表2　成因別内科的救命率の変遷(厚生労働省全国統計)

	年代による変遷					臨床病型(1998～2010)	
	'83～'97	'98～'01	'02～'04	'05～'07	'08～'10	急性型	亜急性型
A型肝炎	57.8	76.5	54.5	54.5	66.7	72.7	40.0
B型肝炎	29.3	29.6	36.1	38.8	12.8	36.9	18.1
自己免疫性	-	16.1	0	25.0	17.6	30.0	14.1
薬物性	23.6	41.0	30.6	39.1	16.7	50.9	18.7
成因不明	17.1	27.4	24.4	24.2	26.7	42.5	17.8
全体	25.9	32.6	31.5	35.2	20.8	42.2	18.8

%

表3　B型肝炎ウイルスによる劇症肝炎の分類．頻度，救命率(厚生労働省全国統計)

	例数	臨床病型頻度(%)		救命	
		急性	亜急性	救命例	救命率(%)
急性感染	254	84.3	15.7	105	41.3
キャリア例	151	35.1	64.9	21	13.9
判定不能	38	73.7	26.3	9	23.7
計	443	66.6	33.4	135	30.5

急性型と11日以上の亜急性型に分類している(表1)．この基準は後の全国統計に基づくReceiver-oriented curve (ROC) 解析(図1)でも再確認されている[3]．発症-昏睡期間のさらに長い遅発性肝不全は，極めて予後不良で，急性肝不全の類縁疾患とされる．

米国では，予後を規定しているのは成因であり，発症ー昏睡期間(臨床病型)が本質ではないとし，臨床病型が一見予後と関連しているようにみえるのは，各病型に含まれる成因に偏りがあるからだとしている[4]．

しかし，わが国の全国統計による内科的救命率をみると，急性型，亜急性型それぞれ40%，20%程度であり，いずれの成因においても急性型は亜急性型に比して救命率が高い(表2)．このことは，少なくとも肝炎や成因不明例においては，臨床病型は予後を規定する重要な因子であることを示している．

米国の見解とのくい違いの原因は，対象とする疾患の違いによると考えられる．すなわち，わが国では肝炎による昏睡型急性肝不全つまり劇症肝炎といういわば単一疾患を対象としてきたのに対し，欧米では肝炎，非肝炎を含めた急性肝不全という症候群を対象にしてきたためと考えられる．その意味でも，わが国の急性肝不全の新定義に即した昏睡型急性肝不全の予後の解析が待たれる．

2. 成因と予後

米国のALF study groupが主張するように，成因は臨床病型と並んで重要な予後因子であることはいうまでもない．表に示すように，内科的救命率は成因により大きく異なり，A型肝炎が比較的良好で自己免疫性，成因不明が予後不良である(表2)．

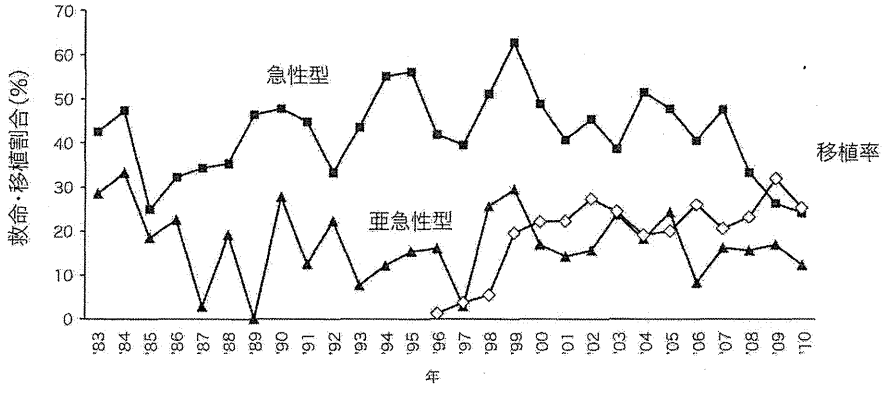

図2　わが国における劇症肝炎の内科的救命率と肝移植率の変遷(厚生労働省全国統計)

B型肝炎の予後は急性感染とキャリアからの発症では大きく異なる．急性感染の約85％は急性型で救命率は約40％と比較的良好であるが，キャリアからの発症では約65％が亜急性型で，救命率は約15％と低い(表3)．さらに近年ではHBV既感染からの再活性化いわゆる*de novo* B型肝炎による劇症肝炎が問題となっており[5]，これまで昏睡発現(劇症化)に至った症例はいずれも内科的救命ができていない．*de novo* B型肝炎は免疫抑制療法に際して，再活性化予防のガイドライン[6]を踏襲することで完全に予防しうるので，今後発生数の減少が期待される．

1997年以前は自己免疫性肝炎としての成因分類がなかったため，非A非B肝炎として集計されており[7]，成因不明に含まれていたと考えられる．このため，1998年以降[8]と単純な比較はできないが，A型，B型，薬物性に関しては比較が可能と考えられ，これらの救命率に大きな改善はみられない(表2)．

3. 予後の変遷と背景・治療因子

わが国における劇症肝炎の内科的救命率の変遷と肝移植率を図2，表に示す．内科的救命率は，1990年代後半から急性型でやや改善している傾向がみられる．この時期は，人工肝補助療法として，血液濾過透析(HDF)が導入され普及した時期にほぼ一致することから，HDFの効果と考えられる．これに対し，亜急性型の救命率は依然として低く，改善がみられていない．代わって，2000年代から亜急性型を中心に主に生体肝移植による救命が図られるようになり，劇症肝炎の25％前後に施行されている．

先にも述べたように，各成因とも年代による大きな改善はみられず，むしろ近年(2008～2010年)はB型肝炎，自己免疫性，薬物性ともに救命率が低下している(表2)．この原因は不明であるが，劇症肝炎患者の高齢化がいわれており，基礎疾患保有率，薬物内服率などの複合的な影響の解析が必要と思われる．

一方で，専門施設ではHDFにより少なくとも急性型劇症肝炎の救命率が格段に向上し，昏睡からの覚醒率も飛躍的に改善したとする報告が多い[9～11]．しかし，疾患の性質上無作為化比較試験(RCT)が困難であり，実証は得られていない．今後は，人工肝補助療法を標準化したうえで多施設の臨床研究により，効果を実証していくことが望まれる．

4. 移植成績

わが国では劇症肝炎の約25％に肝移植が

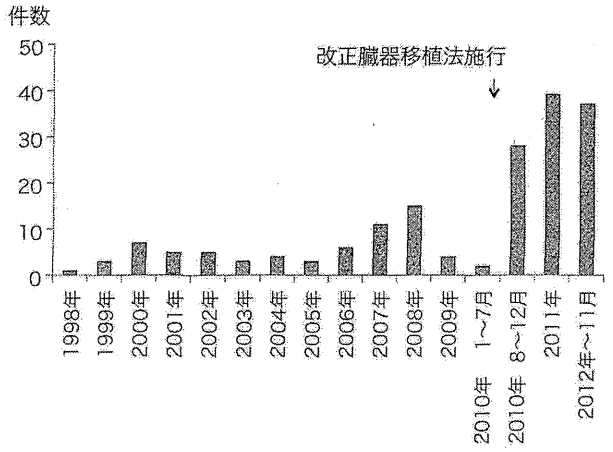

図3　わが国における脳死肝移植件数の変遷(日本臓器移植ネットワークホームページ)

