほぼ等量混合物であると考えられる。TAG 及び DAG からは 2-MAG も生成する

が,これは熱力学的により安定な (S)-1-MAG と (R)-1-MAG に容易に異性化 (ラセミ化) する¹⁶⁾. MAG のヒドロキシル基の脱水により, (S)-1-MAG からは (S)-GE が, (R)-1-MAG からは (R)-GE が生成すると推定される (経路 a). したがって, (R)-異性体と (S)-異性体のほぼ等量混合物である MAG からは GE のほぼラセミ体が生成する. 一方, (S)-1-MAG からは (S)-アシルオキシニウムイオンを経て (S)-GE が, (R)-1-MAG からは (R)-アシルオキシニウムイオンを経て (R)-GE がそれぞれ生成すると推定される (経路 b). したがって, この場合も生成する GE はほぼラセミ体である. 2-MAG から生成するアシルオキシニウムイオンは (R)-異性体と (S)-異性体のほぼ等量混合物であると推定されることから (経路 c), 得られる GE もほぼラセミ体となる. これらのことから, DAG 油に含まれる GE は, R 配置と S 配置の異性体のほぼ等量混合物から構成されるものと考えられる.

4 結 言

本研究ではキラル逆相 HPLC を用いて, 炭素数と二重結合数の異なる GE の光学異性体分離に初めて成功した. この方法に APCLMS を併用して DAG 油中に含まれる微量の GE 光学異性体をあらかじめ濃縮することなしに SIM モードで直接分析した結果, 5 種の GE (16:0-GE, 18:0-GE, 18:1-GE, 18:2-GE, 18:3-GE) の光学異性体がそれぞれ明瞭に分離され, 10 成分の一斉分析が可能となった. 各光学異性体含量は DAG 油の脂肪酸組成を反映して 18:2-GE が最も高く, 次いで 18:1-GE であった. いずれの GE も R 配置と S 配置の異性体のほぼ等量混合物であったが, このことは, GE の前駆体である 1-MAG がほぼラセミ体であることに起因すると考えられる.

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Analysis of Enantiomeric Glycidyl Fatty Acid Esters by Chiral-phase HPLC/MS with Atmospheric Pressure Chemical Ionization

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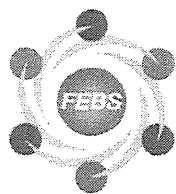
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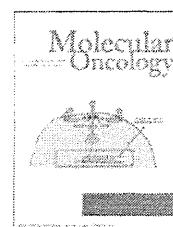
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A simple and selective method was developed for the determination of enantiomeric glycidyl fatty acid esters (GE) in refined edible oils, which are undesirable trace contaminants formed during refining processes. For this purpose, chiral-phase high-performance liquid chromatography (HPLC) was performed with a column containing amylose tris(3,5-dimethylphenylcarbamate) as a chiral selector, using an acetonitrile/methanol mixture as the mobile phase, which gave clear enantiomer resolution of synthetic saturated and unsaturated GE racemates. This was followed by on-line atmospheric pressure chemical ionization mass spectrometry (APCI/MS) to obtain prominent protonated $[M+H]^+$ molecules, by which individual GE molecules could be identified and quantified. The quantification of GE enantiomers was performed in the selected ion monitoring (SIM) of $[M+H]^+$. This chiral-phase HPLC/APCI-MS-SIM method was standardized with synthetic GE racemates, and applied to the separation, identification and quantification of individual GE enantiomers in a commercial diacylglycerol (DAG)-rich oil produced originally from soybean oil and rapeseed oil. The results clearly showed that the DAG-rich oil contained almost equal amounts of *R*- and *S*-enantiomers of glycidyl palmitate (16:0-GE), stearate (18:0-GE), oleate (18:1-GE), linoleate (18:2-GE), and linolenate (18:3-GE), suggesting that both enantiomers would be formed from the corresponding almost racemic 1-monoacylglycerols. The most predominant GE was 18:2-GE, followed by 18:1-GE, which accounted for 107 and 25 $\mu\text{g g}^{-1}$, respectively, reflecting the fatty acid composition of the DAG oil.

Keywords : glycidyl fatty acid ester ; enantiomer ; diacylglycerol-rich oil ; chiral-phase HPLC ; atmospheric pressure chemical ionization mass spectrometry.

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Review

Somatic evolution of head and neck cancer – Biological robustness and latent vulnerability



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ABSTRACT

Despite recent advancements in multidisciplinary treatments, the overall survival and quality of life of patients with advanced head and neck squamous cell carcinoma (HNSCC) have not improved significantly over the past decade. Molecular targeted therapies, which have been addressed and advanced by the concept of “oncogene addiction”, have demonstrated only limited successes so far. To explore a novel clue for clinically effective targeted therapies, we analyzed the molecular circuitry of HNSCC through the lens that HNSCC is an evolving system. In the trajectory of this somatic evolution, HNSCC acquires biological robustness under a variety of selective pressures including genetic, epigenetic, micro-environmental and metabolic stressors, which well explains the major mechanism of “escaping from oncogene addiction”. On the other hand, this systemic view appears to instruct us approaches to target latent vulnerability of HNSCC that is masked behind the plasticity and evolvability of this complex adaptive system.

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1. Introduction

Head and neck squamous cell carcinoma (HNSCC), the sixth most common cancer worldwide, often generates from organs

including the larynx, pharynx, oral cavity, and tongue that play critical roles in respiratory, nutritional, social and communicative functions (Hunter et al., 2005; Masuda et al., 2010). Surgical intervention in these organs often leads to a considerable

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Abbreviations

HNSCC	head and neck squamous cell carcinoma
EMT	epithelial–mesenchymal transition
MET	mesenchymal epithelial transition
CSC	cancer stem cell
EGFR	epidermal growth factor receptor
HPV	human papilloma virus
CML	chronic myeloid leukemia
PDGFR	platelet derived growth factor receptor
IGFR	insulin like growth factor receptor
Stat3	signal transducers and activators of transcription
MAPK	mitogen activated protein kinase
PI3K	phosphoinositide-3-kinase
CIN85	c-Cbl interacting protein of 85 kDa
OGG	oxoguanine DNA glycosylase
LOH	loss of heterozygosity
SNP	single nucleotide polymorphism
IAP	inhibitor of apoptosis
CDC	cell division cycle
COX	cyclooxygenase
HIF	hypoxia inducible factor
PDK	pyruvate dehydrogenase kinase
TKLT	transketolase-like protein
ZEB1	zink finger E-box-binding homeobox-1
HMGA2	high-mobility group AT-hook2
IL	interleukin
ALDH	aldehyde dehydrogenase
eIF	eukaryotic initiation factor
HSP	heat shock protein
TGF	transforming growth factor
HGF	hepatocellular growth factor
IGF	insulin like growth factor
PDGF	platelet derived growth factor
IL	interleukin
MMP	matrix metalloproteinase
VEGF	vascular endothelial growth factor
PDGF	platelet derived growth factor
IP-10	interferon-gamma inducible 10 kD protein
TNF	tumor necrosis factor
IFN	interferon

