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今月のテーマ　進行肝癌治療の現状と今後

進行肝細胞癌に対するソラフェニブの現状と今後の展望

池田公史　光永修一　清水　怜
大野　泉　高橋秀明　奥山浩之
桑原明子[1)]　奥坂拓志[2)]

要旨：ソラフェニブは，プラセボと比較した２つのランダム化比較試験において明らかな延命効果が示され，進行肝細胞癌に対する標準治療として位置付けられた．これまで局所療法を駆使して患者の延命を図ってきたが，ソラフェニブの登場で生存期間の更なる延長が期待できるようになった．ソラフェニブを上手く活用するために，適応，治療成績や副作用を熟知しておくことが重要である．また，ソラフェニブ登場前から日本でよく行われている肝動注化学療法との棲み分けを明らかにすることも重要である．現在，ソラフェニブは局所療法後の補助療法，肝動脈化学塞栓術との併用療法の開発が行われており，早期の対象にも適応が広がることが期待されている．

索引用語：肝細胞癌，ソラフェニブ，化学療法

はじめに

ソラフェニブは，癌細胞の増殖に関与するRAFと，癌周囲の血管新生に関与する血管内皮細胞増殖因子受容体（vascular endothelial growth factor receptor；VEGFR）や，血小板由来増殖因子受容体（platelet derived growth factor receptor；PDGFR）などに対するマルチキナーゼ阻害剤[1)2)]である．このソラフェニブは，欧米を中心に，切除不能肝細胞癌患者を対象としてプラセボと比較したランダム化比較試験（Sorafenib Hepatocellular Carcinoma Assessment Randomized Protocol（SHARP）試験）が行われ，有意に良好な病勢制御割合，増悪までの期間，生存期間が報告された[3)]．また，中国，韓国，台湾のAsia-Pacific諸国を中心に，SHARP試験と同様の対象に対してソラフェニブとプラセボを比較したランダム化比較試験[4)]が行われ（Asia-Pacific試験），有意に良好な病勢制御割合，増悪までの期間，生存期間が報告された．この２つのランダム化比較試験の結果，ソラフェニブは進行肝細胞癌に対する標準的な化学療法として位置付けられた．日本でも2009年５月に切除不能の肝細胞癌患者に対して保険適応となり，これまでに13000人を超える肝細胞癌の患者がソラフェニブで治療されている．この進行肝細胞癌に対するソラフェニブ治療の現状（適応，治療成績，副作用マネージメントなど）[3)~11)]と今後の展望について，国立がん研究センターのデータも交えながら概説する．

1）独立行政法人国立がん研究センター東病院肝胆膵内科　2）独立行政法人国立がん研究センター中央病院肝胆膵内科
Current status and future perspective of sorafenib for advanced hepatocellular carcinoma
Masafumi IKEDA, Shuichi MITSUNAGA, Satoshi SHIMIZU, Izumi OHNO, Hideaki TAKAHASHI, Hiroyuki OKUYAMA, Akiko KUWAHARA[1)] and Takuji OKUSAKA[2)]
1) Division of Hepatobiliary and Pancreatic Oncology, National Cancer Center Hospital East, 2) Hepatobiliary and Pancreatic Oncology Division, National Cancer Center Hospital
Corresponding author：池田　公史（masikeda@east.ncc.go.jp）

I ソラフェニブ治療の現状

1. ソラフェニブの適応

ソラフェニブは，SHARP試験[3]やAsia-Pacific試験[4]において延命効果が示され，標準治療として位置付けられたため，これらの試験の対象がソラフェニブの適応と考えられる．これらの試験の主な適格規準は，切除やラジオ波焼灼術（radiofrequency ablation；RFA），肝動脈化学塞栓術（transcatheter arterial chemoembolization；TACE）などの局所療法の適応がない高度進行例，またはTACEなどの局所療法に抵抗性を示す進行肝細胞癌例であり，高度進行例として肝外転移や脈管浸潤を有する例が多数登録されていた．

Barcelona Clinic Liver Cancer Study Group（BCLC）の治療方針[1]では，Stage C（Advanced stage）の肝外転移を有する例，脈管浸潤を有する例，performance status(PS)1～2の症例，Stage B（Intermediate stage）のTACEに不応の多発例がソラフェニブ治療の適応と記載されている．また，肝癌診療マニュアル第2版によるコンセンサスに基づく肝細胞癌治療アルゴリズム2010年改訂版[12]では，肝外転移を有する例，高度脈管浸潤を有する例，TACE不応例がソラフェニブ治療の対象となるとアルゴリズム上に記載されている．したがって，ソラフェニブの主な適応としては，TACE不応例，脈管浸潤を有する例，肝外転移を有する例が挙げられる．

1）TACE不応例

ソラフェニブ登場前，TACE不応例には日本では肝動注化学療法が中心に行われてきた．国立がん研究センター東病院と中央病院で検討したTACE不応例（n＝84）に対するシスプラチンの肝動注化学療法では，奏効割合3.6%，無増悪生存期間（中央値）1.7カ月，生存期間（中央値）7.1カ月であった[13]．切除不能肝細胞癌に対するシスプラチンの肝動注化学療法の第II相試験（n＝80）の結果は，奏効割合33.8%，1年生存割合67.5%と良好な治療効果であったが[14]，TACE不応例に限定するとあまり良好な治療効果は期待できないようである．

TACE不応例は，SHARP試験[3]やAsia-Pacific試験[4]の適格規準に含まれているため，ソラフェニブの適応と記載されている．しかし，SHARP試験やAsia-Pacific試験のサブグループ解析での結果も示されておらず，これまでにTACE不応に限定した前向き試験の結果も報告されていない．今後，TACE不応例に対する治療成績を確認することが必要である．

2）脈管浸潤を有する例

SHARP試験[3]やAsia-Pacific試験[4]において，脈管浸潤の有無でサブグループ解析を実施した成績が報告されており［ハザード比（95% 信頼区間（confidence interval；CI）：SHARP試験：脈管浸潤あり0.68（0.49～0.93），脈管浸潤なし0.74（0.54～1.00），Asia-Pacific試験：脈管浸潤あり0.63（0.39～1.03），脈管浸潤なし0.64（0.42～0.96）］，脈管浸潤の有無にかかわらず，ソラフェニブは有効であることが示唆されている．

脈管浸潤を有する例に対しては，従来より日本では肝動注化学療法が行われ，良好な抗腫瘍効果が報告されている．これまでにシスプラチン[13)～16)]，フルオロウラシル（5-fluorouracil；5-FU）＋シスプラチン[17)～20)]や5-FU＋インターフェロン[21)～24)]などで高い奏効割合や良好な遠隔成績が報告されている（Table 1）が，大規模な前向き研究やランダム化比較試験などは行われておらず，延命効果が明らかにされていないため，標準治療としてのコンセンサスは得られていない[12]．しかし，肝動注化学療法の腫瘍縮小効果が良好なことや奏効例での長期生存が得られていることから，ソラフェニブが承認された現在でも，日本では肝動注化学療法はしばしば行われている．現在，ソラフェニブ＋シスプラチン動注併用療法とソラフェニブ単独とのランダム化比較試験，シスプラチン動注療法先行ソラフェニブ療法とソラフェニブ単独とのランダム化比較試験，ソラフェニブ＋5-FU＋シスプラチン動注療法とソラフェニブ単独とのランダム化比較試験，5-FU動注療法＋インターフェロン療法とソラフェニブ単独とのランダム化比較試験など，ソラフェニブと肝動注化学療法の比較試験がいくつか行われており

Table 1. 進行肝細胞癌に対するソラフェニブと肝動注化学療法の治療成績（抜粋）

抗癌剤	症例数	奏効割合（%）	病勢制御割合（%）	増悪までの期間/無増悪生存期間（中央値：月）	生存期間（中央値：月）	備考	報告者（文献）	報告年
Sorafenib	137	2.2	36	4.2	9.2	Phase II	Abou-Alfa GK (5)	2006
Sorafenib	51	8.0	26	3.0	5	Phase II	Yau T (6)	2009
Sorafenib	296	8.1	81	9.2	10.5	Phase II	Iavarone M (7)	2011
Sorafenib	299	2.0	43	5.5	10.7	Phase III	Llovet JM (3)	2008
Sorafenib	150	3.0	35	2.8	6.5	Phase III	Cheng AL (4)	2009
Sorafenib	27	4.0	82	4.9	15.6	Phase I	Furuse J (8)	2008
Sorafenib	201	2.5	NA	2.5	5.3	Retrospective	Baek KK (9)	2011
Sorafenib	267	1.5	47	2.6	7.9	Retrospective	Kim JE (10)	2011
Sorafenib	108	3.7	21	3.2	6.1	Retrospective	Chiu J (11)	2012
CDDP	84	4	49	1.7	7.1	Retrospective	Iwasa S (13)	2011
CDDP	80	34	80	NA	67.5%（1年）	Phase II	Yoshikawa M(14)	2008
CDDP	67	37	NA	NA	10.7	Retrospective	Court WS (15)	2002
CDDP*	25	28	72	3.6	7.1	Phase II	Furuse J (16)	2008
5-FU/CDDP	41	22	56	7.0	12.0	Phase II	Park JY (17)	2007
5-FU/CDDP*	52	39	65	4.1	15.9	Phase II	Ueshima K (18)	2010
5-FU/CDDP	97	28	62	7.0	12.0	Retrospective	Kim BK (19)	2011
5-FU/CDDP	114	36	75	NA	10.2	Retrospective	Yamasaki T (20)	2012
5-FU/IFNα*	55	44	51	5.2	11.8	Retrospective	Ota H (21)	2005
5-FU/IFNα*	116	52	54	NA	34%（1年）	Retrospective	Obi S (22)	2006
5-FU/IFNα*	55	29	58	7.5	9.0	Retrospective	Uka K (23)	2007
5-FU/IFNα*	102	39	47	2.0	9.0	Retrospective	Nagano H (24)	2011

*対象：門脈腫瘍栓を有する肝細胞癌，5-FU：5-fluorouracil，CDDP：cisplatin，IFN：interferon，NA：not available.