表4　非肝炎急性肝不全の特徴(文献16)

	例数	昏睡発現				昏睡発現割合(%)	死亡割合(%)	肝不全死の割合(%)	移植割合(%)
		急性型	亜急性型	LOHF	ACLF				
中毒性	3	2	0	0	0	66.7	0	-	0
アルコール性	24	5	2	0	9	45.8	37.5	66.7	0
循環障害	18	4	1	0	2	33.3	56.3	11.1	0
悪性腫瘍浸潤	5	0	3	1	0	80.0	60.0	33.3	0
代謝性	6	2	1	0	1	50.0	16.7	0	16.7
術後肝不全	2	0	0	0	2	100	100	0	0
その他	4	1	1	0	0	50.0	75.0	0	0

行われており，そのほとんどは生体肝移植である．1年，5年，10年生存率はそれぞれ74%，70%，68%と報告されており，内科治療の成績より遙かに高い[12]．しかも，肝移植は原則として内科的治療による救命を断念した患者に対して行われることから，その意義は極めて大きい．

わが国における脳死肝移植は，1997年に脳死臓器移植法が成立し，1998年に第1例目が行われたが，2009年までの10年間で66例が行われたにすぎなかった．そのうち，劇症肝炎は8例(14%)である．しかし，2010年7月に改正臓器移植法が施行されてからは，28カ月で104例の脳死肝移植が行われている(図3)．劇症肝炎は，レシピエントの医学的緊急度が最上位にあることから，ドナーの増加により劇症肝炎に対する脳死肝移植が増加することが期待される．

欧米においても，急性肝不全における肝移植の救命効果についてはRCTがなされていない．しかし，無作為ではないが移植例と内科治療例との救命率の比較から，肝移植が急性肝不全の救命率を有意に向上すると考えられている[13]．米国での急性肝不全に対する脳死肝移植の1年生存率は近年82%に向上したといわれる[14]．英国では1年，5年生存率は

表5　急性肝障害のPTの値と昏睡発現割合(岩手医科大学消化器・肝臓内科，北東北肝炎調査・治療研究会)

	PT(%)		
	<40	40～60	60～80
例数	67	86	112
昏睡発現例	19	1	1
昏睡発現率(%)	28.4	1.2	0.9
	13.1		
		7.9	

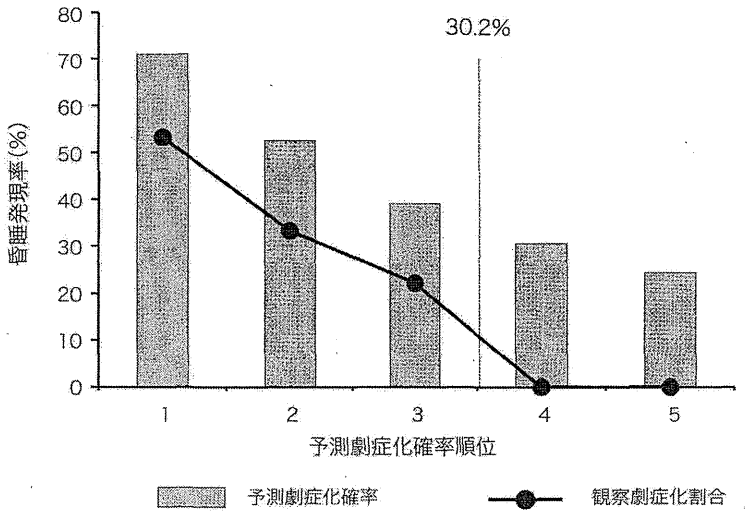

図4　劇症化予知精度：Hosmer-Lemeshow test（予測劇症化確率 20% 以上）（文献19）

それぞれ81%，73%と報告されている[15]．日本と異なり，欧米では脳死肝移植がほとんどを占め，米国では急性肝不全に対する生体肝移植は，移植全体のわずか2%である．

3　非肝炎急性肝不全の予後

1．わが国の成績

先にも述べた通り，劇症肝炎の対象成因すなわち肝炎および成因不明以外の急性肝不全に関する全国調査は2010年に開始されたばかりで，まだ，データの集積が十分ではない．2011年の急性肝不全の定義策定のための基礎資料として，プロトロンビン時間50%以下，PT-INR 1.5以上を示した急性肝障害を暫定的な急性肝不全として2008～2009年，全国調査を行った[16]．この資料を基に，非肝炎による急性肝不全の予後の特徴を述べる．

肝炎も含めて124例の暫定的急性肝不全が集計されたが，このうち非肝炎急性肝不全72例の特徴を表4に示す．この中で最も多いアルコール性はacute-on-chronic liver failure (ACLF) の病型をとることが多いため，急性肝不全の定義では対象疾患から除外されている．アルコール性に次いで多いのは循環障害

によるもので，これには心原性ショックや急性Budd-Chiari症候群，敗血症，DICなどが含まれる．死亡割合に比し肝不全死の割合が低いのが特徴で，原疾患や多臓器不全による死亡が多いといわれる．合併症を有することも多く，肝移植に至ることは稀である．

2. 米国の成績

米国のALF study groupにより，多施設共同研究が行われ昏睡型急性肝不全1,000例以上の解析が発表されている[17]．成因の46％をアセトアミノフェン中毒が占めており，日本の肝炎に相当するのは，成因不明14％，A型肝炎2.6％，B型肝炎7.7％，自己免疫性肝炎5.9％および特異体質による薬物性肝障害（アレルギーと中毒を含む）12％の計42.2％である．

米国の診断基準が肝性昏睡I度も昏睡型に含めていることと，日本ほど人工肝補助を行わないことなどのため，内科治療成績を比較するのは困難だが，内科的救命率は全体で46％であり，アセトアミノフェン65％，A型肝炎58％と良好で，薬物29％，B型肝炎25％，成因不明25％と日本の成績に類似している[18]．また，肝移植が全体の25％に施行され，3年生存率が約80％である点も同様である．

4 予知システムからみた急性肝障害の予後

わが国の急性肝不全の定義が，非昏睡型も規定したことから，PTが40％以下あるいはPT-INRが1.5.以上を示した急性肝障害の昏睡発現率が算出されるようになった．全国統計での登録では，非昏睡型の把握がいまだ不十分なため．みかけ上高い昏睡発現率になっているが，岩手医科大学の劇症化予知システム[19,20]では，PT 80％以下の段階から症例を登録しており，PTに応じたほぼ正確な昏睡発現率が計算されていると思われる(表5)．

このデータからも，PT 40％以下で昏睡発現率が急激に増加することが示されており，1982年に定められた劇症肝炎の診断基準[21]が極めて優れていることが改めて確認された．