impairment of the patient's quality of life, although remarkable progress has been made in reconstructive surgery. Thus, head and neck surgeons and oncologists have often struggled to balance potentially competing goals of therapy, i.e., organ preservation and survival. Mainly to spare surgical intervention, several dose-intensified novel treatment protocols have been introduced in the last decade, including concurrent chemoradiotherapy (e.g. clinical trials led by the Radiation Therapy Oncology Group), "super-selective" intra-arterial chemoradiotherapy (e.g., RADPLAT), and induction chemotherapy followed by radiation or concurrent chemoradiation (e.g., Tax 323 and Tax 324 protocols) reviewed in refs. Alkureishi et al. (2006) and Haddad and Shin (2008). Preliminary results of these treatments appear promising; however, it remains to be seen whether these dose-intensified types of approaches will have a definitive and positive impact on both

long-term overall survival and "functional" organ preservation. Moreover these treatments can be associated with considerable complications (e.g., requirement for feeding tube following severe laryngeal and pharyngeal dysfunction) and potential treatment-related death (Corry et al., 2010; Machtay et al., 2008; Rosenthal et al., 2006). Thus, although there have been recent advances in both surgical and non-surgical therapy, the overall survival and quality of life of patients with HNSCC have not improved significantly over the past decade, especially for patients with advanced stage.

To resolve this dilemma, growing interest has focused on the development of novel treatment strategies based on the HNSCC cell biology. It is generally accepted that the development and progression of HNSCC occur through the stepwise and progressive accumulation of genetic and epigenetic alterations, following direct and repeated exposures to carcinogens including tobacco, alcohol, and viral infections (Hunter et al., 2005; Leemans et al., 2010). Thus, HNSCC represents the classical models of both multistage carcinogenesis and field cancerization (Hunter et al., 2005). Several investigators (Forastiere et al., 2001; Haddad and Shin, 2008; Leemans et al., 2010; Lippman and Hong, 2001) have proposed HNSCC progression models, in which the normal epithelium undergoes an orderly transformation through hyperplasia, dysplasia, carcinoma *in situ* and invasive carcinoma in accordance with cumulative genetic and epigenetic alterations including loss of 9p21 (p16-INK4a), 3p21, 17p13, 13q21, 14q24, 4q26–28, 6p and 8p23 and gain of 11q13 (cyclin D1). Epigenetic silencing of p16-INK4a and loss-of-function mutation of TP53 can also be involved in this process. However, these linear progression models based on intrinsic abnormalities may be overly simplistic, because it has become apparent that malignant phenotypes of cancer are maintained by far more complex system. Numerous intrinsic abnormalities have been reported to be involved in HNSCC pathogenesis and the number of these abnormalities is expanding rapidly (Leemans et al., 2010; Matta and Ralhan, 2009; Molinolo et al., 2009). Moreover, tumor development and progression are not autonomous processes that are achieved by cancer cells alone. The tumor microenvironment profoundly modulates tumor cell biology through interactions between the tumor and the surrounding matrix and non-cancerous cells therein (e.g., bone marrow derived cells), leading to the development of tumor favorable eco-systems or niches, in which cancer cells acquire peculiar abilities to survive metabolic stressors including acidosis, hypoxia and malnutrition, to circumvent immune surveillance, and to invade and metastasize to distant organs (De Boeck et al., 2010; Hanahan and Weinberg, 2011; Hoogsteen et al., 2007; Joyce and Pollard, 2009; Kroemer and Pouyssegur, 2008). Other concepts that expanded the complexity of HNSCC carcinogenesis include epithelial–mesenchymal transition (EMT) (Polyak and Weinberg, 2009) and cancer stem cell (CSC) (Brabletz et al., 2005; Chaffer and Weinberg, 2011; Rosen and Jordan, 2009; Visvader and Lindeman, 2008), which are mainly caused by drastic reprogramming of gene expression profile. In addition, the heterogenic cells in HNSCC might mutually influence their internal circuitry. In order to organize our current knowledge on HNSCC biology, we have numerated potential biomarkers related to HNSCC pathogenesis, according to their main biological functions in Tables 1 and 2.

In general, this overflowing information does not lead to the identification of the critical molecular target or biomarker of HNSCC, except for the recent finding of human papilloma virus (HPV)-positive oropharyngeal cancer. Since a majority of these cancers demonstrate quite favorable response to organ-preserving treatments, they are now recognized as a distinct entity of HNSCC that can be cured within the framework of conventional treatments (Ang et al., 2010; Chung and Gillison, 2009; Leemans et al., 2010). In contrast, ongoing targeted therapy for HPV-negative HNSCC has had limited success, mainly with the use of epidermal growth factor receptor (EGFR) inhibitors, either used alone or in combination with radiation (Bernier et al., 2009; Specenier and Vermorken, 2012). This is partly because the predominant driver mutation or genetic abnormality in oncogene (e.g., BCR-ABL in CML or BRAF in malignant melanoma) (Chapman et al., 2011; Hughes et al., 2003) has not been discovered in HNSCC (Agrawal et al., 2011; Stransky et al., 2011). Nevertheless, it is obvious that this limited success mainly results from our paucity of insight into the molecular mechanism that drives HNSCC development and progression. For example, the major mechanism that causes the frequent (>80%) activation of EGFR signaling in HNSCC is not known, although its significant role in HNSCC development and progression has been highlighted for more than two decade (Masuda et al., 2011). The purpose of this

perspective review is to illuminate current obstacles to and future directions for clinical effective targeted therapies based on the concept that HNSCC is an evolving system.

2. Molecular circuitry of HNSCC and oncogene addiction

It is now apparent that the development and progression of HNSCC are not a consequence of the simple summation and accumulation of the intrinsic and extrinsic abnormalities mentioned above (Tables 1 and 2). In cancer cells, many of these abnormalities interact with each other through complex networks and form a highly integrated “molecular circuitry” in order to maintain the malignant phenotypes of cancer during tumor progression (Molinolo et al., 2009; Weinstein, 2000; Weinstein et al., 1997). Despite this complexity, reversal of only one or a few genetic abnormalities can profoundly inhibit cancer cell growth and in some cases translate to improved clinical survival rates; a phenomenon termed “oncogene addiction” (as well as tumor suppressor gene hypersensitivity) proposed by our mentor Dr. Weinstein (Weinstein et al., 1997). Examples of this phenomenon were observed in mechanistic studies in human cancer cell lines, genetically engineered mouse models of human cancer, and clinical trials

Table 1 – Intrinsic and cell surface factors associated with HNSCC pathogenesis.