(Table 4)，今後，肝細胞癌に対する肝動注化学療法の延命効果が検証され，さらには，ソラフェニブとの棲み分けについても検討されることが期待されている．

3）肝外転移を有する例

肝外転移を有する例に対しては，全身化学療法が最も良い適応と思われるが，SHARP 試験[3]やAsia-Pacific 試験[4]のサブグループ解析においてはあまり良好な結果は得られておらず［ハザード比(95%CI)：肝外転移あり：SHARP 試験：0.85(0.64～1.14)，Asia-Pacific 試験：0.82（0.57～1.18)］，むしろ肝外転移のない症例の方が良好な傾向である［ハザード比（95%CI)：肝外転移なし：SHARP 試験：0.55(0.39～0.44)，Asia-Pacific 試験：0.45（0.25～0.84)］．この理由は明らかにされていないが，肝外転移を有するほど進行した症例に対しては，投与期間が短くなり，有意な差を見出せなかったのではないかと考えている．肝外転移を有する例に対しては，他に有効な全身化学療法もないことから，今後もソラフェニブによる治療が行われると思われるが，さらに有効性の高い新たな治療法を開発することも必要と考えられる．

このように腫瘍側因子からみたソラフェニブの適応は，TACE 不応例，脈管浸潤を有する例，肝外転移を有する例が挙げられているが，肝予備能からみたソラフェニブの適応はどうだろうか？SHARP 試験や Asia-Pacific 試験では，Child Pugh A の症例のみを対象として有効性と安全性が確認されているため，Child Pugh A がソラフェニブの良い適応とされている．海外で行われたソラフェニブの第 II 相試験[5]では，Child Pugh B において，ビリルビンの上昇，腹水や脳症など有害事象が出現しやすく，増悪までの期間［Child Pugh

Table 2. ソラフェニブの有害事象の発現頻度

著者（文献）	発表年	試験実施国	n	手足症候群		高血圧		AST		ALT		備考
				All Grade	Grade 3	All Grade	Grade 3	All Grade	Grade 3	All Grade	Grade 3	
Abou-Alfa GK (5)	2006	海外（欧米）	137	30.7%	5.1%	NA	NA	NA	NA	NA	NA	Phase II
Iavarone M (7)	2011	海外（欧米）	296	28.0%	9.0%	18.0%	7.0%	NA	NA	NA	NA	Prospective study
Llovet JM (3)	2008	海外（欧米）	297	21%	8%	5%	2%	1.7%	1.7%	0.7%	0.7%	Phase III（SHARP 試験）
Cheng AL (4)	2009	海外（アジア）	149	45.0%	10.7%	18.8%	2.0%	NA	NA	NA	NA	Phase III（Asia-Pacific 試験）
Chiu J (11)	2012	韓国	172	40.4%	13.5%	24.4%	3.5%	NA	NA	67.7%	12.4%	Retrospective
Furuse J (8)	2008	日本	27	44.4%	7.4%	18.5%	18.5%	3.7%	3.7%	7.4%	7.4%	Phase I
Kudo M (27)	2011	日本と韓国	229	82.0%	35.0%	31.0%	15.0%	25.0%	12.0%	21.0%	8.0%	Phase III（TACE 補助療法）
東病院	2012	日本	127	69.0%	7.0%	35.0%	12.0%	57.0%	48.0%	49.0%	24.0%	Retrospective

NA, not available.
AST, aspartate aminotransferase ; ALT, alanine aminotransferase ; TACE, transcatheter arterial chemoembolization.

A（中央値）：21週，B：13週］や生存期間［Child Pugh A（中央値）：41週，B：14週］もChild Pugh Aと比べて不良であるとの報告もある．また，切除不能な肝細胞癌患者におけるソラフェニブの治療成績を検討する多国間非介入調査試験（Global Investigation of therapeutic DEcisions in unresectable HCC and Of its treatment with sorafeNib；GIDEON）の中間解析[25]では，Child Pugh Bは個々の有害事象ではChild Pugh Aと有意な差を認めなかったが，重篤な有害事象が高率であり，生存期間［Child Pugh A（中央値）：10.3カ月，B：4.8カ月］も不良であったと報告されている．このように，Child Pugh Aに分類される肝予備能が良好な症例がソラフェニブの適応と考えられている．

2．ソラフェニブの抗腫瘍効果と遠隔成績

ソラフェニブの奏効割合［完全奏効（complete response；CR）と部分奏効（partial response；PR）の割合］はこれまでの治療成績をみると，5%前後であり（Table 1），国立がん研究センター東病院（n＝127）での検討でも同様で［奏効割合5%（95% CI：2～10%）］，腫瘍縮小効果は期待できない．ただし，安定（stable disease；SD）まで含めた病勢制御割合では50%近い報告が多く，東病院の成績も57%（95%CI：48～65%）であった．このことから，ソラフェニブは，腫瘍の縮小は期待できないが，癌の進行を抑制することで延命効果が得られると考えられている．また，ソラフェニブによって，腫瘍径は縮小しなくても腫瘍濃染が消失する例が認められており，この腫瘍濃染の消失を壊死と考え，治療効果の指標として評価することも重要である．Lencioniらはこの腫瘍濃染の消失を考慮したmodified response evaluation criteria in solid tumor（modified RECIST）の概念を発表した[26]．このmodified RECISTでは腫瘍濃染が消失した部分を壊死とみなし，壊死の部分を測定しないように最長径を測定し，腫瘍縮小効果を判定する．この概念を導入することで，ソラフェニブによって効果が得られている症例をより多く見出すことが可能となった．

ソラフェニブの増悪までの期間は，おおよそ3～5カ月程度とまだ限られている（Table 1）．東病院の治療成績でも3.6カ月と厳しい現状である．また，ソラフェニブ治療開始後の生存期間はおおよそ7～10カ月程度であり（Table 1），東病院の生存期間中央値も10.6カ月と，依然，厳しい状況である．今後，他剤との併用療法や新規抗癌剤の開発などによりさらなる治療成績の向上を

Table 3. ソラフェニブ診療における日本と海外の違い（GIDEONの中間解析の結果）

	総計 (n=1571)	USA (n=313)	EU (n=588)	Latin America (n=59)	Asia (n=450)	日本 (n=161)
患者背景因子						
年齢（中央値）	62	60	67	65	53	69
Performance status 0	40	28	46	25	30	73
1	43	42	39	63	51	26
BCLC stage A	7	12	9	20	2	3
B	19	12	24	36	10	30
C	54	39	53	31	68	57
Child Pugh A	61	38	66	34	65	84
B	23	32	22	47	20	12
前切除歴	19	11	14	7	24	40
前局所療法歴	55	49	44	29	69	84
肝動脈化学塞栓術	46	37	32	15	64	76
ラジオ波焼灼術	15	10	15	15	12	38
初回治療からの時間（月）	4	3	3	1	3	30
ソラフェニブ治療						
治療期間（中央値）	12	12	14	25	9	13
1日投与量（中央値）	693	575	746	800	763	489
初回投与量：800mg	74	57	81	98	78	62
：400mg	22	34	15	2	20	36
有害事象での治療中止割合	19	22	20	3	15	32
全Gradeの有害反応	64	71	66	44	51	89
Grade 3～4の有害反応	25	26	28	8	14	44
重篤な有害反応	9	9	10	8	4	17
生存期間（中央値：月）	−	8.4	9.4	12.5	7.9	9.3

GIDEON, Global Investigation of therapeutic DEcisions in unresectable HCC and Of its treatment with sorafeNib；USA, United States of America；EU, European Union；BCLC, Barcelona Clinic Liver Cancer Group.
Kudo et al., Poster presented at AASLD2011, November 4-8, 2011, San Francisco, CA, USA.

期待したい．

3．ソラフェニブの有害事象

ソラフェニブには，手足症候群，高血圧，下痢，肝機能障害，膵酵素の上昇などの特有の有害事象がある．これらの有害事象の発現割合は，特に日本などのアジアでは，欧米と比較して高率である[4,8,11,27]（Table 2）．その原因は不明だが，アジア諸国では特定の有害事象に対するより一層の注意が必要である．また，これらの有害事象はしばしば治療の休止や中止の一因となるため，副作用をきちんとマネージメントして治療を長期に継続できるように心がけることも重要である．

東病院では，「チームネクサバール」を立ち上げ，チーム医療でソラフェニブの副作用マネージメントに取り組んでいる[28]．このチームネクサバールの中で，薬剤師や看護師は，ソラフェニブの服薬指導だけでなく，手足症候群の予防や対処方法，血圧測定の重要性，高血圧，下痢，皮疹などさまざまな副作用の対応方法について患者に説明する．外来診察日にはソラフェニブのアドヒアランスを確認し，副作用のモニタリングを行い，担当医へフィードバックする．また，次回外来診察までの間に，電話にて患者の状態を確認し（テレフォンフォローアップ），在宅でのソラフェニブのアドヒアランス確認や副作用モニタリングを行う．このように，薬剤師や看護師は，医師の診療に関わり，副作用マネージメントを施し，治療の継続性を高め，最大限の治療効果を引き出すこ

Table 4. 現在進行中のソラフェニブ関連のランダム化比較試験

BCLC stage		治療法	レジメン	相	試験/グループ名	登録番号
Stage A	Early stage	切除/RFA 後補助療法	ソラフェニブ vs. プラセボ	第 III 相試験	STORM	NCT00692770
Stage B	Intermediate stage	TACE との併用療法	ソラフェニブ vs. 経過観察	第 II 相試験	TACTICS	NCT01217034
			ソラフェニブ vs. プラセボ	第 III 相試験	ECOG	NCT01004978
			ソラフェニブ vs. プラセボ	第 III 相試験	TACE-2	NCT01324076
Stage C	Advanced stage	ソラフェニブとの併用療法	ソラフェニブ＋ドキソルビシン vs. ソラフェニブ	第 III 相試験	CALGB	NCT01015833
			ソラフェニブ＋プラバスタチン vs. ソラフェニブ	第 III 相試験	FFCD	NCT01075555
			ソラフェニブ＋エルロチニブ vs. ソラフェニブ	第 III 相試験	SEARCH	NCT00901901
			ソラフェニブ＋エベロリムス vs. ソラフェニブ	第 II 相試験	SGCCR	NCT01005199
			ソラフェニブ＋ベバシズマブ vs. ソラフェニブ	第 I/II 相試験	NCCTG	NCT00867321
		ソラフェニブと動注の併用療法	ソラフェニブ＋シスプラチン動注 vs. ソラフェニブ	第 II 相試験	奥坂班	UMIN000005703
			シスプラチン動注＋先行ソラフェニブ vs. ソラフェニブ	第 II 相試験	SCOOP-II trial	UMIN000006147
			ソラフェニブ＋5-FU/シスプラチン動注 vs. ソラフェニブ	第 III 相試験	SILIUS	NCT01214343
			5-FU 動注＋インターフェロン vs. ソラフェニブ	第 III 相試験	杏雲堂	UMIN00000240

RFA, radiofrequency ablation ; ECOG, Eastern Cooperative Oncology Group ; CALGB, Cancer and Leukemia Group B ; FFD, Federation Francophone de Cancerologie Digestive ; SGCCR, Swiss Group for Clinical Cancer Research ; NCCTG, North Central Cancer Treatment Group.