また，この予知システムでは，予測劇症化確率に比して観察劇症化割合が低下しており(図4)，早期に予知して専門施設に搬送することが劇症化予防に有効となる可能性を示唆している．

5 おわりに

急性肝不全の予後改善のために，移植の推進，強力な人工肝補助の開発，細胞移植や再生治療法など，さまざまなアプローチがなされている．しかし，いまだに予後改善効果が実証された治療法はない．劇症化予知・予防も含めたこれらのアプローチが，急性肝不全による死亡の減少に繋がることを期待する．

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《トピックス》

10 急性肝不全の診断基準

滝川康裕*
たきかわやすひろ

ポイント

- 急性肝不全はさまざまな成因による肝障害を包含する症候群である.
- "初発症状発現から8週間以内"をもって,"急性"を定義する.
- 肝予備能低下のない慢性の基礎肝疾患の存在を除外しない.
- 肝細胞機能障害の客観的指標として,肝の蛋白合成能を表すプロトロンビン時間を採用している.
- 肝性脳症Ⅱ度以上を伴う昏睡型と伴わない非昏睡型に分類する.
- 基礎に肝硬変を有するいわゆるAcute-on-chronic liver failureは除外する.

キーワード 急性肝不全,劇症肝炎,肝性脳症,プロトロンビン時間,診断基準

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わが国の急性肝不全の診断基準は,2011年厚生労働省科学研究費補助金(難治性疾患克服研究事業)「難治性の肝・胆道疾患に関する調査研究班」で定められた[1].それまで,わが国では急性肝不全の一部である「劇症肝炎」を定義した,犬山シンポジウムの診断基準[2](1982年)を急性肝不全の代表的診断基準として長年用いてきた経緯がある.新たに「急性肝不全」として診断基準を定めた背景には,欧米の疾患概念との整合性を得る目的があり,ポイントは成因の範囲とプロトロンビン時間(PT)の表記方法である.この点を中心に,わが国の新しい基準について解説する.

◎疾患概念

急性肝不全(Acute liver failure:ALF)とは急激かつ高度の肝細胞機能障害に基づいて肝不全症候あるいは所見(脳症,黄疸,腹水,プロトロンビン時間延長など)をきたす予後不良の疾患群である[3].多くの場合,ウイルス性肝炎や薬物性肝障害による広汎あるいは亜広汎肝細胞死によって引き起こされるが,Reye症候群や急性妊娠性脂肪肝のように壊死・炎症がほとんどない病態でも起こりうる.

肝細胞死の機序は,これまで炎症を主体とした場合(ウイルス性,薬物アレルギー性,自己免疫性肝炎)とその他(薬物中毒,循環障害など)の場合にわけて考えられてきたが,発端が非炎症性の肝細胞死であっても組織障害に伴う炎症機構が障害を増幅することもあり,両者は明瞭に区別できないこともある.

これまで種々の定義あるいは診断基準が定められたが,それらの間の相違点は,①肝不全の症候あるいは所見を肝性脳症に限定するか他の所見も含めるか,②成因を肝炎のみに限定するか,肝炎以外の原因も包含するか,③発症時期を初発症状(自覚症状)の発現時点とするか,黄疸等の客観的症候の確認時点とするか,などである.

◎わが国の急性肝不全の診断基準

表1にわが国の急性肝不全の診断基準を示す.

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表 1　急性肝不全の定義

正常肝ないし肝予備能が正常と考えられる肝に肝障害が生じ，初発症状出現から 8 週以内に，高度の肝機能障害に基づいてプロトロンビン時間が 40%以下ないしは INR 値 1.5 以上を示すものを「急性肝不全」と診断する．急性肝不全は肝性脳症が認められない，ないしは昏睡度がⅠ度までの「非昏睡型」と，昏睡Ⅱ度以上の肝性脳症を呈する「昏睡型」に分類する．また，「昏睡型急性肝不全」は初発症状出現から昏睡Ⅱ度以上の肝性脳症が出現するまでの期間が 10 日以内の「急性型」と，11 日以降 56 日以内の「亜急性型」に分類する．

（注 1）B 型肝炎ウイルスの無症候性キャリアからの急性増悪例は「急性肝不全」に含める．また，自己免疫性で先行する慢性肝疾患の有無が不明の症例は，肝機能障害を発症する前の肝機能に明らかな低下が認められない場合は「急性肝不全」に含めて扱う．

（注 2）アルコール性肝炎は原則的に慢性肝疾患を基盤として発症する病態であり，「急性肝不全」から除外する．ただし，先行する慢性肝疾患が肥満ないしアルコールによる脂肪肝の症例は，肝機能障害の原因がアルコール摂取ではなく，その発症前の肝予備能に明らかな低下が認められない場合は「急性肝不全」として扱う．

（注 3）薬物中毒，循環不全，妊娠脂肪肝，代謝異常など肝臓の炎症を伴わない肝不全も「急性肝不全」に含める．ウイルス性，自己免疫性，薬物アレルギーなど肝臓に炎症を伴う肝不全は「劇症肝炎」として扱う．

（注 4）肝性脳症の昏睡度分類は犬山分類（1972 年）に基づく．ただし，小児では「第 5 回小児肝臓ワークショップ（1988 年）による小児肝性昏睡の分類」を用いる．

（注 5）成因分類は「難治性の肝疾患に関する研究班」の指針（2002 年）を改変した新指針に基づく．

（注 6）プロトロンビン時間が 40%以下ないしは INR 値 1.5 以上で，初発症状出現から 8 週以降 24 週以内に昏睡Ⅱ度以上の脳症を発現する症例は「遅発性肝不全」と診断し，「急性肝不全」の類縁疾患として扱う．

（持田　智，他：肝臓 52：393-398，2011[1]より引用）

この基準では，①先行する慢性肝障害の除外基準を明確にしている．②「急激かつ高度の肝細胞機能障害に基づく肝不全症候あるいは所見」を PT 延長として客観的所見に定義している．③肝不全症候の代表である肝性脳症を客観的判断が可能なⅡ度以上に定義し，昏睡型と非昏睡型に分類している．④「急性」の意味を，初発症状発現から 8 週以内に肝不全症状（徴候）が出現する場合と定義し，一般的に「慢性肝障害」の定義に用いられる 6ヵ月（24 週）以上続く肝障害との中間に位置する，8 週から 24 週の間に肝不全症候あるいは所見が出現した場合を「遅発性肝不全（Late onset hepatic failure：LOHF）」と定義し，類縁疾患として扱うことを定めている．

劇症肝炎は肝炎（ウイルス性肝炎，薬物アレルギー性肝障害，急性発症自己免疫性肝炎）のみを対象とし，しかもⅡ度以上の肝性昏睡発現例と定義していた．したがって，急性肝不全の診断基準のなかで，劇症肝炎は「肝炎による昏睡型急性肝不全」に相当する．