Function	Factors	Refs. no in foot note
1. Agonist dependent or independent signal transduction	EGFR, erbb2 (HER2), PDGFR, IGFR, c-Met, src, Ras, Stat3, MAPK, NF- κ B, PI3K-PTEN-AktmTOR, Wnt/ β -catenin, TGF- β /smad, CXCR, p38, C1N85	1
2. DNA replication and repair	p53, nucleotide excision repair (NER), OGG	2
3. Genetic and chromosomal instability	chromosomal number variation, DNA aneuploidy, LOH, SNP, chromosomal rearrangemen,	3
4. Cell fate, survival, differentiation, senescence, and apoptosis	Telomerase, Survivin, Bcl-2/XL, Bax, IAPs, p53, Myc	4
5. Mitotic regulation	aurora kinase A	5
6. Cell cycle and check point control	cyclin D1, p14, p16, p21, p27, pRB, CDC25A, CDC25B	6
7. Cell adhesion and motility	integrins, E-cadherin, CD44, moesin, laminin	7
8. Transcription, translation post-translation regulation and chromatin remodeling	DNA methylation, miRNA, eIF4, S6, HSP, BMI1, EZH2	8
9. Viral infection	human papilloma virus, Epstein-Barr virus	9
10. Intracellular metabolism	aerobic glycolysis (Warburg effect), hypoxia, xenobiotics, prostaglandin synthesis, Cox-2, reactive oxygen species (ROS), HIF-1, mitochondrial DNA mutation, PDK1, LDH, TKLT-1, Myc, AktmTOR	10
11. Epithelial-mesenchymal transition	Src, BMI1, ZEB1, TrkB, Cox-2, HMGA2, IL-1 β , Snail, Twist, E-cadherin, Vimentin	11
12. Cancer stemness	CD44, Notch, Wnt, Oct, Nanog, Stat3, CD133, ALDH1	12

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Table 2 – Extrinsic factors associated with HNSCC pathogenesis.

Function	Factors	Refs no in foot note
1. Mitogens, adhesion and motility molecules, and proteases	TGF- α , Amphiregulin, HGF, IGF, PDGF, ILs, MMPs, chemokines	[1]
2. Non-cancerous cells in stroma	Bone marrow derived cells, cancer associated fibroblast, tumor associated macrophage, Treg	[2]
3. Angiogenesis	VEGF, PDGF	[3]
4. Extracellular metabolism	acidosis, hypoxia, ROS	[4]
5. Immune response	Rantes, IP-10, TNF- α , IFN- γ , VEGF	[5]

References: 1 – Barnes et al. (2007), Bran et al. (2009), Hunter et al. (2005), Knowles et al. (2003), Reuter et al. (2007); 2 – De Boeck et al. (2010), Strauss et al. (2007), Tabachnyk et al. (2012); 3 – Bran et al. (2009), Matta and Ralhan (2009); 4 – Hoogsteen et al. (2007); 5 – Jewett et al. (2006)

with molecular targeted agents (Weinstein, 2000, 2002; Weinstein and Joe, 2008, 2006). The concept of oncogene addiction has been generally accepted as a rationale for molecular targeted therapy, and an increasing number of studies and reviews on this subject have been published (Baylín and Ohm, 2006; Felsher, 2008; Garraway and Sellers, 2006b; Grant, 2008; Kaelin, 2005; Kroemer and Pouyssegur, 2008; Luo et al., 2009; Masuda et al., 2010; Mills et al., 2001; Sharma and Settleman, 2007). Although the precise mechanism of oncogene addiction is not fully known, Dr. Weinstein speculated that tumor specific homeostasis that is maintained by “bizarre” molecular circuitry might play a fundamental role in the development of this phenomenon (Weinstein, 2000; Weinstein and Joe, 2006). For example, probably to encounter the effects of growth enhancing molecules, paradoxical increases of tumor suppressive molecules are frequently observed in this bizarre circuitry (Weinstein, 2000). This situation would be analogous to a car driving at high speeds with both the accelerator and brake engaged simultaneously. Hence, when the accelerator is released (i.e., the effects of tumor enhancing molecules are inhibited), the car comes to a sudden stop (i.e., cell ceases to proliferate or undergoes apoptosis). In the concepts of “oncogene shock” and “oncogene amnesia”, Sharma and Settleman (2007), and Felsher (2008) also proposed this homeostatic feedback theory to explain the mechanism of oncogene addiction. In addition to this feedback theory, there are several possible explanations for the mechanisms of oncogene addiction such as genetic streamlining, or synthetic lethality (Kaelin, 2005; Mills et al., 2001). Due to the genetic streamlining model, once a cancer cell becomes highly and continuously dependent on a given proliferative signal, other collateral growth signals regress and eventually the remaining signal predominates proliferation (Mills et al., 2001). As a result, if this predominant signal is inhibited, the cancer cell cannot survive any more losing the driving force for proliferation. The synthetic lethality concept describes a condition in which combinational mutations in two genes leads to cell death, whereas mutation in one of two genes is compatible with survival (Kaelin, 2005). A clinical manifestation of this phenomenon was discovered in a recent study (Fong et al., 2009). Inhibition of poly (ADP-ribose) polymerase (PARP) has been shown specifically kill cancer cells including ovarian, breast and prostate cancer that have mutations in BRCA1 and BRCA2. In this setting, PARP and BRCA, are paralogous genes in their ability to repair DNA damage by the

homologous recombinant pathway. Simultaneous inhibition of these two paralogs results in synthetic lethality. The theory of “molecular house of cards” (Kaelin, 2005) also explain the mechanism of synthetic lethality. In the molecular circuitry of cancer that proceeds with accumulative genetic aberrations, each additional mutation must be beneficial (adaptive) or at least neutral to pre-existing mutations for the maintenance of cellular integrity. Due to this biological restriction, the molecular circuitry of cancer presumably falls into the state of molecular house of cards in accordance with tumor progression. Thus, extraction of just one card (i.e., reversal of one genetic abnormality) might lead to the collapse of the house. Of note, the clinical example of synthetic lethality has not yet been discovered in HNSCC.