とを目標として，日々の診療に取り組んでいる．実際，東病院でのソラフェニブの有害事象はこれまでの報告と同様に，欧米と比べて高頻度に認められたが（Table 2），有害事象によるソラフェニブの中止割合は 10% と少なかった．また，その有害事象の内訳は，肝障害 5%，皮疹 3% などで，手足症候群で中止になった症例は 1 例も認めておらず，より良いソラフェニブ治療を施すことが可能となった．

4. ソラフェニブ診療における日本と海外の比較

日本を含めた世界 35 カ国以上，350 施設以上が参加した国際共同前向き非介入試験である GIDEON では，日常診療でのソラフェニブの安全性や有効性などの情報を収集して解析されており，日本と海外を比較した中間解析の結果も報告されている[29]（Table 3）．この中間解析の患者背景から判断される日本人のソラフェニブ治療例の特徴は，高齢者，PS 0 の症例が海外と比べて多

く認められたが，ソラフェニブ開始前のStageはC（Advanced stage）が57%で，海外と同様に進行した症例が治療対象であった．前治療としては，切除やRFA，TACEなどが行われている症例が海外と比べて高率であり，肝細胞癌の診断からソラフェニブ治療開始までの期間が30カ月と最も長いことが特徴的で，日本では肝細胞癌を早期に診断し，切除やRFA，TACEなどの局所療法が施された上で，ソラフェニブの治療が行われていることが示唆された．ソラフェニブの投与期間（中央値）は13週と海外と同様であったが，重篤な有害事象の発現割合は海外と比べて高率であり，有害事象で中止された症例も32%と高率であった．生存期間の中央値は9.3カ月と海外と比べてほぼ同等であった．したがって，日本でのソラフェニブ治療の現状は，前治療歴が多く，有害事象にも注意が必要であるが，治療成績に関しては海外と遜色ない結果であり，同等の効果が期待できると考えられた．

II ソラフェニブ治療の今後の展望

切除，局所療法の適応のない，または効果が期待できない進行肝細胞癌で，肝予備能はChild Pugh Aの症例に対して，ソラフェニブは標準治療として位置付けられた．さらなる治療効果を期待して，進行癌に対しては，エルロチニブやドキソルビシン[30]など他の抗癌剤との併用療法や，前述の肝動注化学療法との併用療法の開発が進行中である（Table 4）．また，切除やRFA後の補助療法，TACEとの併用療法[27)31)]も試みられており（Table 4），ソラフェニブの適応は進行癌のみならず，より早期のStageにまで適応が広がる可能性がある．

おわりに

肝細胞癌の全身化学療法は，ソラフェニブの登場により，大きく様変わりしている．ソラフェニブの腫瘍縮小効果はあまり期待できないが，病勢制御割合は高く，増悪までの期間と生存期間を有意に延長させることが明らかになり，肝細胞癌に対する標準治療として位置付けられた．このソラフェニブを上手く活用するために，ソラフェニブの適応，治療成績や副作用マネージメントを熟知しておくことが重要である．また，ソラフェニブ登場前から日本でよく行われている肝動注化学療法との棲み分けも明らかにする必要がある．現在，ソラフェニブは肝切除やRFA後の補助療法，TACEとの併用療法の開発が行われており，より早期の対象にも適応が広がることが期待されている．

本論文内容に関連する著者の利益相反
：池田公史（バイエル薬品株式会社），光永修一（中外製薬株式会社），奥坂拓志（日本イーライリリー株式会社，中外製薬株式会社，大鵬薬品工業株式会社）

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ORIGINAL ARTICLE

Elimination of esophageal multiple precancerous lesions by chemotherapy: potential chemoprevention of metachronous multiple cancer development after curative treatment

Yoshiyuki Yukawa · Manabu Muto · Yusuke Amanuma · Yasumasa Ezoe · Yoko Mashimo · Kimiko Hori · Takahiro Horimatsu · Shuko Morita · Shin'ichi Miyamoto · Sachiko Minamiguchi · Tsutomu Chiba

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Abstract
Background Dysplastic squamous epithelium is a precancerous lesion for squamous cell carcinoma. It is often present in the esophagus and head and neck region, and can be visualized as a Lugol-voiding lesion (LVL) by iodine chromoendoscopy. However, effective treatment for such dysplastic epithelia has not yet been developed.
Methods Between March 2008 and July 2011, 40 consecutive patients with advanced esophageal squamous cell carcinoma were treated by two cycles of neoadjuvant chemotherapy (NAC) (5-fluorouracil, 800 mg/m^2, d 1–5; cisplatin, 80 mg/m^2, d 1: q 21 days) at Kyoto University Hospital, and received iodine chromoendoscopy both before and after NAC. Iodine chromoendoscopy findings were divided into 4 groups: group A, absence of LVLs; group B, several ($\leq$10/one endoscopic view) small ($\leq$5 mm) LVLs; group C, many ($\geq$10/one endoscopic view) small ($\leq$5 mm) LVLs; group D, numerous irregular-shaped multiform LVLs. Group C and D are defined as multiple LVLs. Endoscopic changes of LVLs before and after NAC were investigated retrospectively.
Results Before NAC, 6, 12, 9, and 13 cases were classified in group A, B, C, and D, respectively. All cases in group A before NAC remained in group A after NAC. Multiple LVLs (group C and D) were significantly improved in 17 of 22 patients (77.3 %), while several small LVLs (group B) were improved in only 4 of 12 cases (33.3 %) ($p = 0.025$ by Fisher's exact test).
Conclusions Multiple dysplastic lesions tended to improve by chemotherapy. In contrast, there was little change in the mucosa with fewer dysplastic lesions after chemotherapy. These data show that chemotherapy has the potential to eliminate precancerous lesions.

Keywords Esophageal cancer · Precancerous conditions · Chemoprevention

Y. Yukawa · M. Muto (✉) · Y. Amanuma · Y. Mashimo · K. Hori · T. Horimatsu · S. Morita · S. Miyamoto · T. Chiba
Department of Gastroenterology and Hepatology, Kyoto University Graduate School of Medicine, 54 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan
e-mail: mmuto@kuhp.kyoto-u.ac.jp

Y. Ezoe
Department of Multidisciplinary Cancer Treatment, Kyoto University Graduate School of Medicine, Kyoto, Japan

S. Minamiguchi
Department of Pathology, Kyoto University Graduate School of Medicine, Kyoto, Japan

Introduction

There were 462,000 new cases of esophageal cancer and 386,000 patients died of the disease in 2002 worldwide [1]. Head and neck (H&N) cancer accounted for 643,000 new cases and 349,000 deaths in 2002 worldwide [1]. The prognosis of these cancers remains poor because of lack of symptoms at early stages and hence difficulty of early detection, resulting in presentation as advanced cancer.

Esophageal cancer is divided into two major histological types, squamous cell carcinoma (SCC) and adenocarcinoma, with the former being the most common histological type worldwide. The most common histological type of H&N cancer is also SCC. Alcohol and tobacco are definite human carcinogens for both esophageal SCC (ESCC) and H&N SCC (HNSCC) [2]. Furthermore, acetaldehyde derived from ethanol potentially causes the phenomenon of

"field cancerization" [3], whereby multiple SCCs develop in not only the esophagus but also the H&N regions.

Dysplastic squamous epithelium is recognized as a precancerous lesion for SCC. These dysplastic lesions are not visible at routine endoscopy. However, because these lesions do not stain with Lugol's iodine solution, they are visualized as Lugol-voiding lesions (LVLs) by iodine chromoendoscopy and easily detected [4]. Multiple dysplastic lesions often appear in the esophageal mucosa and have been revealed to be a risk factor for multiple development of SCC in both the esophagus and the H&N regions [5, 6]. Clinically, multiple development of SCC causes the following critical problems: (1) difficulty in treatment, (2) poor prognosis, and (3) recurrence after treatment. If these dysplastic lesions could be eliminated, multiple SCC development would be prevented. However, effective treatment for these precancerous lesions has not yet been established.

Anticancer drugs have strong cytotoxic activity against cells with malignant potential. 5-Fluorouracil (5-FU) and cisplatin (CDDP) are key drugs for treatment of ESCC [7]. If these drugs have chemopreventive effect on precancerous lesions, they would be candidate chemopreventive agents.

In this study, we investigated morphological changes of Lugol staining patterns in the background esophageal mucosa before and after neoadjuvant chemotherapy (NAC) for ESCC and assessed the chemotherapeutic effect on dysplastic squamous epithelia.

Patients and methods

Between March 2008 and July 2011, 43 patients with advanced ESCC received NAC at Kyoto University Hospital. NAC comprised 2 cycles of the FP regimen (5-FU, 800 mg/m^2, d 1–5; CDDP, 80 mg/m^2, d 1: q 21 days) [7]. Among them, three patients did not receive iodine chromoendoscopy after NAC. Thus, we analyzed 40 patients in this study.