◉成因

急性肝不全の成因は多岐にわたる（**表 2**）．「劇症肝炎」の診断基準に基づいて全国集計が行われていた時代は，**表 2** のⅠウイルス性，Ⅱ自己免疫性，Ⅲ-①薬物アレルギー，Ⅸ成因不明を肝炎として集計してきた．しかし，成因の約 40%を締める「成因不明」が果たして「肝炎」であるのかの確証がないまま劇症肝炎としてきたことも事実である．

2010 年の症例から，新たに定められた急性肝不全の診断基準に基づいて全国集計が開始され，従来の肝炎による急性肝不全と非肝炎による急性肝不全の臨床的特徴の差異が，今後明らかになると思われる．また，クラスター分析から成因ごとの臨床データの特徴を割り出し，成因不明例の成因推定を行う試みもなされている．

◉プロトロンビン時間（PT）の表記

凝固因子のほとんどは肝細胞で合成される半減期の短い糖タンパクであることから，その血中濃度を反映する PT は肝機能の優れた指標として古くから用いられている．しかし，その測定法は標準化されておらず，延長秒数，対照比，国際標準比（international normalized ratio：INR）等が用いられている．INR は経口抗凝固薬（ワルファリン）服用患者の管理のために設定され，世界的に普及しているが，これを肝疾患患者に対し

表 2　急性肝不全の成因分類

Ⅰ．ウイルス性
　Ⅰ-①A型
　Ⅰ-②B型
　　Ⅰ-②-1．急性感染例
　　Ⅰ-②-2．キャリア例*
　　Ⅰ-②-2-ⅰ．無症候性キャリア例（誘因なし）
　　Ⅰ-②-2-ⅱ．無症候性キャリアの再活性化例
　　Ⅰ-②-2-ⅲ．既往感染の再活性化例（de novo 肝炎）
　　Ⅰ-②-3．判定不能例
　Ⅰ-③C型
　Ⅰ-④E型
　Ⅰ-⑤その他
Ⅱ．自己免疫性
Ⅲ．薬物性
　Ⅲ-①薬物アレルギー
　Ⅲ-②薬物中毒
Ⅳ．循環障害
Ⅴ．悪性腫瘍の肝浸潤
Ⅵ．代謝性
Ⅶ．術後肝不全
Ⅷ．その他
Ⅸ．成因不明
Ⅹ．分類不能

Ⅰ，Ⅱ，Ⅲ-①およびⅨは「劇症肝炎」に相当する急性肝不全の成因である．一方，Ⅲ-②，Ⅳ～Ⅷは肝臓に炎症を伴わない急性肝不全に相当する．なお，これら分類に際して用いる診断基準は別途定める．
*無症候性キャリアで免疫抑制・化学療法が誘因で発症した場合は再活性化例として扱う．また，HBs抗原陰性の既応感染例も再活性化した場合はキャリア例として扱うが，その位置づけに関しては，今後検討することにする．

てそのまま適用すると，試薬間，施設間差が大きいことがすでに指摘されている．しかし，米国ではINRを急性肝不全の定義に加え，移植適応の優先順位の指標（Model for Endstage Liver Disease：MELD）にも採用しており，広く普及しているという現実がある．

したがって，わが国の急性肝不全の診断基準では，「劇症肝炎」の診断基準の時代から用いられているPT 40%以下の基準と併記して，米国の基準であるINR 1.5を採用している．両者は等価でないことはすでに判明しており，基準としては不備といわざるを得ない．経口抗凝固薬として今後もワルファリンが使用され続けるのか不透明な状況であり，INRが今後，測定法として消滅する可能性も考慮すると，肝疾患独自のPT標準化が望まれる．

◉昏睡型の臨床病型

昏睡型の急性肝不全の予後は発症あるいは黄疸の発現から肝性昏睡の発現までの期間により異なることが知られており，この期間によっていくつかの臨床病型にわけられている．この病型の設定は，各基準まちまちである．わが国では劇症肝炎の全国集計をもとに，初発症状から昏睡までの期間が10日以内の急性型と11日以上の亜急性型に分類している（**表1**）．発症–昏睡期間のさらに長いLOHFは，さらに予後不良である．

この傾向は肝炎に限った「劇症肝炎」でのみ成立している可能性があり，米国では，予後を規定しているのは成因であり，発症－昏睡期間（臨床病型）が本質ではないとしている．臨床病型が一見予後と関連しているようにみえるのは，各病型

に含まれる成因に偏りがあるからだとしている[4]．この臨床病型分類が，成因対象を拡大した急性肝不全全体でも意味を持つかどうかは今後の大きな課題である．

まとめ

新たに定められたわが国の急性肝不全の診断基準について解説した．今後，この基準に基づいて全国統計が進められ，非肝炎による急性肝不全の臨床的特徴が明らかになる．現在，急性肝不全のなかで劇症肝炎のみが特定疾患として医療費補助の対象となっているが，今後，この拡大の是非が重要な課題となると思われる．

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Non-alcoholic steatohepatitis and preneoplastic lesions develop in the liver of obese and hypertensive rats: Suppressing effects of EGCG on the development of liver lesions

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ABSTRACT

Non-alcoholic steatohepatitis (NASH), which involves hepatic inflammation and fibrosis, is associated with liver carcinogenesis. The activation of the renin-angiotensin system (RAS), which plays a key role in blood pressure regulation, promotes hepatic fibrogenesis. In this study, we investigated the effects of (−)-epigallocatechin-3-gallate (EGCG), a major component of green tea catechins, on the development of glutathione *S*-transferase placental form (GST-P)-positive (GST-P$^+$) foci, a hepatic preneoplastic lesion, in SHRSP.Z-*Lepr*^{fa}/IzmDmcr (SHRSP-ZF) obese and hypertensive rats. Male 7-week-old SHRSP-ZF rats and control non-obese and normotensive WKY rats were fed a high fat diet and received intraperitoneal injections of carbon tetrachloride twice a week for 8 weeks. The rats were also provided tap water containing 0.1% EGCG during the experiment. SHRSP-ZF rats presented with obesity, insulin resistance, dyslipidemia, an imbalance of adipokines in the serum, and hepatic steatosis. The development of GST-P$^+$ foci and liver fibrosis was markedly accelerated in SHRSP-ZF rats compared to that in control rats. Additionally, in SHRSP-ZF rats, RAS was activated and inflammation and oxidative stress were induced. Administration of EGCG, however, inhibited the development of hepatic premalignant lesions by improving liver fibrosis, inhibiting RAS activation, and attenuating inflammation and oxidative stress in SHRSP-ZF rats. In conclusion, obese and hypertensive SHRSP-ZF rats treated with a high fat diet and carbon tetrachloride displayed the histopathological and pathophysiological characteristics of NASH and developed GST-P$^+$ foci hepatic premalignant lesions, suggesting the model might be useful for the evaluation of NASH-related liver tumorigenesis. EGCG might also be able to prevent NASH-related liver fibrosis and tumorigenesis.