Indeed, examples of bizarre molecular circuitry of HNSCC have been demonstrated in clinical samples (Homma et al., 1999; Lo Muzio et al., 2005; Naresh et al., 2001; Pena et al., 1999; Wilson et al., 2001). In several studies, the increased Bcl-2 expression or low-apoptotic index paradoxically associated with favorable outcomes in patients with early stage HNSCC (Homma et al., 1999; Lo Muzio et al., 2005; Naresh et al., 2001; Pena et al., 1999; Wilson et al., 2001). These counterintuitive findings may imply that in a subset of early stage HNSCC, anti-apoptotic signals protect cancer cells from apoptosis in order to maintain a net volume of tumor mass that is mainly composed of less progressive and therefore slow-growing cell population. Sathyan et al. (2007) also demonstrated an intriguing finding in 151 cases of oral cancer obtained from patients in India, which were known to have a rare TP53 mutation. They found that in these samples, the activating H-ras mutations significantly correlated with lower expression of cyclin D1 and CDK4, higher expression of pRB and p16^{INK4A}, and thereby a better prognosis. Although the precise mechanism of these paradoxical associations between oncogenic and tumor suppressive proteins is not clear, this setting, at least in part, mimics the findings observed in HPV-positive oropharyngeal cancer patients. They demonstrate a significantly favorable response to chemotherapy or chemoradiation due to the distinctive molecular profiling: wild type but inactivated p53 protein by viral E6, inactivated pRB protein by viral E7 and high p16^{INK4A} expression (Ang et al., 2010; Chung and Gillison, 2009; Leemans et al., 2010). It might be also relevant to this favorable outcome that HPV-positive HNSCC is genetically more stable than HPV-negative HNSCC: 2.28 vs 4.38 mutations per mega base in whole-

exome sequencing and 19 vs 119 somatic rearrangements in whole-genome sequencing (Leemans et al., 2010; Stransky et al., 2011). In a recent commentary article to the study by Ang and colleagues (Ang et al., 2010), Lowy and Munger postulated an interesting theory that in HPV-positive HNSCCs chemoradiation might reactivate the function of wild type p53 and pRB by reducing the levels of viral E6 and E7 protein, and thereby induce G1 check point control and consequent apoptosis, resulting in the better response (Lowy and Munger, 2010). This scenario well exemplify an example of oncogene addiction in HNSCC, thus HPV-positive HNSCC is highly dependent on (e.g., addicted to) E6 and E7 proteins for the maintenance of malignant phenotype.

3. Cancer evolution and the mechanisms of escaping from “oncogene addiction”

Despite the fact that the concept of oncogene addiction has gained wide acceptance as a rationale for molecular targeting, a majority of these therapies have demonstrated only marginal effects so far on a variety of malignancies including HPV-negative HNSCC. Sharma and Settleman described this frustrating situation referring to the “one–two punch” – “oncogene addiction has set the stage for the knock-out by the first punch but is holding the devastating second” (Sharma and Settleman, 2007). Based on the recent conceptual framework on cancer biology, the main reason of this limited success could be attributed to the cancer evolution theory, in which cancer is considered as a peculiar organ or an adaptive system composed of heterogeneous cell population, because this complex machinery can demonstrates amazing plasticity and robustness in this evolving process (Greaves and Maley, 2012; Kitano, 2004; Merlo et al., 2006; Nowell, 1976; Tian et al., 2011). Consistent with the Darwinian principle, this somatic evolution proceeds under harsh selective pressures including a variety of genetic, epigenetic, metabolic, immunologic and micro-environmental stressors that are primarily deleterious for the maintenance of cellular integrity (Luo et al., 2009). To survive and overcome these stressors, cancer cell must continuously reprogram its internal wiring diagram of molecular circuitry by cunningly orchestrating cancer causing (adaptive), non-cancer causing (non-adaptive), and housekeeping maneuvers or nodes to avoid systemic failure (i.e., cell death), and thereby achieve a state of cancer specific homeostasis or equilibrium. This intracellular molecular landscape or gene regulatory network is termed “cancer attractor”, borrowing visual representation of the normal cell differentiation trajectory originally proposed by Waddington in 1957 (Brock et al., 2009; Huang et al., 2009; Marusyk et al., 2012). In Figure 1, we schematically proposed a model of evolutionary trajectory of HNSCC based on the concept of cancer attractor. As opposed to the normal cell differentiation, in which developing cells flow down the surface of rugged slope, cancer evolutionary trajectory is supposed to be uphill movement in terms of biological potential (i.e., fitness). A clone acquired novel driver mutation, epigenetic abnormality, or altered gene expression profile in response to selective pressures can go up to a higher attractor state toward de-differentiation. In accordance with tumor evolution, it is

obvious that each attractor become more complex, because the internal molecular circuitry has to process exponentially growing information, as exemplified by the accumulated number of mutations or drastic change in gene expression profile caused by chromatin remodeling, which is often observed in advanced stage tumor. Due to principals of systems biology (Kitano, 2004; Tian et al., 2011), this complexity provides cancer cells with biological robustness by making the basin of attractor deeper (i.e., stable), although, in parallel, the hurdle to the next attractor becomes higher (Figure 1). Consequently, the molecular circuitry of cancer cells in higher attractor demonstrates remarkable resilience or adaptability to more severe selective pressures (Brock et al., 2009; Gillies et al., 2012; Marusyk et al., 2012), whereas that of lower attractors tends to be unstable. This is consistent with clinical evidence that a majority of HPV-negative HNSCCs with advanced stage show remarkable resistance to chemo/radiation. It is of note that an individual tumor is composed of heterogeneous cancer cells, which inhabit different attractors. We linked tumor evolutionary trajectory to hallmarks of HNSCC in Figure 1, which is discussed later.

This scenario well explains the mechanism of oncogene addiction as well as that of “escaping from oncogene addiction” (i.e., tolerance) – a dismal phenomenon that is frequently observed in molecular targeting therapies. As cancer proceeds in its evolutionary trajectory, a tumor mass becomes more heterogeneous involving diverse clones in higher attractors, which demonstrate stronger resistance to molecular targeting and chemo/radiation through either acquired (i.e., pre-existing) or *de novo* mechanism achieved by additional mutation or non-mutagenic adaptation (Brock et al., 2009; Gillies et al., 2012; Marusyk et al., 2012; Stratton, 2011). As a result, even if a given molecular targeting therapy or chemo/radiation can kill a majority of clones, the increased emergence of these refractory clones, particularly in advanced HNSCC, leads to the re-growth of tumor.

Taken together, it should be emphasized that for evolving cancers, molecular targeting as well as conventional DNA-damaging treatment might be just another selective pressure for further evolution, because they might promote selective sweep and/or emergence of resilient cells.