Dysplastic epithelia in the esophagus were evaluated by iodine chromoendoscopy. At the endoscopic examination, approximately 10 ml 1.5 % iodine solution was sprayed along the whole length of the esophagus using a spraying catheter. A well-demarcated unstained area was defined as a LVL. More than 10 in number LVLs in one endoscopic view were defined as many occurrences, and ten or fewer in number as several occurrences. LVLs less than 5 mm in diameter were defined as small. When several sizes of LVL with diameters greater than 5 mm were observed, the esophagus exhibited an irregular-shaped multiform pattern of LVLs. Lesions diagnosed as definite esophageal cancer by biopsy were excluded from LVL counts. Filed endoscopic images were reviewed by two experienced endoscopists (Y.Y. and M.M.), and endoscopic findings were categorized into the following 4 groups according to the previous report [8] (Fig. 1): group A, absence of LVLs; group B, several ($\leq$10/one endoscopic view) small ($\leq$5 mm in diameter) LVLs; group C, many ($\geq$10/one endoscopic view) small ($\leq$5 mm in diameter) LVLs; group D, numerous irregular-shaped multiform LVLs. Group C and D are defined as multiple LVLs. When the LVLs decreased and/or shrunk to former groups (i.e., group D to group A, B, or C, group C to group A or B, and group B to group A) after NAC, it was defined that LVLs were improved.

TNM classification (version 6) was used for clinical staging. Histological evaluation of the pathological criteria for the effects of NAC followed the Japanese classification of esophageal cancer 10th edition as follows: grade 0, no recognizable cytological or histological therapeutic effect; grade 1, apparently visible cancer cells account for 1/3 or more of the tissue, but there is some effective evidence of degeneration of cancer tissue or cells; grade 2, visible cancer cells account for less than 1/3 of the tissue, while other cancer cells are severely degenerated or necrotic; grade 3, no viable cancer cells are evident [9].

Statistical analysis was performed using SPSS Statistics version 17 software (SPSS Inc., Chicago, IL). Continuous variables are expressed as median and range. The Kruskal–Wallis test and Fisher's exact test were used to compare continuous data and categorical data, respectively. When post hoc paired comparisons were performed, Bonferroni's correction was used to adjust the p value for multiplicity. p-Value less than 0.05 was considered significant.

Results

For the 40 patients, median age was 65 years (range 50–76 years); 34 (85.0 %) were male and 6 (15.0 %) were female. Clinical stages of primary ESCC were as follows: 10 (25.0 %) patients at IIA, 17 (42.5 %) at IIB, 11 (27.5 %) at III, 1 (2.5 %) at IVA, and 1 (2.5 %) at stage IVB. Nine patients (22.5 %) had another synchronous ESCC, and five patients (12.5 %) had synchronous pharyngeal SCC. Of them, 2 patients had both another synchronous ESCC and synchronous pharyngeal SCC. Thirty-five patients (87.5 %) were habitual drinkers, and median ethanol amount consumed was 60 g/day (range 0–450 g/day). Thirty-five patients (87.5 %) were smokers, and median Brinkman index was 600 (range 0–1600).

The dose of 5-FU and CDDP was reduced (80 %) at the second cycle of the FP regimen in one case, and the second cycle of the FP regimen was postponed by 2 weeks in another three cases.

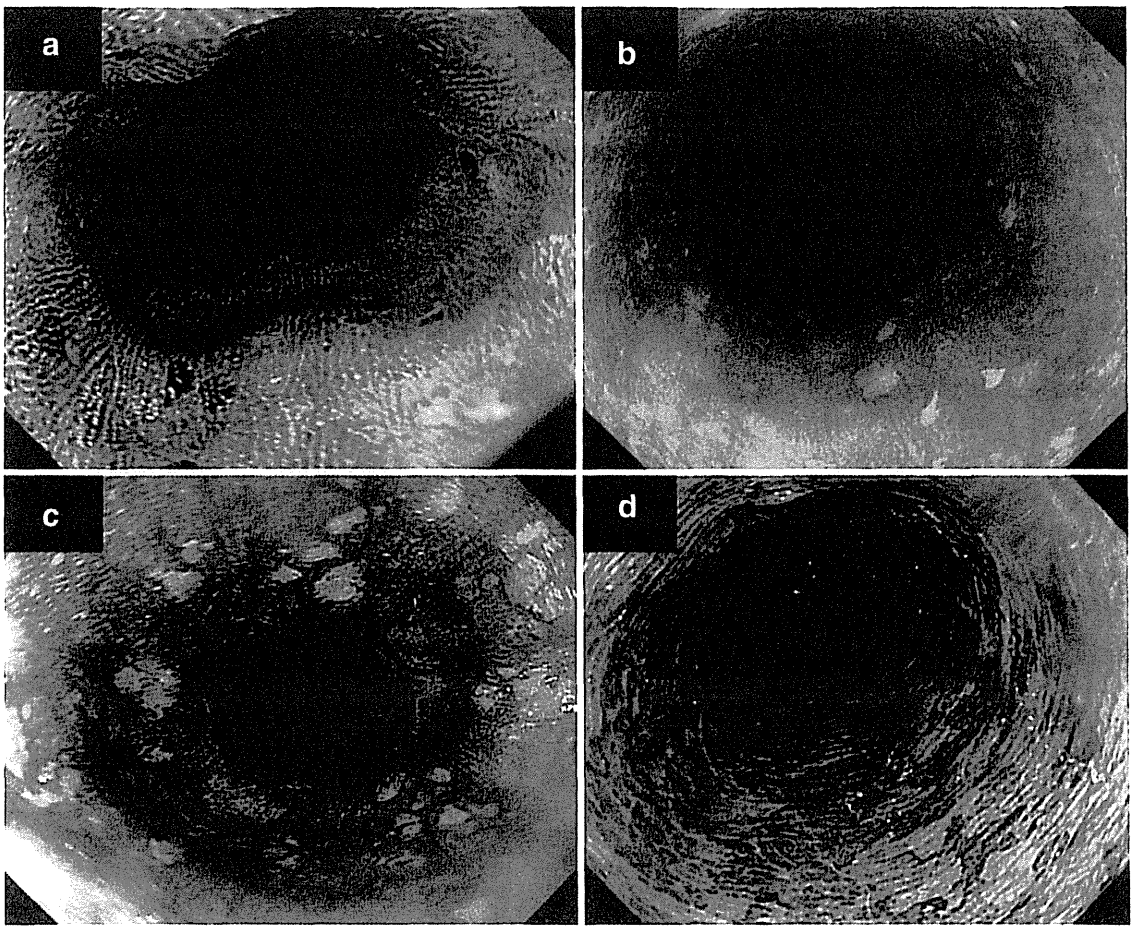

Fig. 1 Classification of iodine staining patterns of esophageal mucosa. A well-demarcated unstained area by iodine chromoendoscopy was defined as a LVL: **a** group A, absence of LVLs; **b** group B, several (≤10/one endoscopic view) small (≤5 mm) LVLs; **c** group C, many (≥10/one endoscopic view) small (≤5 mm) LVLs; **d** group D, numerous irregular-shaped multiform LVLs. Group C and D are defined as multiple LVLs. *LVL* Lugol-voiding lesion

Among 40 patients, before NAC, 6 cases were classified in group A, 12 cases in group B, 9 cases in group C, and 13 cases in group D. All 5 patents with synchronous HNSCC were in group D, and there was significant difference in the frequency of synchronous HNSCC between the 4 groups ($p = 0.008$ by Fisher's exact test); however, by post hoc paired comparisons, there was no significant difference (Table 1).

Endoscopic evaluation for changes in LVLs before and after NAC are summarized in Table 2. All 6 cases in group A before NAC remained in that group after NAC. Of 12 cases in group B before NAC, 4 improved and were reassigned to group A, while 8 remained in group B after NAC. Of 9 cases in group C before NAC, 8 improved and were reassigned to group B, and only one remained in group C after NAC. Of 13 cases in group D before NAC, 9 improved (2 to group B and 7 to group C) after NAC.

Multiple LVLs (group C and D) significantly improved in 17 of 22 patients (77.3 %), while only 4 of 12 cases (33.3 %) improved in group B ($p = 0.025$ by Fisher's exact test) (Fig. 2). Figure 3 shows a representative case. In this case, primary ESCC shrunk after 2 cycles of NAC. Furthermore, while the surrounding esophageal mucosa was classified in group D before NAC, it was apparently improved and reassigned to group B after NAC. In another case, similar to the endoscopic improvement of LVLs, one of the LVLs was histologically improved from high-grade intraepithelial neoplasia to mild atypical squamous epithelium indefinite for low-grade intraepithelial neoplasia or reactive atypia (Fig. 4).

In 6 patients who were classified into group A before NAC, all cases received esophagectomy, and pathological effects of NAC on the primary ESCC were grade 1 in 4 cases and grade 2 in 2 cases. In 40 patients who had LVLs (group B, C, and D) before NAC, 3 patients could not receive esophagectomy after NAC because of distant metastasis [liver metastasis (1), peritoneal dissemination (1)] and aortic invasion (1), and 31 patients received

Table 1 Demographics of the patients ($n = 40$)

	Group A	Group B	Group C	Group D	p
Patients, n	6	12	9	13	–
Sex					
Male	3	11	9	11	0.075
Female	3	1	0	2	
Age (years), median (range)	68.0 (54–72)	63.5 (56–75)	66.0 (50–76)	65.0 (50–74)	0.893
Another synchronous ESCC, n (%)	1 (16.7 %)	2 (16.7 %)	1 (11.1 %)	5[b] (38.5 %)	0.513
Synchronous HNSCC, n (%)	0 (0 %)	0 (0 %)	0 (0 %)	5[a, b] (38.5 %)	0.008
Habitual drinkers, n (%)	4 (66.7 %)	11 (91.7 %)	8 (88.9 %)	12 (92.3 %) 1; unknown	0.155
Ethanol amount (g/day), median (range)	35 (0–180)	65 (0–150)	50 (0–165)	105 (25–450)	0.135
Smokers, n (%)	4 (66.7 %)	10 (83.3 %)	9 (100 %)	12 (92.3 %)	0.244
Brinkman index, median (range)	518 (0–1500)	600 (0–900)	800 (40–1600)	600 (0–1600)	0.458

[a] All of 5 cases had synchronous pharyngeal cancer

[b] 2 patients had both synchronous another ESCC and synchronous pharyngeal cancer