1. Introduction

Non-alcoholic fatty liver disease (NAFLD), which is strongly associated with obesity, diabetes mellitus, and the metabolic syndrome, is becoming one of the most common liver diseases worldwide. NAFLD ranges from simple steatosis to non-alcoholic steatohepatitis (NASH), which is a severe condition of inflamed fatty liver that can progress to hepatic fibrosis, cirrhosis, or even hepatocellular carcinoma (HCC) [1,2]. HCC often occurs in patients with NASH, especially in those with advanced fibrosis and cirrhosis, and the occurrence of HCC is the strongest predictor of mortality in patients with advanced fibrosis [3]. Therefore, in order to improve the prognosis of the patients with NASH, it is necessary to elucidate the pathological mechanisms implicated in the progression of liver fibrosis and HCC development. Several pathophysiological mechanisms explaining the development of HCC in NASH have been described, including the emergence of insulin resistance, induction of chronic inflammation and oxidative stress, and an imbalance of adipokines [1–6]. However, appropriate animal models to evaluate NASH-related liver fibrosis and carcinogenesis have not yet been generated.

Recently, angiotensin-II (AT-II) has been implicated as an important molecule in the progression of liver fibrosis and steatosis [7–9]. AT-II is a component of the renin-angiotensin system (RAS), a key regulator of arterial pressure, and has been shown to induce the contractility and proliferation of hepatic stellate cells (HSCs), which play a pivotal role in liver fibrogenesis [7–9]. RAS is frequently activated in patients with hepatic cirrhosis [8]. Activation of RAS has also been implicated in the etiology of hypertension, obesity, and metabolic syndrome [10]. These findings are significant when considering NASH-related liver carcinogenesis because most patients with NASH that develop HCC experience complications with obesity, diabetes, hypertension, and cirrhosis

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[11]. In addition, AT-II might play a role in the induction of oxidative stress and chronic inflammation in the liver [12,13], both of which are critically involved in the pathogenesis and progression of NASH and the related development of HCC [1–5]. These reports indicate that targeting RAS activation, which is associated with obesity and hypertension, might be an effective strategy to inhibit NASH-related liver carcinogenesis.

The SHRSP.Z-*Lepr*^{fa}/IzmDmcr (SHRSP-ZF) rat is an obese and hypertensive rat, established by crossing stroke-prone spontaneously hypertensive rats (SHRSP) with Zucker Fatty (ZF) rats [14]. SHRSP-ZF rats inherit the leptin receptor *OB-ob* gene mutation found in ZF rats and become obese while developing hypertension. Therefore, the phenotype resembles that of human metabolic syndrome. The rats may thus be a useful tool for investigating the molecular mechanisms underlying metabolic syndrome [15,16]. We therefore considered that appropriate treatment(s) to the SHRSP-ZF rats enable us to establish a novel animal model of NASH and NASH-related hepatocarcinogenesis that mimics those of humans and to use as a preclinical animal model for chemoprevention studies for the diseases.

In the present study, we aimed to create a new NASH-related liver tumorigenesis rat model that appropriately reflects the pathological conditions of human NASH by using SHRSP-ZF rats. We also investigated the potential preventive effects of (−)-epigallocatechin-3-gallate (EGCG), a green tea catechin (GTC), on liver fibrosis, steatosis, and tumorigenesis using this rodent model because green tea is considered to prevent metabolic disorders, including obesity, insulin resistance, hypertension, and NAFLD [17–19], as well as possesses anticancer and cancer chemopreventive properties in various organs, including the liver [20–23]. Glutathione *S*-transferase placental form (GST-P)-positive (GST-P$^+$) foci are frequently used as an indicator of preneoplastic lesions for HCC of rats, since this biomarker shows good correlations with long term carcinogenicity results [24]. We evaluated liver tumorigenesis and chemopreventive efficacy of EGCG in the SHRSP-ZF rats using GST-P$^+$ foci as a biomarker.

2. Materials and methods

2.1. Animals and chemicals

Six-week-old male SHRSP-ZF rats and control Wister Kyoto (WKY) rats, which are normotensive and do not present with obesity, were obtained from Japan SLC (Shizuoka, Japan) and humanely maintained at Gifu University Life Science Research Center in accordance with the Institutional Animal Care Guidelines. High-fat diet 32 (HFD, 507.6 kcal/100 g) with 56.7% fat derived calories was purchased from CLEA Japan (Tokyo, Japan). Carbon tetrachloride (CCl_4) was purchased from Sigma (St. Louis, MO, USA). EGCG was obtained from Mitsui Norin (Tokyo, Japan).

2.2. Experimental procedure

In a preliminary study, we confirmed that the development of preneoplastic lesions, GST-P$^+$ foci, was observed in the liver of WKY and SHRSP-ZF rats only when they were treated with both HFD and CCl_4 (data not shown). Therefore, all rats were fed a pelleted HFD throughout the experiment and received CCl_4 in the present study. After 1 week of acclimatization, 20 WKY rats (Groups 1 and 2; 10 rats for each group) and 20 SHRSP-ZF rats (Groups 3 and 4; 10 rats for each group) were randomly divided into 2 groups. All rats received an intraperitoneal injection of CCl_4 (0.5 mL/kg body weight) twice a week for 8 weeks. At the start of the intraperitoneal injections, the rats in Groups 2 and 4 were provided tap water containing 0.1% EGCG, while the rats in Groups 1 and 3 were provided tap water throughout the experiment. The concentration of EGCG (0.1%), which was established according to the findings of previous chemopreventive studies [22,23] was within the physiological range observed in humans after daily intake of GTCs on a per unit body weight basis [25]. At the end of the experiment (15 weeks of age), all rats were killed by CO_2 asphyxiation, and the development of hepatic steatosis, fibrosis, and GST-P$^+$ foci was determined.

2.3. Histopathological and immunohistochemical examinations

Maximum sagittal sections of 3 sublobes were used for histopathological examination. For all experimental groups, 4 μm-thick sections of formalin-fixed and paraffin-embedded livers were stained with hematoxylin & eosin (H&E) for conventional histopathology or with Azan stain to observe liver fibrosis [26]. The histological features of the livers were evaluated using the NAFLD activity score (NAS) system [27]. The immunohistochemistry of α-smooth muscle actin (α-SMA) [26] and GST-P [28] was performed using primary anti-α-SMA (DAKO, Glostrup, Denmark) and anti-GST-P (MBL, Nagoya, Japan) antibodies, respectively, by using paraffin-embedded sections. In order to evaluate the oxidative stress and lipid peroxidation in the liver, immunohistochemical staining for 8-hydroxy-2′-deoxyguanosine (8-OHdG, NIKKEN SEIL, Shizuoka, Japan) and 4-hydroxy-2′-nonenal (4-HNE, NIKKEN SEIL) of paraffin-embedded sections was performed. Immunohistochemical staining for Mac-1 (Abcam, Cambridge, MA, USA) was also performed on the paraffin-embedded sections to evaluate the infiltration of macrophages in the liver. The Azan- and α-SMA-positive areas were quantified using BZ-Analyzer-II software (KEYENCE, Osaka, Japan) [29]. GST-P$^+$ foci, which consisted of 3 or more positive cells, were counted as hepatic preneoplastic lesions, as previously described [30], and its multiplicity was assessed on a unit area basis (per cm^2). The assessment for GST-P$^+$ foci development and the NAS scoring system were blinded from each other.