4. Targeting hallmarks of HNSCC

4.1. Twelve hallmarks of HNSCC

In view of this evolutionary trait, it seems quite difficult to timely identify the Achilles’ heel of HNSCC within the complex and dramatically changing molecular circuitry. However, despite a myriad of intrinsic and extrinsic alterations, cancer evolution proceed with the acquisition of a series of malignant phenotypes that can be boiled down into several properties, as first categorized by Hanahan and Weinberg into six hallmarks of cancer (Hanahan and Weinberg, 2000). To date, several additional traits were proposed as additional hallmarks by four research groups including a recent revision by Hanahan and Weinberg (Colotta et al., 2009; Hanahan and Weinberg, 2011; Kroemer and Pouyssegur, 2008; Luo et al., 2009). Moreover, in

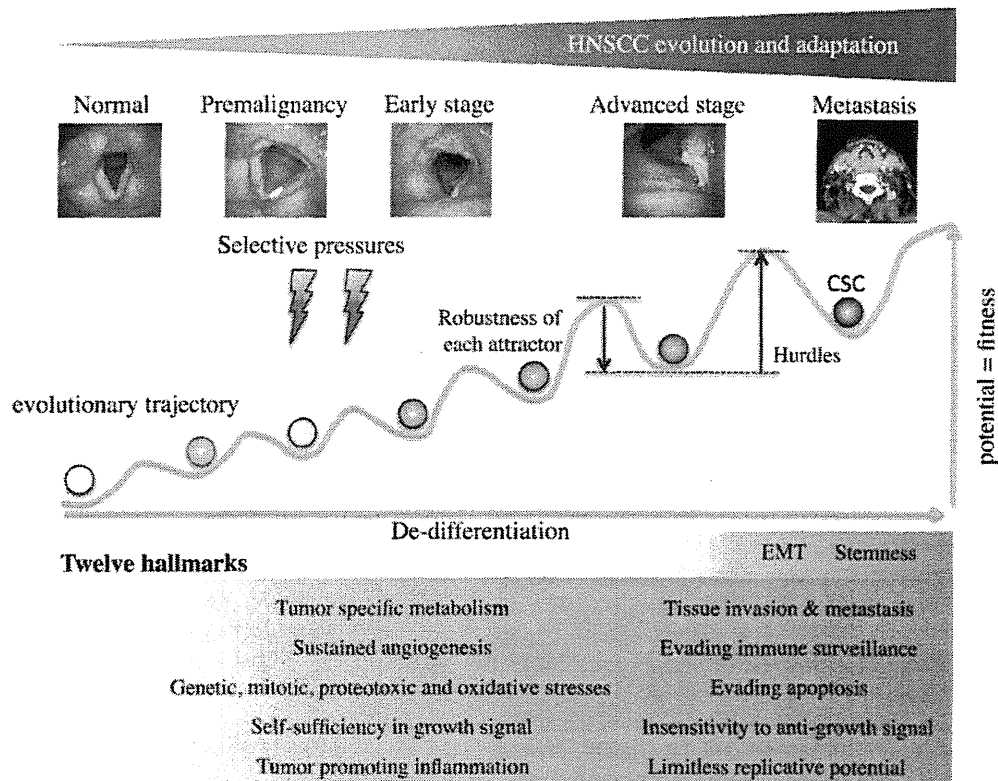


Figure 1 – Tumor evolutionary trajectory and twelve hallmarks of HNSCC. HNSCC evolves from normal epithelium to advanced tumors. In this process, intracellular molecular circuitry or gene regulatory network achieves transient equilibrium state, “cancer attractor”, which is depicted as a basin in the trajectory. A variety of genetic, epigenetic, micro-environmental and metabolic stressors work as selective pressures. To acquire advanced fitness, immature clone goes up to the higher attractor state. In accordance with evolution, the state of each attractor becomes more robust, although, in parallel, the hurdle to the next attractor becomes higher. When compared to the normal cell differentiation trajectory for which visual representation of attractor was originally developed, cancer evolutionary trajectory seems to be the reverse movement, i.e., de-differentiation. In our opinion (see Figure 2), cancer stem cell (CSC) of HNSCC (purple) lies in the highest attractor. Of note, for simplification we depicted the trajectory of HNSCC in a two-dimensional format, but it should be multi-dimensional and the trajectory is not a single route. We postulate that in this evolutionary trajectory HNSCC acquires twelve hallmarks. As discussed in Figure 2 and text, we categorized the epithelial–mesenchymal transition (EMT) and CSC as the distinctive properties that the most advanced HNSCC display.

view of the impact of two fundamental discoveries, EMT and CSC, we propose a model of the 12 hallmarks of solid tumors that might be applicable to HNSCC (Figure 1): (1) self sufficiency in growth signals, (2) insensitivity to growth inhibitory signals, (3) evasion of programmed cell death, (4) limitless replicative potentials, (5) sustained angiogenesis, (6) tissue invasion and metastasis (7) evading immune surveillance, (8) cancer promoting inflammation, (9) genetic instability, mitotic stress, proteotoxic stress, and oxidative stress, (10) cancer specific metabolism, (11) EMT, and (12) CSC. Rather than the perspective of the reductionist that has governed cancer researches for a long time, these hallmarks-oriented views might better provide us a clue to identify the Achilles’ heel of an evolving system.

4.2. Targeting EMT and CSC for improved chemo/radioselection

In our categorization of HNSCC hallmarks, we ranked the EMT and CSC at the top of the hierarchy (i.e., the most

malignant phenotypes) (Figure 1). This view is based on our hypothesis depicted in Figure 2. The CSC population of HNSCC is likely to originate from clonally expanding cancer cells (Figure 1) at a relatively advanced phase of tumorigenesis, because a majority of early stage HNSCCs, in particular, Stage I and II laryngeal and oropharyngeal cancers, are cured by chemoradiation or radiation alone and seldom recur or metastasize (Cohan et al., 2009; Hirasawa et al., 2010; Kumamoto et al., 2002; Selek et al., 2004). Therefore, early stage HNSCC apparently lacks the distinctive properties of CSC: strong resistance to chemoradiation (Baumann et al., 2008; Dean et al., 2005). Hence, the emergence of the CSC in HNSCC appears to occur at a relatively advanced phase of tumor progression. However, this model does not exclude the possibility that CSCs of HNSCC originate from normal stem cells that have experienced genetic and epigenetic alterations (Figure 2). Our definition of CSC in HNSCC is akin to that proposed by Baumann et al (Baumann et al., 2008): a CSC is the cell that has capacity to cause tumor recurrence when left after irradiation in its natural environment.