ESCC esophageal squamous cell carcinoma, *HNSCC* head and neck squamous cell carcinoma

Table 2 Endoscopic changes of LVLs before and after NAC

Before NAC	After NAC				Improvement (%)	
	Group A	Group B	Group C	Group D		
LVLs (−)						
Group A ($n = 6$)	6	0	0	0	–	
LVLs (+)						
Group B ($n = 12$)	4	8	0	0	33.3 (4/12)	
Group C ($n = 9$)	0	8	1	0	88.9 (8/9)	61.8 (21/34)
Group D ($n = 13$)	0	2	7	4	69.2 (9/13)	
Total ($n = 40$)	10	18	8	4	–	

Number of patients is shown. Before NAC, among 40 patients, LVLs were absent in 6 cases (group A), and LVLs were found in 34 cases (group B, C, and D). After NAC, LVLs were visually improved in 21 cases (61.8 %) of 34 patients who had LVLs

LVLs Lugol-voiding lesions, *NAC* neoadjuvant chemotherapy

esophagectomy. In 31 patients who had LVLs before NAC and received esophagectomy after NAC, the relationship between the pathological effects of NAC on the primary ESCC and the endoscopic effects of NAC on the LVLs was assessed. Among 18 patients who achieved improvement of LVLs after NAC, pathological effects of NAC on the primary ESCC were grade 1 in 10 cases, grade 2 in 5 cases, and grade 3 in 3 cases. Among 13 patients who did not achieve improvement of LVLs after NAC, pathological effects of NAC on the primary ESCC were grade 0 in 1 case, grade 1 in 9 cases, and grade 2 in 3 cases, but grade 3 in no case. Improvement of LVLs was not associated with chemotherapeutic effects on primary ESCC after NAC ($p = 0.312$ by Fisher's exact test).

Discussion

The present study is the first report to show that multiple LVLs tend to endoscopically improve by chemotherapy. In contrast, there was little change in the mucosa of patients with fewer dysplastic lesions by chemotherapy. Although not all LVLs actually represent precancerous lesions, these data show that chemotherapy has potential to prevent squamous carcinogenesis.

Since the first report by Voegeli et al. [10], iodine chromoendoscopy has been the standard method for detection of early ESCC and evaluation of its extension and multiplicity. In addition to these uses, iodine chromoendoscopy is useful not only to detect precancerous lesions

with malignant potential to develop into ESCC, but also to predict risk of ESCC and HNSCC [5, 6, 8, 12, 13].

We previously assessed 434 LVLs in cases with HNSCC histopathologically, and diagnosed as follows: SCC (17.3 %), severe dysplasia (2.5 %), moderate dysplasia (11.5 %), mild dysplasia (19.6 %), parakeratosis (37.3 %), and nondysplastic and nonparakeratotic (11.8 %) [8]. Kouzu et al. [11] examined 159 Lugol unstained lesions in cases with ESCC histopathologically, and reported that 69 % of unstained lesions of more than 10 mm were diagnosed as cancer, while 72 % of unstained lesions less than 5 mm in diameter were diagnosed as esophagitis or normal mucosa. Hori et al. [6] examined 456 LVLs in cases with ESCC, HNSCC, or both ESCC and HNSCC histopathologically, and reported that, as LVL size increased, high-grade intraepithelial neoplasia was significantly more frequently detected than nonneoplastic lesions and low-grade intraepithelial neoplasia. Therefore, we speculated that, in this study, because a part of small LVLs less than 5 mm in diameter in group B and group C were precancerous and improved by chemotherapy, many small LVLs (group C) improved to several small LVLs (group B), but several small LVLs (group B) remained in group B.

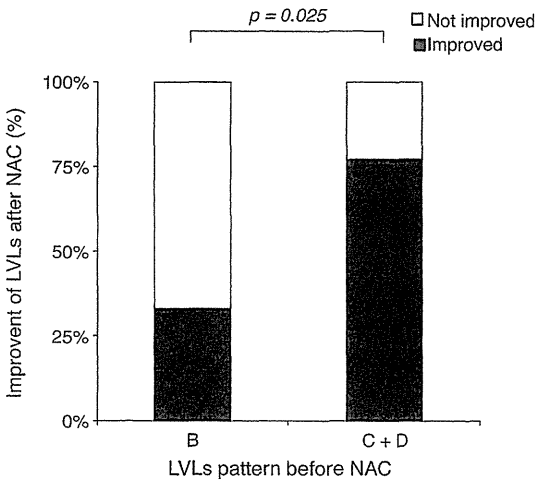

Fig. 2 Improvement of LVLs of esophageal mucosa before and after NAC. *Black bar* improved (%), *white bar* not improved (%). Multiple LVLs in group C and D significantly improved in 17 of 22 patients (77.3 %), while only 4 of 12 cases (33.3 %) improved in group B ($p = 0.025$ by Fisher's exact test). *LVL* Lugol-voiding lesion, *NAC* neoadjuvant chemotherapy

We previously reported that presence of multiple irregular-shaped Lugol unstained lesions in the esophagus was a significant and independent risk factor for multiple SCCs in both the esophagus and the head and neck region [5] and a significant risk factor for synchronous and

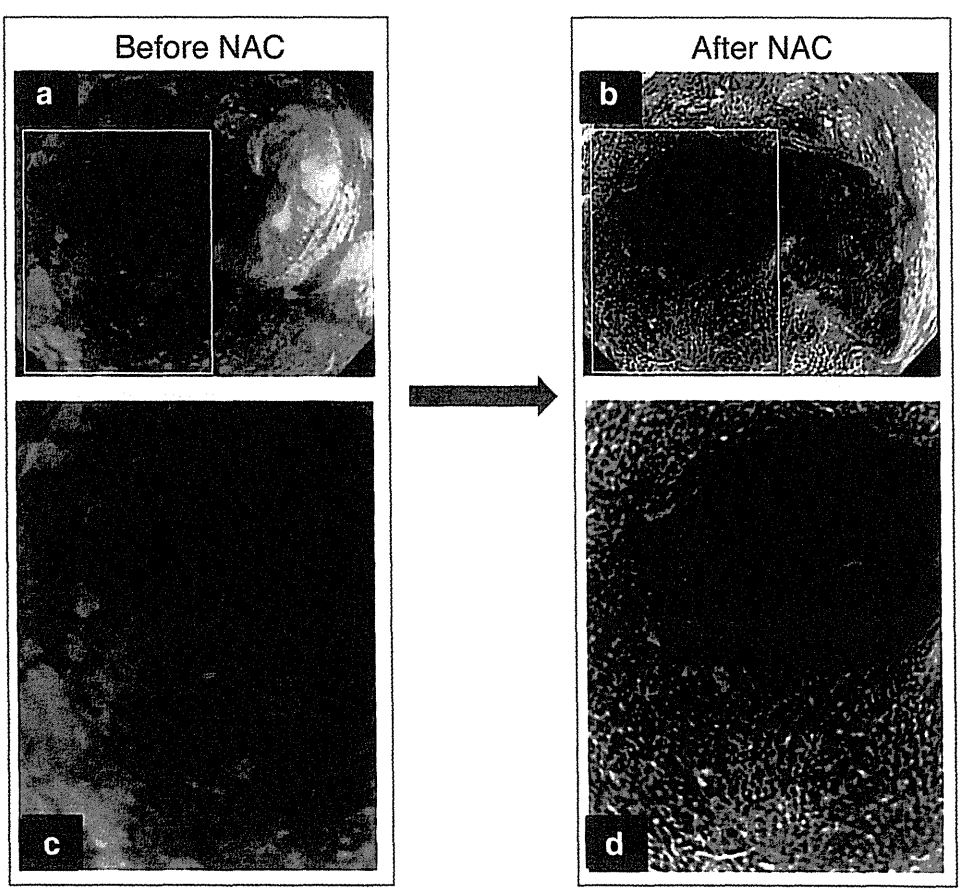

Fig. 3 Comparison of iodine chromoendoscopic images before and after NAC. A representative endoscopic view of primary esophageal cancer site before NAC (**a**) and after NAC (**b**). Before NAC, this case was classified in group D (**c**). After NAC, primary tumor showed dramatic shrinkage (**b**) and the multiple LVLs pattern was improved to group B (**d**). *LVL* Lugol-voiding lesion, *NAC* neoadjuvant chemotherapy