2.4. RNA extraction and quantitative real-time reverse transcription-polymerase chain reaction analysis

Total RNA was isolated from the livers of experimental rats using the RNAqueous-4PCR kit (Ambion Applied Biosystems, Austin, TX, USA). cDNA was amplified from 0.2 μg of total RNA using the SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). Quantitative real-time reverse transcription-PCR (RT-PCR) analysis was performed using specific primers that amplify *tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, monocyte chemoattractant protein-1 (MCP-1), plasminogen activator inhibitor-1 (PAI-1), transforming growth factor (TGF)-β1, α-SMA, procollagen-1, tissue inhibitor of metalloproteinases (TIMP)-1, TIMP-2, matrix metalloproteinases (MMP)-2, MMP-9, angiotensin-converting enzyme (ACE), AT-II type 1 receptor (AT-1R), glutathione peroxidase (GPx), catalase (CAT),* and *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* genes. The sequences of *TNF-α, IL-1β, IL-6, MCP-1, PAI-1, TIMP-1, TIMP-2, MMP-2, MMP-9, ACE,* and *AT-1R* primers, which were obtained from Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/), are shown in Supplemental Table S1. The sequences of other primers are described in a previous report [31]. Each sample was analyzed on a LightCycler Nano (Roche Diagnostics, GmbH, Mannheim, Germany) with FastStart Essential DNA Green Master (Roche Diagnostics). Parallel amplification of *GAPDH* was used as the internal control.

2.5. Protein extraction and western blot analysis

Total protein was extracted from the livers of experimental rats and equivalent amounts of proteins (20 μg/lane) were examined by western blot analysis [23]. The primary antibody for cytochrome P450 2E1 (CYP2E1) was purchased from Abcam. Primary antibodies for c-Jun NH2-terminal kinase (JNK), phosphorylated JNK (p-JNK), and GAPDH were obtained from Cell Signaling Technology (Beverly, MA, USA). The antibody to GAPDH served as the loading control.

2.6. Clinical chemistry

The blood samples collected from the inferior vena cava of the rats at the time of killing after 6 h of fasting were used for chemical analyses. The serum levels of TNF-α (R&D Systems, Minneapolis, MN, USA), IL-6 (R&D Systems), insulin (Shibayagi, Gunma, Japan), glucose (BioVision Research Products, Mountain View, CA, USA), adiponectin (Shibayagi), leptin (Shibayagi), total cholesterol (Wako Pure Chemical, Osaka, Japan), triglyceride (Wako Pure Chemical), non-esterified fatty acid (NEFA) (Wako Pure Chemical), and AT-II (USCN Life Science Inc, Wuhan, China) were determined by enzyme immunoassay according to the manufacturers' protocols. The serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured using a standard clinical automatic analyzer (type 7180; Hitachi, Tokyo, Japan).

2.7. Hepatic hydroxyproline analysis

The hepatic hydroxyproline content (μmol/g wet liver) was quantified colorimetrically in duplicate samples from approximately 200 mg wet-weight of liver tissues [32].

2.8. Oxidative stress analysis

Serum hydroperoxide levels, one of the markers for oxidative stress, were determined using the derivatives of reactive oxygen metabolites (d-ROM) test (FREE Carpe Diem; Diacron s.r.l., Grosseto, Italy). After equalizing the protein contents, hepatic levels of malondialdehyde (MDA) were evaluated using an MDA assay kit (Northwest Life Science Specialties, Vancouver, WA, USA).

Table 1
Body, liver, and adipose tissue weights and BMI of the experimental rats.

Group no.	Strain	EGCG	No. of rats	Body weight (g)	Relative organ weight (g/100 g body weight)		BMI[b]
					Liver	Adipose[a]	
G1	WKY	–	10	312.5 ± 13.3[c]	4.1 ± 1.0	1.9 ± 0.4	6.0 ± 0.4
G2	WKY	+	10	296.8 ± 19.4	3.7 ± 0.2	2.0 ± 0.2	6.0 ± 0.3
G3	SHRSP-ZF	–	10	352.9 ± 37.9[d]	5.6 ± 0.6[d]	2.8 ± 0.2[d]	8.3 ± 0.9[d]
G4	SHRSP-ZF	+	10	421.1 ± 38.7[e,f]	6.4 ± 0.4[e]	2.8 ± 0.1[e]	9.4 ± 0.7[e,f]

[a] White adipose tissue of the periorchis and retroperitoneum.
[b] Body mass index.
[c] Mean ± SD.
[d] Significantly different from group 1 by Tukey–Kramer multiple comparison test ($P < 0.05$).
[e] Significantly different from group 2 by Tukey–Kramer multiple comparison test ($P < 0.05$).
[f] Significantly different from group 3 by Tukey–Kramer multiple comparison test ($P < 0.01$).

2.9. Statistical analysis

All data are presented as mean ± SD and were analyzed using the GraphPad In-Stat software program version 3.05 (GraphPad Software, San Diego, CA) for Macintosh. One-way analysis of variance (ANOVA) was used to make comparison between the groups. If the ANOVA analysis indicated significant differences, the Tukey–Kramer multiple comparisons test was performed to compare the mean values among the groups. The differences were considered significant when the two-sided P value was less than 0.05.

3. Results

3.1. General observations

The body weights, relative weights of liver and adipose tissues, and body mass index (BMI) of the SHRSP-ZF rats were significantly higher than those of the WKY rats, regardless of EGCG treatment (Table 1; $P < 0.05$). In SHRSP-ZF rats, the body weights and BMI of the EGCG-treated rats were significantly higher than those of untreated rats ($P < 0.01$), suggesting that EGCG might prevent body weight loss caused by liver fibrosis. During the experiment, EGCG in the drinking water did not cause any clinical symptoms for toxicity. Histopathological examinations also revealed the absence of toxicity from EGCG in the liver, kidney, and spleen (data not shown).

3.2. Effects of EGCG on the development of hepatic preneoplastic lesions and histopathology in the experimental rats

Irrespective of the rat strain, GST-P$^+$ foci were observed in the livers of rats from all groups at the termination of the experiment (Fig. 1A). However, the number of foci was significantly increased, by approximately 5.2-fold, in SHRSP-ZF rats compared to that in WKY rats (Fig. 1B; $P < 0.001$), indicating that obesity and hypertension play a critical role in accelerating the development of hepatic preneoplastic lesions. On the other hand, EGCG treatment significantly inhibited the development of GST-P$^+$ foci in obese and hypertensive SHRSP-ZF rats ($P < 0.001$).