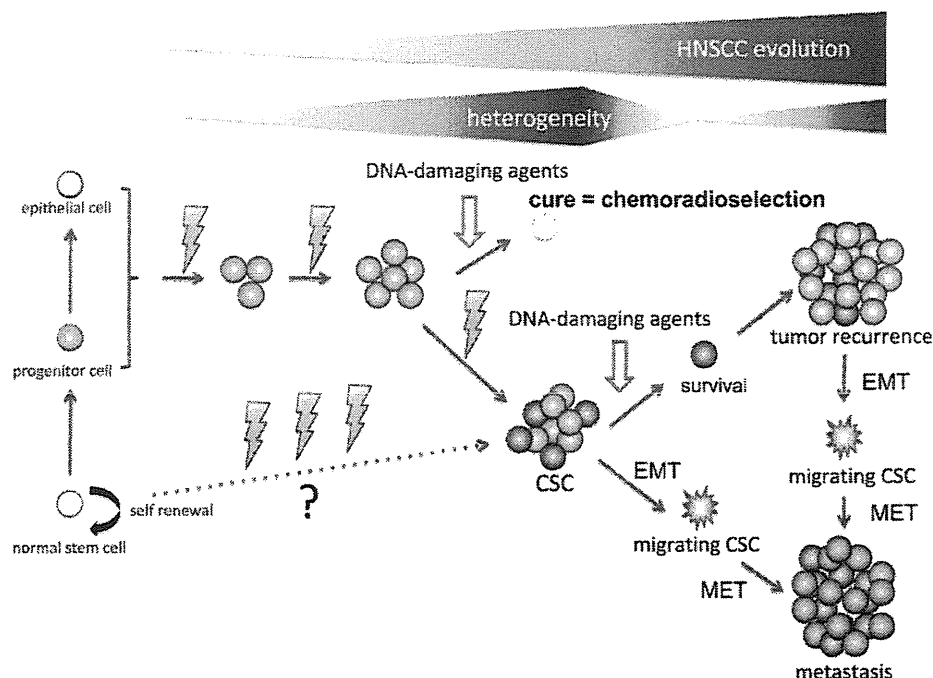


Figure 2 – Proposed mechanism of the development of cancer stem cell (CSC) (purple) in HNSCC and its relation to sensitivity to DNA-damaging agents, and tumor recurrence and metastasis. CSCs of HNSCC appear to originate from cancer cells that have undergone processes of multistage carcinogenesis (orange and green) at the advanced stage of tumor progression, since a majority of early stage tumors are cured by chemoradiation or irradiation alone (i.e., chemoradioselection) lacking the CSC phenotype. Thus, the origin of HNSCC is not likely CSC. However, this model does not exclude the possibility that CSCs of HNSCC originate from normal stem cells (white) that accumulated a series of abnormalities. CSCs can survive treatments with DNA-damaging agents and propagate recurrent tumor. CSCs that have undergone epithelial–mesenchymal transition (EMT) can migrate as “migrating CSCs” (jagged purple) and propagate metastatic tumors after they have, in turn, undergone mesenchymal epithelial transition (MET). Recurrent and metastatic tumors appear to be composed of relatively homogeneous cell population that has two distinctive properties: insensitivity to DNA-damaging therapy and ability to rapidly expand gross tumor volume. For more details see text.

Intriguingly, recurrent and metastatic tumors seldom respond to chemotherapeutic agents and often grow at an amazingly rapid speed leading to the death of the host on the order of months. Thus, these tumors are likely composed of relatively homogeneous cells that have two distinctive traits: insensitivity to DNA-damaging therapies and ability to rapidly expand gross tumor volume (Figure 2). Presumably recurrent or metastatic tumor cells are mainly composed of advanced cancer cells in the higher cancer attractors, which originate (i.e., fall down) from the CSC attractor (Figure 1). Thus, these cells can grow fast escaping from quiescent state of CSC, but still retain the distinctive feature of “stemness”, strong protection from apoptosis (Visvader and Lindeman, 2008).

As described in recent reviews (Brabletz et al., 2005; Chaffer and Weinberg, 2011; Hanahan and Weinberg, 2011; Polyak and Weinberg, 2009), the concepts of EMT and the CSC are closely related and the dynamically changing phenotypes displayed by individual cancer cells during EMT or MET appear to be compatible with stem cell or multi-potential progenitor cells. Thus, in this review we support the migrating CSC theory: CSCs that undergo EMT acquire the abilities to migrate, metastasize and propagate metastatic tumors (Figure 2) (Brabletz et al., 2005). Taken together, we consider

EMT and stemness as distinctive properties displayed by the most evolved cancer cells that have acquired invulnerability as well as pluripotency to transform into phenotypically diverse cells (Figure 1).

Within the last decade, the mainstay of definitive treatment for advanced HNSCC has shifted from radical surgery plus post-operative irradiation to organ-preserving strategies, employing dose-intensified chemotherapy and/or chemoradiotherapy (Haddad and Shin, 2008). During this shift, the novel concept of chemoselection has emerged as a rationale for organ preservation (Urba et al., 2006; Worden et al., 2008, 2009). This concept highlights the observation that those patients who display favorable responses to induction chemotherapy tend to be cured by additional chemoradiotherapy without surgical intervention, thereby facilitating organ preservation. In line with this concept, we have developed a program of concomitant chemoradiation, including a course of 30–40 Gy, as a means of “selection” (i.e., chemoradioselection) for more than 30 years (Kumamoto et al., 2002; Masuda et al., 2012). Accordingly, if we could reverse the EMT and CSC hallmarks in HNSCC, which is now considered to be the major reason for chemo/radio resistance, an increasing number of patients might benefit from chemoradioselection (Figure 2), achieving organ preservation.

Recent studies on CSC and EMT in HNSCC are summarized in Table 1. Several markers that could be used in the isolation of CSCs of HNSCC have been identified including CD44, CD133 and ALDH (Chen, 2009; Clay et al., 2010; Prince and Ailles, 2008). However, it is of note that the reliability of CD44 as a marker of HNSCC stemness was questioned by Mack and Gires (2008), because frequent CD44 expression can be observed in both normal and carcinoma tissues of the head and neck. Consistent with our idea that the process of HNSCC evolution is that of de-differentiation (Figure 1), recent studies of HNSCC have demonstrated constitutive activation of several embryonic markers or molecules that play critical roles during organogenesis, maintenance of cellular lineage, wound healing, and differentiation. These include Stat3, Oct-4, Nanog, BMI1, Wnt, and Notch (Agrawal et al., 2011; Chen et al., 2009; Chiou et al., 2008; Masuda et al., 2010; Prince et al., 2007; Song et al., 2010; Stransky et al., 2011; Zhang et al., 2010). In general, our understanding of the molecular mechanisms of CSC development in HNSCC remains limited.