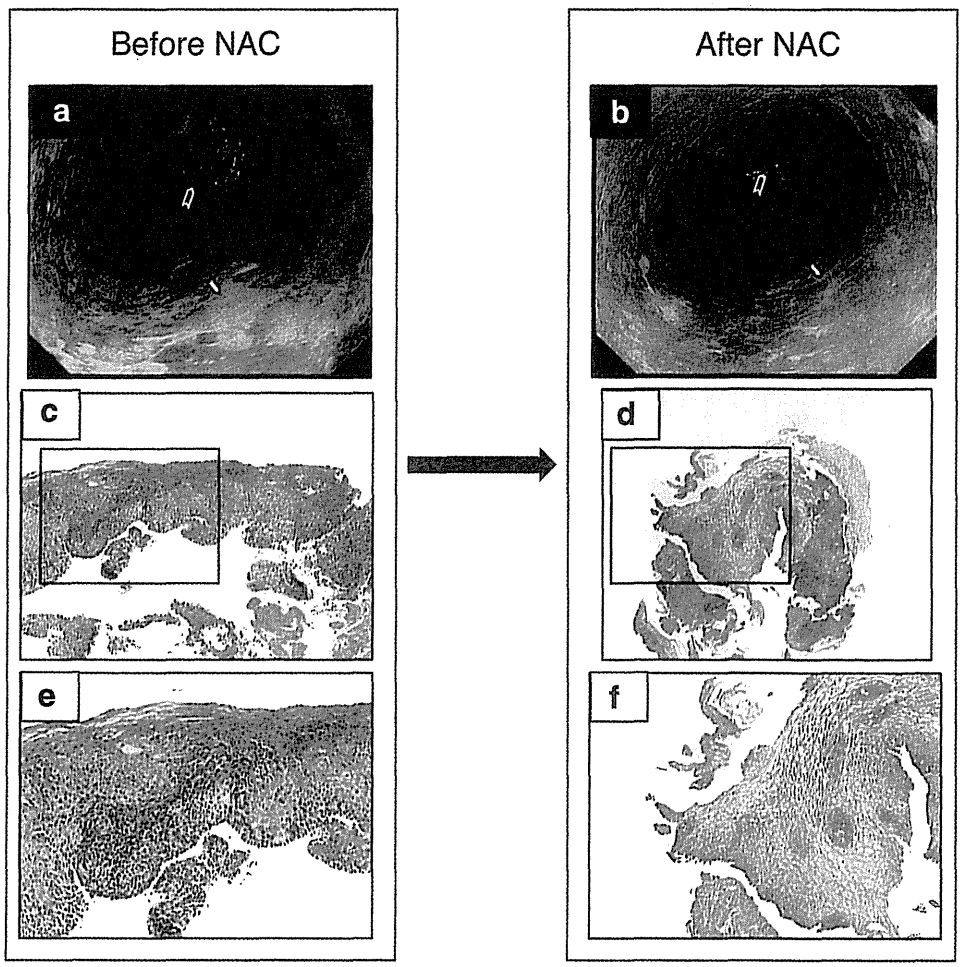

Fig. 4 Relationship between iodine chromoendoscopic improvement and histopathological improvement of LVLs before and after NAC. LVL pattern was improved from group D to group C; moreover, histopathological finding of LVL was improved from high-grade intraepithelial neoplasia to mild atypical squamous epithelium indefinite for low-grade intraepithelial neoplasia or reactive atypia. **a** Endoscopic view before NAC; *black arrowhead* primary ESCC site, *white arrowhead* biopsy site of a LVL. **b** Endoscopic view after NAC; *black arrowhead* primary ESCC site, *white arrowhead* biopsy site of a LVL. **c** Microscopic view of a LVL before NAC (low power). **d** Microscopic view of a LVL after NAC (low power). **e** Microscopic view of a LVL before NAC (high power). **f** Microscopic view of a LVL after NAC (high power). *LVL* Lugol-voiding lesion, *NAC* neoadjuvant chemotherapy

metachronous ESCC in patients with HNSCC [8]. Shimizu et al. [12, 13] also reported that presence of numerous minute noncancerous Lugol unstained areas was a significant risk factor for both metachronous ESCC and metachronous HNSCC after endoscopic resection of primary superficial ESCC. Recently, Hori et al. [6] reported that larger number and size of LVLs in both patients with ESCC and those with HNSCC increased the risk for second primary cancer in the other organ. They concluded that patients with LVLs must be followed closely for development of second primary cancer. These results might strongly indicate that, if patients had multiple LVLs, they were good candidates to prevent development of SCC or multiple SCCs.

Furthermore, it is well known that *p53* is a tumor-suppressor gene that plays an important role in ESCC carcinogenesis. Its point mutation was already present in the esophageal dysplastic lesions. Therefore, from the biological point of view, dysplastic lesions have been considered to be neoplastic and precancerous [14]. Accordingly, reduction or elimination of multiple LVLs potentially paves the way to cancer prevention.

Surgical resection of the organ per se is one possible approach to prevent development of multiple SCCs. However, this approach seems to be too invasive, because these target lesions are not cancerous but precancerous. As a different approach, endoscopic removal of all multiple dysplastic lesions is possible. However, excess removal of the mucosa causes severe stricture, resulting in dysphagia. Chemoprevention may be a feasible approach to prevent multiple SCCs. One randomized controlled chemoprevention trial found that selenomethionine supplementation for 10 months favorably effected a change in esophageal dysplasia grade among participants who started the trial with mild dysplasia. However, in this trial, moderate dysplasia did not show the preventive effect [15]. This result may show that regression of cell populations already harboring high malignant potential is difficult by chemopreventive supplements.

While 5-FU, CDDP [7, 16, 17], docetaxel [18], etc., have been used as anticancer drugs in ESCC, a combination of 5-FU and CDDP remains the standard regimen. However, adverse events of grade 3 or worse by the Common Terminology Criteria for Adverse Events

(CTCAE) are observed in 18–27 % of patients [16, 17]. Such treatment is intolerable for cancer prevention rather than treatment, because of this severe toxicity. In contrast, capecitabine and S1 are oral agents and relatively easy to use; however, their toxicities are also not negligible [19, 20]. As a low-toxicity agent, multivitamin and mineral supplement use has been tried to prevent esophageal cancer. However, data showing chemopreventive efficacy in esophageal cancer are scarce [15, 21]. Thus, if we consider the multiple dysplastic lesions as the target to eliminate, active and low-toxicity anticancer drugs could be candidate chemopreventive agents.

There is another potential explanation for the present results. One possibility is that the iodine solution itself changes the staining properties and results in a reduction of the number of unstained lesions because iodine solution irritates the mucosa and causes erosions and edematous changes. We could not deny the possibility that iodine solution itself changes the staining properties and results in a reduction of the number of unstained lesions. However, we thought that iodine staining itself could not eliminate multiple LVLs, because all of the cases could not show the disappearance of multiple LVLs in spite of the iodine staining.

This study has some limitations. One is its retrospective nature, and the other is that this study did not examine the histological change in all cases. However, because the iodine staining pattern was very sensitive to histological status, improvement of LVL pattern could be well associated with histological improvement. To investigate the difference of changes in the LVLs after NAC, further large-scale prospective study with histological evaluation is required.

In summary, in this study, chemotherapy by 5-FU and CDDP decreased multiple dysplastic lesions in the esophagus. The present results provide a possibility to prevent development of multiple SCC in the esophagus.

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Conflict of interest The authors have no conflict of interest.

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DIAGNOSIS IN ONCOLOGY

Cerebral Air Embolism Caused by Chemoradiotherapy for Esophageal Cancer

Case Report

A 58-year-old man who presented with a persistent cough was admitted to our hospital. Esophagogastroduodenoscopy showed a slightly depressed type of tumor in the middle esophagus. A biopsy specimen revealed the presence of squamous cell carcinoma. Enhanced computed tomography (CT) showed multiple lymph node metastases, including cervical, mediastinal, and perigastric lymph nodes. Notably, a metastatic mediastinal lymph node had invaded the left main bronchus, pericardium, and aorta (Fig 1, arrowheads). A diagnosis of stage III (TNM classification, cT1bN3M0) advanced esophageal cancer was made. The patient received concurrent chemotherapy (cisplatin 80 mg/m^2 on day 1 and fluorouracil 800 mg/m^2 on days 1 through 5) and radiation therapy (RT) using 40 Gy at 2.5 Gy per fraction. On day 22 after the initiation of treatment, the patient had an episode of high fever. CT findings showed that the patient had developed pneumonia and mediastinitis caused by an esophagobronchial fistula that developed through necrosis of the metastatic lymph node. A bronchial stent was inserted into the left main bronchus to control the pneumonia, and a second course of chemotherapy and RT for the cervical lymph node (30 Gy at 3.0 Gy per fraction) was introduced. On day 91 after treatment initiation, the patient exhibited a sudden onset of dysarthria followed by disturbance of consciousness. Diffusion-weighted magnetic resonance imaging of the brain revealed the presence of multiple high-intensity spots, which were consistent with acute cerebral infarction. A brain CT clearly indicated the presence of multiple cerebral air embolisms (Fig 2, arrows). A left atrial–esophageal fistula was suspected from the CT findings. This fistula may have provided a route of entry for the air into the general circulation (Fig 3, arrow), which might have caused the cerebral air embolism. The intraesophageal pressure varied from −10 to −5 mmHg at rest and increased to 20 to 30 mmHg during swallowing. The left atrial pressure was 4 to 12 mmHg. These results suggested that the increase in intraesophageal pressure during swallowing caused the

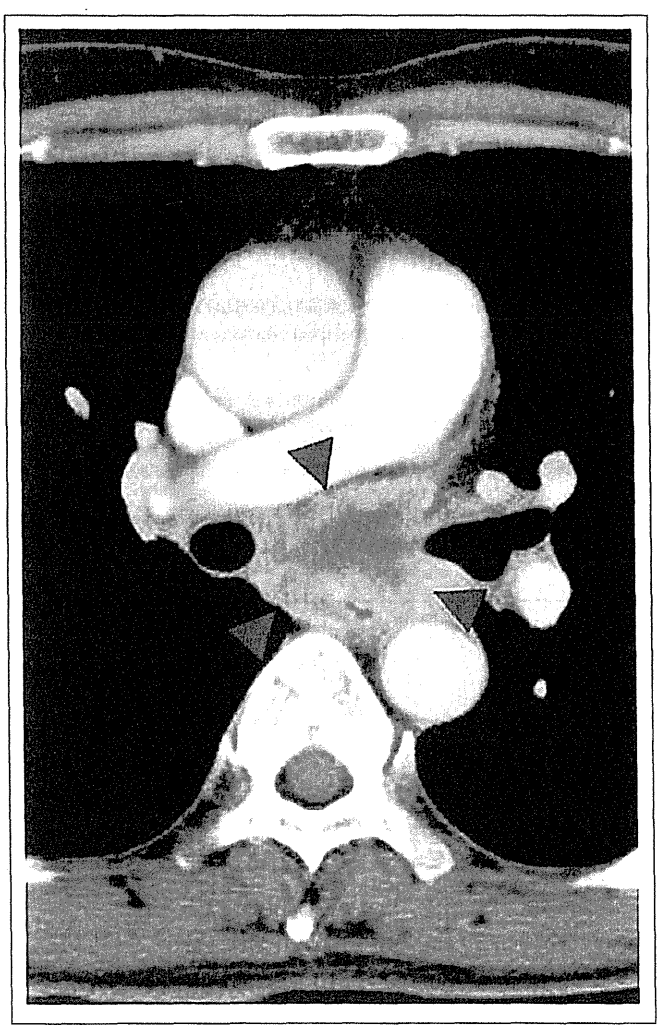

Fig 1.