Steatosis with ballooning and/or Mallory-Deng body (Fig. 1C and D), and the infiltration of macrophages (Fig. 1E), which are a recognized feature of alcoholic hepatitis and NASH [27], were observed in the liver of both strains of rats that received CCl_4. However, the NAS scores, which reflect the sum of steatosis, hepatocyte ballooning, and lobular inflammation [27], were significantly higher in the SHRSP-ZF rats than in the WKY rats (Fig. 1F; $P < 0.01$). When given EGCG, the NAS score was improved in SHRSP-ZF rats ($P < 0.01$).

3.3. Effects of EGCG on liver fibrosis in the experimental rats

Azan-stained sections indicated that SHRSP-ZF and WKY rats developed liver fibrosis after CCl_4 injection. However, the degree of fibrosis was more severe in SHRSP-ZF rats; densitometric analysis showed that the hepatic fibrosis area in SHRSP-ZF rats was significantly larger than that in WKY rats (Fig. 2A; $P < 0.001$). Densitometric analysis of α-SMA immunohistochemistry also showed that the α-SMA-immunoreactive areas, which reflect the activation of HSCs, were remarkably increased in the livers of SHRSP-ZF rats in comparison with those in the livers of WKY rats (Fig. 2B; $P < 0.001$). However, administration of EGCG through drinking water significantly improved CCl_4-induced liver fibrosis and inhibited the activation of HSCs in SHRSP-ZF rats (Fig. 2A and B; $P < 0.001$).

Similar findings were observed in the measurements of the hepatic hydroxyproline contents. The amount of hydroxyproline in the liver, which was approximately 7.2-fold higher in SHRSP-ZF rats than in WKY rats ($P < 0.001$), decreased significantly after EGCG treatment (Fig. 2C; $P < 0.01$). Moreover, quantitative real-time RT-PCR analysis revealed that, in the livers of SHRSP-ZF rats, EGCG significantly decreased the expression levels of *MMP-2*, *MMP-9*, *TIMP-1*, *TIMP-2*, *α-SMA*, *procollagen-1*, *TGF-β1*, and *PAI-1* mRNA ($P < 0.05$), all of which were remarkably higher in SHRSP-ZF rats than in WKY rats (Fig. 2D; $P < 0.05$).

3.4. Effects of EGCG on serum levels of AT-II and hepatic expression of ACE and AT-1R mRNA in the experimental rats

Hyperactivity of RAS is closely associated with liver fibrosis and carcinogenesis [8,33]. Therefore, the serum levels of AT-II and the expression levels of RAS components, including *ACE* and *AT-1R* mRNA in the liver, were investigated. The serum level of AT-II was markedly elevated in SHRSP-ZF rats compared to that in WKY rats ($P < 0.001$), but was significantly decreased by EGCG treatment (Fig. 3A; $P < 0.05$). In SHRSP-ZF rats, there was a marked increase in the expression levels of *ACE* and *AT-1R* mRNA in the liver ($P < 0.05$); however, EGCG significantly decreased the expression levels of these mRNA (Fig. 3B; $P < 0.05$).

3.5. Effects of EGCG on oxidative stress, lipid peroxidation in the liver, and hepatic expression of CYP2E1, JNK, and p-JNK proteins in the experimental rats

Hepatic oxidative stress and lipid peroxidation are implicated in the hepatic fibrogenesis, progression of fatty livers to NASH, and development of HCC [4,6]. Therefore, the levels of oxidative stress and antioxidant biomarkers in the experimental rats were next assessed. SHRSP-ZF rats showed a significant increase in serum