It has been demonstrated that several signaling axes or molecules can promote EMT in HNSCC including, IL-1 β /Cox-2/ZEB1, src, TrkB/Twist, BMI1/PTEN/PI3K/Akt, and BMI1/Twist, causing the downregulation of E-cadherin (Table 1) (Dohadwala et al., 2010; Grille et al., 2003; Kupferman et al., 2010; Mandal et al., 2008; Song et al., 2009; St John et al., 2009). Overexpression of the mesenchymal marker, HMG A2 that was observed in more than 70% oral squamous cell carcinoma tissues, was significantly associated with poor outcomes in patients (Miyazawa et al., 2004). A recent study demonstrated Twist1 and BMI1, interacting each other, promote tumor acquisition of both EMT and CSC traits and thereby worsen the prognosis of patients with HNSCC (Yang et al., 2010). Furthermore, the existence of migrating CSCs (Figure 2) in HNSCC was demonstrated by a recent study, in which a highly metastatic subpopulation of cancer cells selected from a xenograft mouse model expressed high levels of CSS markers, including CXCR4 and integrin β 1, and altered levels of EMT markers such as E-cadherin and vimentin (Chen, 2009). Biddle et al. (2011) also revealed that in HNSCC cell line, there is a population of CSC that has undergone EMT. This population, they termed EMT CSCs, display the expression levels of high CD44 and low ESA. These findings suggest that fundamental functions of CSC and EMT indeed overlap in HNSCC.

The processes by which cancer cells acquire the traits of stemness and the ability to dynamically change their morphology and motility through EMT (i.e., formation of migrating CSC) (Figure 2) are presumably a consequence of drastic reprogramming (e.g., chromatin remodeling and transcriptional reprogramming) of molecular circuitry toward a state of de-differentiation, that may resemble embryonic development and wound healing (Bracken and Helin, 2009). This idea was exemplified in studies with high-throughput expression profiling analyses that displayed a “lineage addiction” model of human melanoma (Garraway and Sellers, 2006a, b; Garraway et al., 2005), “wound signature” profile of breast cancer (Chang et al., 2005, 2004; Wong et al., 2008b), and embryonic stem cell-like gene expression signature in several types of cancer (Ben-Porath et al., 2008; Wong et al., 2008a). Consistent with these findings, two whole-exome sequencing

studies recently demonstrated that the mechanism of squamous differentiation might be genetically dysregulated in HNSCC (Agrawal et al., 2011; Stransky et al., 2011). They identically discovered frequent loss-of-function mutation in Notch, thus demonstrating its tumor suppressive role. Stransky et al. (2011) further analyzed their data and speculated that a maturation arrest (i.e., de-differentiation), caused by mutations in several genes that regulate squamous differentiation (e.g., Notch and TP63), may play an important role in HNSCC carcinogenesis. The reported roles of Notch in carcinogenesis (i.e., oncogenic or tumor suppressive) have been controversial so far (Ranganathan et al., 2011). However, these reports, taken together with recent similar findings of frequent Notch mutation in SCC of the skin and lung (Wang et al., 2011b), suggest that, at least in SCC, Notch appears to function as a tumor suppressor. Collectively, further insights into the mechanism of the squamous cell differentiation in HNSCC might lead to the effective molecular targeting that can reverse EMT and CSC hallmarks and thereby improve the effects of chemoradiation (Figures 1 and 2). In addition, it appears to be of great importance to design mechanistic studies to gain insight into the roles of chromatin remodeling in the induction of EMT and CSC in HNSCC.

4.3. Targeting the “CPU” in the molecular circuitry of HNSCC

Despite a myriad of experiments data (Tables 1 and 2) including the two whole-exome sequencing studies mentioned above (Agrawal et al., 2011; Stransky et al., 2011), predominant gain-of-function mutations in oncogenes have not yet been identified in HNSCC. On the other hand, the most prevalent genetic alterations or epigenetically silenced genes are mainly observed in tumor suppressors including TP53, CDKI and Notch (Stransky et al., 2011). These findings raise a fundamental question: who drives HNSCC? A possible explanation may be that throughout the development and progression of HNSCC, the driving force is generated by the dynamic interactions of multiple non-mutated growth enhancing factors, rather than by a few mutated oncogenes. This distinctive characteristic of HNSCC is apparently a major hurdle that prevents clinically effective molecular targeting. However, this setting might be a double-wedged sword, because HNSCC has to constantly and stably generate driving force through the contextual stoichiometry of multiple intracellular molecules under a variety of intrinsic and extrinsic selective pressures. Otherwise HNSCC is not able to climb or rather falls down from the evolutionary trajectory (Figure 1). Theoretically, if we could identify a molecule that function as a central processing unit (CPU) to generate the driving force for a relatively long span of HNSCC evolution, inhibition of this molecule might cause considerable damage to HNSCC. As with computer circuitry, the “CPU molecule” must be a processor of multiple incoming information signals (inputs) and as well as an integrator and a transmitter of outgoing signals (i.e., driving force) to the peripheral devices (outputs). In cancer cell biology inputs correspond to a variety of information including mitogenic stimuli, exposure to carcinogens, cross-talk with cells in surrounding stroma, environmental conditions, and genetic and metabolic stressors. On the other

hand, outputs are comparable to a variety of biological events including cell cycle progression, check point control, immortalization, cell fate determination: senescence, apoptosis, necrosis and proliferation, modulation of angiogenic switch, and enhancement of cellular motility, which are the fundamental components of cancer hallmarks.

Among the various factors that can drive HNSCC development and progression (Tables 1 and 2), Stat3, NF- κ B and mTOR appear to play the role of potential “CPU” molecules. These three signaling axes are frequently activated in subsets of HNSCC and underlie a variety of input signals mentioned above (i.e., CPU inputs). Moreover, they can promote cell proliferation, cell cycle progression, survival, angiogenesis, motility, immune evasion, tumor-promoting inflammation, cellular metabolism, EMT and stemness (i.e., CPU outputs), through modulating the expression levels of a variety of effector proteins (Amornphimoltham et al., 2005; Colotta et al., 2009; Engelman, 2009; Guertin and Sabatini, 2007; Masuda et al., 2010; Molinolo et al., 2007; Pedrero et al., 2005; Ruan et al., 2006; Van Waes, 2007; Yu et al., 2009). As a result, inhibition of Stat3, NF- κ B or mTOR is likely to cause substantial damage to cancer cells in which these signals are constitutively activated. However, it might be optimistic to expect that single inhibition of only one of these signals may lead to complete regression, because HNSCC cells may survive these heavy “blows” by compensating each other as a surrogate CPU. This speculation is based on the findings that these three signaling can modulate a surprisingly overlapping set of downstream molecules (e.g., c-myc, cyclin D1 and IAPs) and as well there is crosstalk or feedback among them (Colotta et al., 2009; Squarize et al., 2006; Yu et al., 2009; Zhou et al., 2007a). In addition, the effects of the single inhibition might be attenuated by the heterogeneity of HNSCC. However, considering the pivotal roles of these three molecules in the molecular circuitry of HNSCC, dual or triple inhibition might surpass the robustness of HNSCC. Since small molecule inhibitors of Stat3, NF- κ B and mTOR are being developed and clinically available (Engelman, 2009; Masuda et al., 2010; Ruan et al., 2006; Specenier and Vermorken, 2012), the timing might be optimal for the development of multiple CPU targeting on condition that the problem of possible toxicities will be addressed.