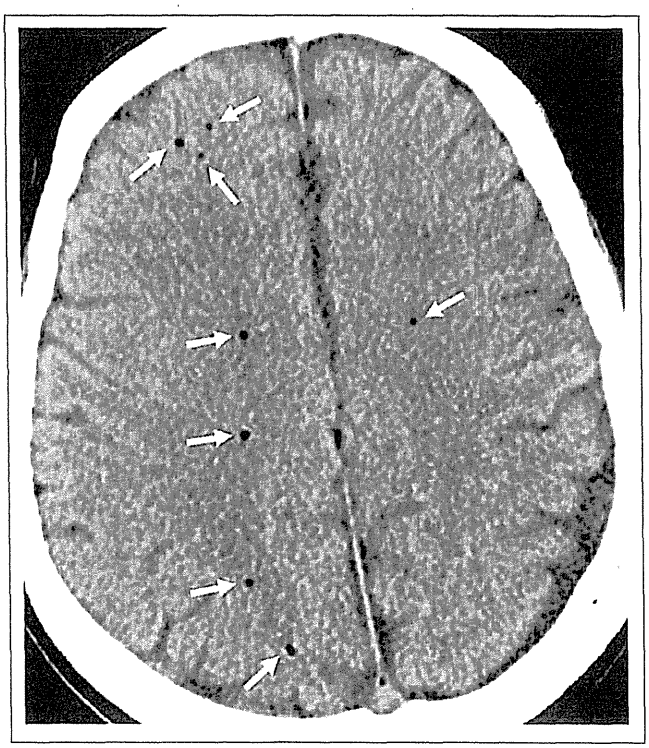

Fig 2.

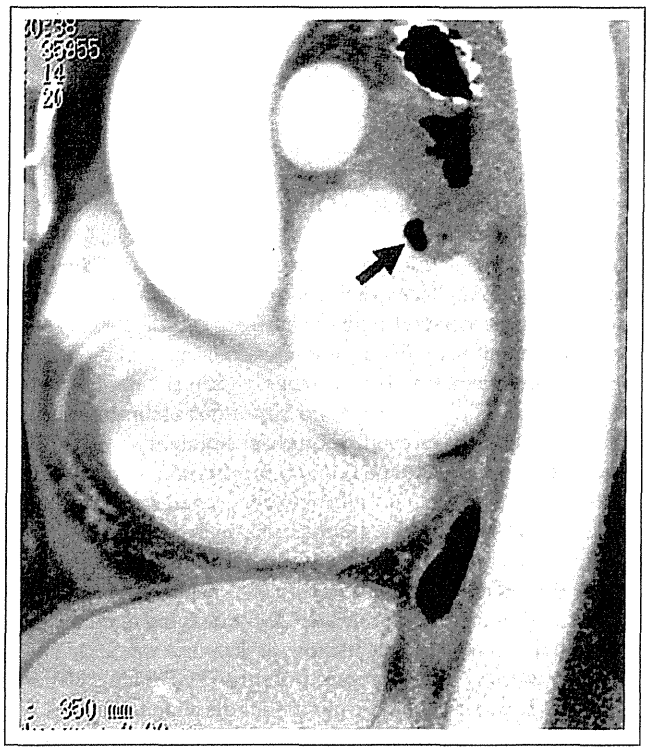

Fig 3.

development of air embolisms because of the presence of the left atrial–esophageal fistula, which had developed because of necrosis of the lymph node. The patient experienced frequent remissions and exacerbations of the disturbance of consciousness, and he died as a result of the disease on day 12 after the onset of the neurologic symptoms. The left atrial—esophageal fistula was identified at autopsy (Fig 4).

Discussion

Cerebral air embolism is an unprecedented complication of chemoradiotherapy for esophageal cancer. To our knowledge, only two similar cases have been reported in the English literature.[1,2] In both cases, cerebral air embolism developed a few years after completion of

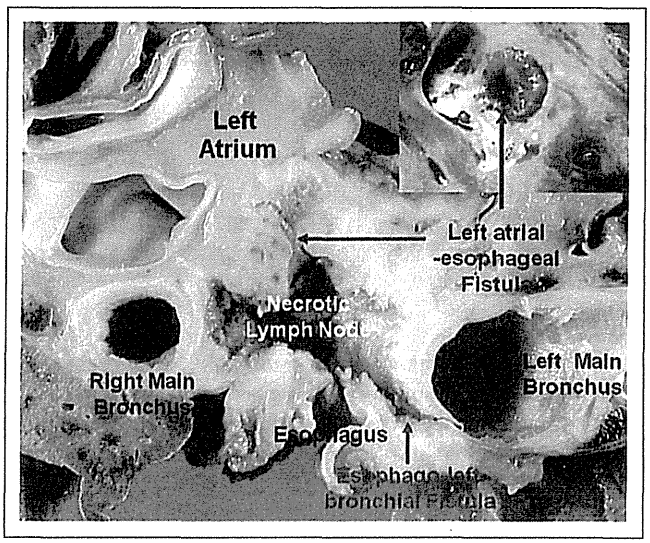

Fig 4.

treatment: one case involved surgery followed by RT, and the other involved stent placement after RT. To our knowledge, this is the first report of a cerebral air embolism that occurred during treatment. Because chemoradiotherapy is a potent treatment option, the clinician should consider the possibility of this lethal complication in patients with tumor invasion of the left atrial wall.

Shin'ichi Miyamoto, Yoko Mashimo, Takahiro Horimatsu, Yasumasa Ezoe, Shuko Morita, Manabu Muto, and Tsutomu Chiba

Graduate School of Medicine, Kyoto University, Kyoto, Japan

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The author(s) indicated no potential conflicts of interest.

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Combination of ADH1B*2/ALDH2*2 polymorphisms alters acetaldehyde-derived DNA damage in the blood of Japanese alcoholics

Yoshiyuki Yukawa,[1] Manabu Muto,[1,4] Kimiko Hori,[1] Haruna Nagayoshi,[2] Akira Yokoyama,[3] Tsutomu Chiba[1] and Tomonari Matsuda[2]

[1]Department of Gastroenterology and Hepatology, Kyoto University Graduate School of Medicine, Kyoto; [2]Research Center for Environmental Quality Management, Kyoto University, Otsu; [3]Department of Internal Medicine, Kurihama Alcoholism Center for National Hospital Organization, Yokosuka, Japan

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The acetaldehyde associated with alcoholic beverages is an evident carcinogen for the esophagus. Genetic polymorphisms of the alcohol dehydrogenase 1B (*ADH1B*) and aldehyde dehydrogenase 2 (*ALDH2*) genes are associated with the risk of esophageal cancer. However, the exact mechanism via which these genetic polymorphisms affect esophageal carcinogenesis has not been elucidated. *ADH1B*2* is involved in overproduction of acetaldehyde due to increased ethanol metabolism into acetaldehyde, and *ALDH2*2* is involved in accumulation of acetaldehyde due to the deficiency of acetaldehyde metabolism. Acetaldehyde can interact with DNA and form DNA adducts, resulting in DNA damage. N^2-ethylidene-2′-deoxyguanosine (N^2-ethylidene-dG) is the most abundant DNA adduct derived from acetaldehyde. Therefore, we quantified N^2-ethylidene-dG levels in blood samples from 66 Japanese alcoholic patients using liquid chromatography/electrospray tandem mass spectrometry, and investigated the relationship between N^2-ethylidene-dG levels and *ADH1B* and *ALDH2* genotypes. The median N^2-ethylidene-dG levels (25th percentile, 75th percentile) in patients with *ADH1B*1/*1* plus *ALDH2*1/*1*, *ADH1B*2* carrier plus *ALDH2*1/*1*, *ADH1B*1/*1* plus *ALDH2*1/*2*, and *ADH1B*2* carrier plus *ALDH2*1/*2* were 2.14 (0.97, 2.37)/10^7 bases, 2.38 (1.18, 2.98)/10^7 bases, 5.38 (3.19, 6.52)/10^7 bases, and 21.04 (12.75, 34.80)/10^7 bases, respectively. In the *ALDH2*1/*2* group, N^2-ethylidene-dG levels were significantly higher in *ADH1B*2* carriers than in the *ADH1B*1/*1* group ($P < 0.01$). N^2-ethylidene-dG levels were significantly higher in the *ALDH2*1/*2* group than in the *ALDH2*1/*1* group, regardless of ADH1B genotype (*ADH1B*1/*1*, $P < 0.05$; *ADH1B*2* carriers, $P < 0.01$) N^2-ethylidene-dG levels in blood DNA of the alcoholics was remarkably higher in individuals with a combination of the *ADH1B*2* and *ALDH2*2* alleles. These results provide a new perspective on the carcinogenicity of the acetaldehyde associated with alcoholic beverages, from the aspect of DNA damage. (*Cancer Sci* 2012; 103: 1651–1655)

Esophageal squamous cell carcinoma (SCC) occurs 3.7–9.5 times more frequently in East-Asian countries, such as Japan, China, Taiwan, and Korea, than in Western countries.[1] Alcohol consumption is a well-established risk factor for esophageal SCC; moreover, in October 2009, the International Agency for Research on Cancer certified that the acetaldehyde associated with alcoholic beverages is an evident carcinogen for the head and neck region and for the esophagus.[2] However, the carcinogenic mechanism of acetaldehyde in these regions remains unclear.

Orally ingested ethanol is metabolized to acetaldehyde mainly by alcohol dehydrogenase 1B (ADH1B) and alcohol dehydrogenase 1C (ADH1C) in the liver. Subsequently, acetaldehyde is metabolized to acetate by aldehyde dehydrogenase 2 (ALDH2) in the liver. The *ADH1B* and *ALDH2* genes contain single-nucleotide polymorphisms (SNPs) that modulate the enzymatic activity of their protein products. The variant *ADH1B*2* increases ethanol metabolism to produce acetaldehyde by 40 times because of an amino acid substitution of histidine for arginine at position 47 of the protein. The variant *ALDH2*2* is deficient in the activity of acetaldehyde catabolism because of an amino acid substitution of lysine for glutamine at position 487. These variants are thought to play protective roles against alcoholism because of the unpleasant flushing response associated with acetaldehydemia after drinking.[3]

In East-Asian countries, *ADH1B*2* and *ALDH2*2* are prevalent genotypes found in approximately 90% and 50% of these populations, respectively.[4] Despite the protection against alcohol intake afforded by these genotypes, continuous alcohol consumption by these individuals leads to increased exposure to carcinogenic acetaldehyde. Therefore, this genetic background is considered a cause of the high incidence of esophageal SCC in East Asia.