4.4. Targeting non-oncogene addiction – tumor specific metabolism

Another clue for an effective molecular targeting was introduced in a recent review by Luo et al. (2009). They extended the cancer addiction concept from oncogene to non-oncogene and described its significance in relation to the five stress phenotypes of cancer. Non-oncogene addiction describes a phenomenon in which cancer cell growth and survival strongly depend on the activities of a wide variety of genes and pathways that are not inherently oncogenic themselves. Intriguingly, activation of these genes or pathways appears to play a far more essential role in the maintenance of cancer specific phenotypes than in that of normal cell viability. This observation seems to be of particular interest in the distinctive molecular circuitry of HNSCC that lacks predominant driver mutation in oncogenes as mentioned above. Among the five stress types of cancer, the tumor specific

metabolism seems to be the most attractive target, because this concept provides a distinctive view that cancer is a peculiar living organ in human body (DeBerardinis et al., 2008; Hanahan and Weinberg, 2011; Kroemer and Pouyssegur, 2008; Tennant et al., 2010). In the 1920s, Warburg found that cancer cells consume glucose at a surprisingly high rate and secrete most glucose-derived carbon as lactate, rather than oxidizing it completely in the tricarboxylic acid (TCA) cycle even under high oxygen condition – a phenomenon known as the “Warburg effect” (Warburg, 1923). The reason why cancer cells employ this inefficient metabolic system as the source of ATP is not clear but must be related to their ability to adapt cancer specific environment. In a recent comprehensive review, Kroemer and Pouyssegur (2008) summarized the attributes of the cancer specific metabolic system into four points. First, in this setting cancer cells can live in conditions of fluctuating oxygen tension. Second, an acidic condition generated by cancer-derived lactic acid is advantageous for tumor invasion and immune suppression. Moreover lactate produced by cancer cells is taken up by stromal cells to generate pyruvate that, in turn, refuels cancer cells. In this microecosystem, anaerobic cancer cells utilize aerobic stromal cells as a source of energy for survival and proliferation through these complementary metabolic systems. Third, nicotinamide adenine dinucleotide phosphate (NADPH) produced through the pentose phosphate pathway (PPP) protects cancer cells against oxidative damage derived from their microenvironment or chemotherapeutic agents. Fourth and perhaps most importantly, cancer cells obtain high demanding biomaterials for *de novo* cell replication (i.e., tumor proliferation) via glucose degradation. To this end, intermediates generated in the glycolytic pathway are used for biosynthesis of nucleotides, lipids, and amino acids rather than oxidative phosphorylation. Collectively, tumor specific aerobic glycolysis (i.e., “Warburg effect”) may describe a much more sophisticated setting than Warburg originally anticipated, clearly reflecting the processes of cancer evolution.

Several recent findings demonstrated that HNSCC is indeed dependent on this peculiar glycolysis pathway. The list of dysregulated molecules in HNSCC metabolism is shown in Table 1. In brief, upregulation of PDK1, LDH, TKTL1, HIF-1 α , and ROS, frequent (about 50%) mitochondrial DNA mutations and constitutive activation of the Akt/mTOR pathway were associated with enhanced cellular growth and in some cases with poor prognosis of patients by advancing Warburg metabolic phenotype (Engelman, 2009; Hoogsteen et al., 2007; Koukourakis et al., 2009; McFate et al., 2008; Molinolo et al., 2009; Schneider et al., 2008; Sun et al., 2010; Tennant et al., 2010; Wigfield et al., 2008; Zhou et al., 2007b). Of note, there were strong interactions within these factors.

Collectively, “glycolysis addiction” might be a highly exploitable target in HNSCC for three reasons. First, Warburg effect might be a necessary condition for the maintenance of other hallmarks of HNSCC, especially, in advanced stage, because without a method to meet the excessive demand for energy and biomaterials, cancer cannot progress beyond a certain point: probably quite early stage. Second, even for highly adapting cancer cells it seems difficult to establish *de novo* alternative metabolic pathways independent of excessive glucose supply, so far as they are constituents of human body.

Third, taken together, inhibition of aerobic glycolysis might surpass the plasticity of HNSCC and thereby can cause the drastic reversion of HNSCCs to early stage tumors, a majority of which could be cured by conventional DNA-damaging modalities – the strategy of chemoradioselection mentioned above. A recent finding that addition of 2-deoxy-D-glucose, an inhibitor of glucose metabolism, to cisplatin demonstrated enhanced cytotoxicity in HNSCC cell lines, at least in part, supports this idea (Simons et al., 2007). The development of anti-Warburg therapy seems promising.

5. Molecular diagnosis for the ideal molecular targeting

In this perspective review, we have discussed about working hypotheses to circumvent the robustness of HNSCC and also to reverse its hallmarks to the curable levels by conventional DNA-damaging agents from a systemic view. However, it is apparent that the development and progression of HPV-negative HNSCC is dependent on the distinctive molecular circuitry that function as a “complex adaptive system” (Merlo et al., 2006; Tian et al., 2011). Thus, in parallel with this strategy, the development of the “molecular diagnosis”, a holistic conceptualization of the entire molecular circuitry of HNSCC, appears to be the most powerful solution that enables us to precisely capture the vulnerability of HNSCC and as well to accurately predict the effects of molecular targeting and the consequent probabilities of adaptation (i.e., further evolution). During the last decade, we have witnessed tremendous progress in the development of molecular techniques to obtain genetic, epigenetic, transcriptomic, proteomic, secretomic and metabolic information on an unprecedented scale, although the assessment of dynamic interplay between the tumor and environmental factors is still underway (Gonzalez-Angulo et al., 2010; Hanahan and Weinberg, 2011; Kreeger and Lauffenburger, 2010). It is expected that by processing this myriad information through powerful and sophisticated methods based on computational biology, including network theory, and systems biology that integrate physics and mathematical approaches into biological and medical insights, we can visualize how cellular behavior of HNSCC is driven by complex molecular circuitry (Gonzalez-Angulo et al., 2010; Kreeger and Lauffenburger, 2010; Merlo et al., 2006; Tian et al., 2011). Compared to other human malignancies, HNSCC has an apparent advantage that tumor specimens can be relatively easily obtained under direct observation. Thus, HNSCC might provide the ideal platform of “molecular diagnosis” for continued global monitoring of the intrinsic molecular circuitry that will allow the timely identification of the Achilles’ heel that is hidden behind the robustness and evolvability of HNSCC.

Conflict of interest

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