Acetaldehyde is a highly reactive electrophile that can interact with DNA to form DNA adducts, such as N^2-ethyl-2′-deoxyguanosine (N^2-Et-dG),[5,6] α-*S*- and α-*R*-methyl-γ-hydroxy-1, N^2-propano-2′-deoxyguanosine (α-*S*-Me-γ-OH-PdG and α-*R*-Me-γ-OH-PdG),[7,8] and N^2-ethylidene-2′-deoxyguanosine (N^2-ethylidene-dG).[7] N^2-Et-dG blocks DNA synthesis and induces DNA mutation.[9–11] α-*S*-Me-γ-OH-PdG and α-*R*-Me-γ-OH-PdG induce DNA-protein or DNA–DNA cross-links and induce DNA mutation.[12] We reported previously that the levels of the N^2-Et-dG, α-*S*-Me-γ-OH-PdG, and α-*R*-Me-γ-OH-PdG adducts were significantly higher in the leukocytes of ALDH2-deficient Japanese alcoholics.[13]

Although N^2-ethylidene-dG was the most abundant of the DNA adducts derived from acetaldehyde,[14] it was unstable at the nucleoside level and difficult to measure.[7] Wang *et al.*[7] established a system to quantify N^2-ethylidene-dG by adding $NaBH_3CN$, a strong reducing agent that converts N^2-ethylidene-dG to the stable N^2-Et-dG. Since then, the quantification of N^2-ethylidene-dG has become possible.[14–20]

We reported previously that N^2-ethylidene-dG levels were significantly higher in the liver and stomach of ALDH2-deficient mice after oral ethanol ingestion.[14,20] In humans, N^2-ethylidene-dG was detected in liver and lung DNA samples[15,18] and N^2-ethylidene-dG levels were significantly higher in the blood DNA of drinkers[17] and significantly lower in the leukocyte DNA of smokers after smoking cessation.[16] However, the

[4]To whom correspondence should be addressed.
E-mail: mmuto@kuhp.kyoto-u.ac.jp

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relationship between N^2-ethylidene-dG levels and SNPs of the *ADH1B* and *ALDH2* genes has not been examined in humans.

N^2-ethylidene-dG is produced from deoxyguanosine and acetaldehyde. Acetaldehyde is ubiquitous in the environment[21] and alcoholic beverages and cigarettes are major sources of acetaldehyde for humans. In particular, alcohol consumption may be the most important route of acetaldehyde exposure in humans, as typical alcohol beverages contain over 10 g of ethanol/glass, much of which may be metabolized to acetaldehyde, whereas the level of acetaldehyde in cigarette smoke ranges from 0.6 to 2.1 mg/cigarette.[22] N^2-ethylidene-dG levels in the blood of drinkers were 5270 ± 8770 fmol/μmol dG,[17] which was several times higher than that observed in the smokers who drink only occasionally or not all (1310 ± 1720 fmol/μmol dG).[16] Therefore, N^2-ethylidene-dG could become a good biomarker of acetaldehyde exposure associated with alcohol beverages.

In this study, we quantified N^2-ethylidene-dG levels in the leukocytes of Japanese alcoholics, as a biomarker of acetaldehyde-derived DNA damage, and investigated the relationship between N^2-ethylidene-dG levels and SNPs of the *ADH1B* and *ALDH2* genes.

Materials and Methods

Patients and ADH1B and ALDH2 genotypes. The participants in this study were 66 Japanese intoxicated alcoholic men who came to the Kurihama Alcoholism Center of the National Hospital Organization. This study was approved by the Ethics Committee of Kurihama Alcoholism Center for the National Hospital Organization and informed consent was obtained from all participating patients. Information regarding drinking profiles and smoking habits was obtained from the patients, as described previously.[23] Blood samples were obtained from patients on the day of admission for alcoholism treatment. *ADH1B* and *ALDH2* genotyping was performed on blood DNA using PCR/RFLP, as described previously.[24,25]

Isolation of DNA. DNA was isolated from 1 mL of whole blood using the QIAamp DNA Blood Midi Kit (Qiagen, Hilden, Germany). The procedure was performed according to the manufacturer's instructions, with the exception of the addition of $NaBH_3CN$ to all solutions (final concentration, 100 mM). After the purification step, DNA was dissolved in distilled water.

DNA adduct standard and its stable isotope. N^2-Et-dG and [U-$^{15}N_5$]-labeled N^2-Et-dG were synthesized as described previously.[6]

DNA digestion. DNA samples (10–15 μg) were digested to their corresponding 2′-deoxyribonucleoside-3′-monophosphates via the addition of 15 μL of 17 mM sodium succinate and 8 mM $CaCl_2$ buffer (pH 6.0) containing micrococcal nuclease (22.5 U) and spleen phosphodiesterase (0.075 U), and [U-$^{15}N_5$]-labeled N^2-Et-dG. Solutions were mixed and incubated for 3 h at 37°C, followed by the addition of alkaline phosphatase (1 U), 10 μL of 0.5 M Tris–HCl (pH 8.5), 5 μL of 20 mM $ZnSO_4$, and 67 μL of distilled water and incubation of solutions for an additional 3 h at 37°C. The mixture was evaporated to 10–20 μL and 100 μL of methanol was added to precipitate proteins. After centrifugation, the methanol fraction (supernatant) was transferred to a new Eppendorf tube. The precipitate was re-extracted using 100 μL of methanol and the methanol fraction was collected into the Eppendorf tube. This methanol solution was evaporated to dryness, resuspended in 50 μL of distilled water, and subjected to liquid chromatography/electrospray tandem mass spectrometry (LC/MS/MS).

Instrumentation. Liquid chromatography/electrospray tandem mass spectrometry analyses were performed using a Shimadzu LC system (Shimadzu, Kyoto, Japan) interfaced with a Quattro Ultima triple-stage quadrupole MS (Waters/Micromass, Manchester, UK). The LC column was eluted over a gradient that began at a ratio of 5% methanol to 95% water and was changed to 40% methanol over a period of 30 min, to 80% methanol from 30 to 35 min, and finally returned to the original starting conditions (5:95) for the remaining 11 min. The total run time was 46 min. Sample injection volumes of 20 μL each were separated on a Shim-pack XR-ODS column (3.0 × 75 mm, 2.2 μm; Shimadzu) and eluted at a flow rate of 0.2 mL/min. Mass spectral analyses were performed in a positive ion mode using nitrogen as the nebulizing gas. The temperature of the ion source was 130°C, the temperature of the desolvation gas was 380°C, and the cone voltage was operated at a constant 35 V. Nitrogen was also used as the desolvation gas (700 L/h) and cone gas (35 L/h), and argon was used as the collision gas, at a collision cell pressure of 1.5×10^{-3} mbar. Positive ions were acquired in multiple-reaction monitoring mode. The multiple-reaction monitoring transitions monitored were as follows: [$^{15}N_5$]-N^2-Et-dG, *m/z* 301 → 185 and N^2-Et-dG, *m/z* 296 → 180. The amount of N^2-Et-dG was quantified using the ratio of the peak area of N^2-Et-dG to that of [U-$^{15}N_5$]-labeled N^2-Et-dG. The QuanLynx software (version 4.0; Waters/Micromass) was used to create a standard curve and to calculate the concentration of N^2-Et-dG. The amount of deoxyguanosine was monitored using a Shimadzu SPD-10A UV–Visible detector that was set in place before the tandem MS.

Statistical analyses. Statistical analyses were performed using SPSS statistics software (version 17; SPSS Inc., Chicago, IL, USA). Values were expressed as means and standard deviations or medians plus 25th and 75th percentiles (Q1, Q3). Analysis of variance (ANOVA) and the Kruskal–Wallis test were used to compare normally distributed data and non-normally distributed data, respectively, among the groups with different *ADH1B* and *ALDH2* genotype combinations; in addition, *post hoc* paired comparisons were performed using Tukey's method for ANOVA and Holm's method for the Kruskal–Wallis test.[23] Because N^2-ethylidene-dG levels were not normally distributed, the Mann–Whitney test was used to compare N^2-ethylidene-dG levels among genotype groups. Holm's method was used to adjust the *P*-values for multiplicity.[23] An adjusted *P*-value < 0.05 was considered significant.

Results

Clinical characteristics. Table 1 lists the patients' characteristics. There were 50 patients in the *ALDH2*1/*1* group, 16 patients in the *ALDH2*1/*2* group, and no patients in the *ALDH2*2/*2* group. Among the 50 patients with the *ALDH2*1/*1* genotype, 13 patients were homozygous for *ADH1B*1* (*ADH1B*1/*1* + *ALDH2*1/*1*; Group 1) and 37 patients were *ADH1B*2* carriers (*ADH1B*1/*2*, 14; *ADH1B*2/*2*, 23) (*ADH1B*2* carriers + *ALDH2*1/*1*; Group 2). Among the 16 patients with the *ALDH2*1/*2* genotype, eight patients were homozygous for *ADH1B*1* (*ADH1B*1/*1* + *ALDH2*1/*2*; Group 3) and eight patients were *ADH1B*2* carriers (*ADH1B*1/*2*, 4; *ADH1B*2/*2*, 4) (*ADH1B*2* carriers + *ALDH2*1/*2*; Group 4).

In the *ALDH2*1/*1* group, the mean body weight was significantly lower in Group 2 than in Group 1 ($P < 0.05$), whereas no such difference was observed in the *ALDH2*1/*2* group. There were no significant differences among the four groups regarding age, height, amount of alcohol consumed in the previous 24 h, interval from the last drink, and number of cigarettes smoked during the previous 24 h.

Level of the N^2-ethylidene-dG adduct and combinations of *ADH1B* and *ALDH2* genotypes. N^2-ethylidene-dG was converted to N^2-Et-dG by adding $NaBH_3CN$ during the DNA isolation step and the amount of N^2-Et-dG was measured. Because the amount of N^2-Et-dG is, by far, lower than that of

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