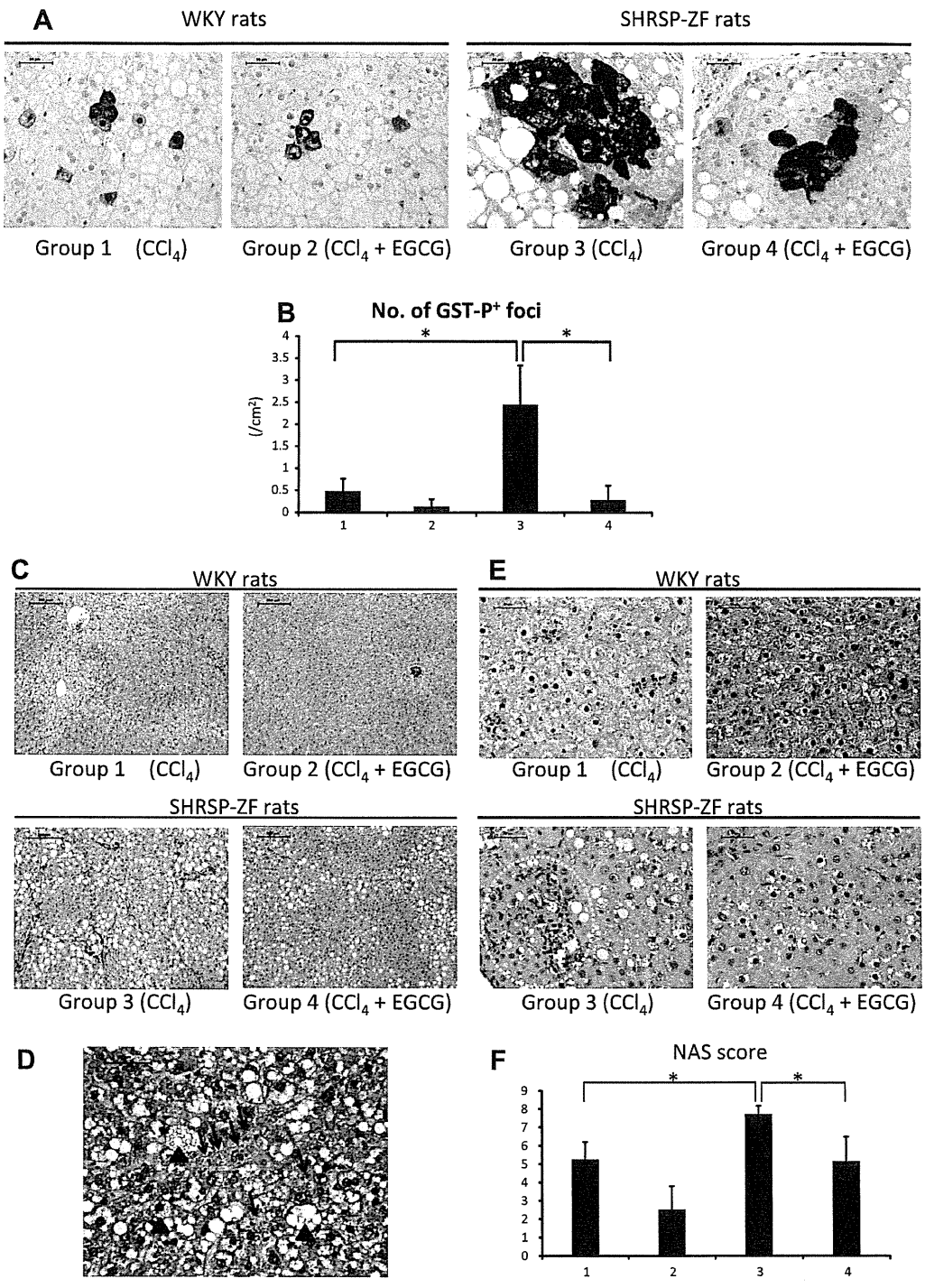

Fig. 1. Effects of EGCG on the development of GST-P$^+$ foci and histopathology in the livers of the experimental rats. (A) Representative photomicrographs of GST-P$^+$ foci and (B) the average number of GST-P$^+$ foci that developed in the livers of the experimental rats. Group 1: WKY rats treated without EGCG, Group 2: WKY rats treated with EGCG, Group 3: SHRSP-ZF rats treated without EGCG, and Group 4: SHRSP-ZF rats treated with EGCG. (C and D) Histopathology of the livers of the experimental rats. H&E staining of liver paraffin sections show steatosis with fibrosis and fatty degeneration in the WKY and SHRSP-ZF rats that were fed HFD and received CCl_4. (D) High magnification of view shows liver cell ballooning (arrow heads) and Mallory-Deng body (arrows) in the liver of a SHRSP-ZF rat from Group 3. (E) The results of the immunohistochemical analysis of Mac-1 in the livers of the experimental rats. Infiltration of macrophages is indicated with circular broken lines. (F) The NAS score (steatosis, inflammation, and ballooning) was determined based on the histopathological analysis. Bars are (A and C) 200 μm and (D and E) 50 μm. The values are expressed as mean ± SD. $^*P < 0.001$.

d-ROM levels, which reflect serum hydroperoxide levels ($P < 0.001$), but this increase was significantly attenuated by EGCG treatment (Fig. 4A; $P < 0.05$). The increased levels of hepatic MDA, a marker of hepatic lipid peroxidation, in SHRSP-ZF rats ($P < 0.05$) were also reduced by EGCG treatment (Fig. 4B; $P < 0.05$). These findings are consistent with the results of immunohistochemical analysis for 8-OHdG, a product of hydroxyl radical-induced oxidative damage in DNA, and 4-HNE, a marker of lipid peroxidation.

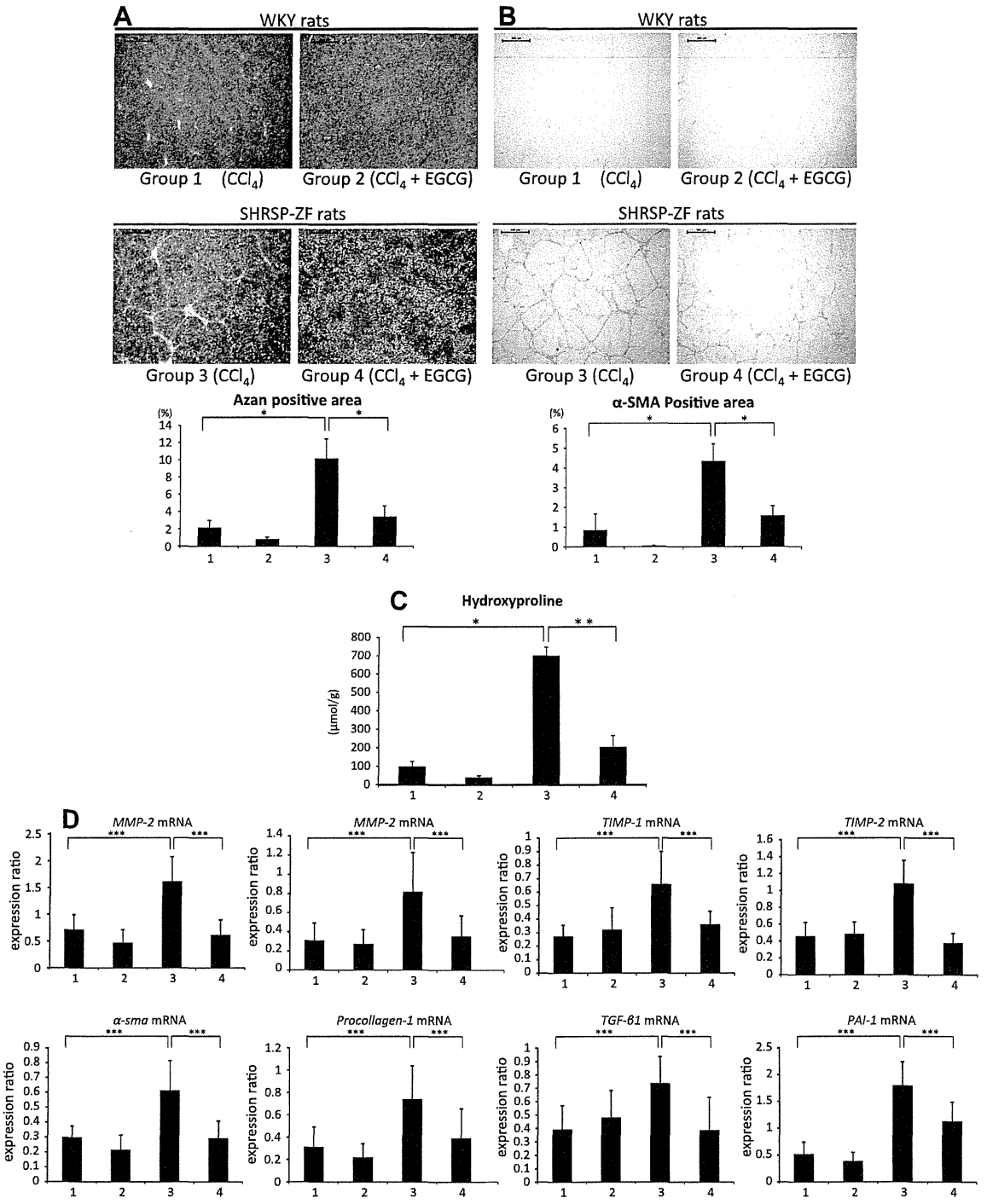

Fig. 2. Effects of EGCG on hepatic fibrosis in the experimental rats. (A) Representative photomicrographs of liver sections stained with Azan stain to show fibrosis (upper panels). The hepatic fibrosis area was evaluated by Azan stain (lower panel). (B) Immunohistochemical detection of α-SMA expression in the livers of the experimental rats (upper panels). The α-SMA-positive area, which shows the activation of HSCs, was evaluated using an image analyzer (lower panel). (C) The hepatic hydroxyproline content was quantified colorimetrically. (D) Total RNA was isolated from the livers of experimental rats, and the expression levels of MMP-2, MMP-9, TIMP-1, TIMP-2, α-SMA, TGF-β1, procollagen-1, and PAI-1 mRNA were examined by quantitative real-time RT-PCR by using specific primers. Bars are 200 μm. The values are expressed as mean ± SD. $^{*}P < 0.001$, $^{**}P < 0.01$, $^{***}P < 0.05$.

The expression levels of 8-OHdG and 4-HNE proteins were markedly increased in the hepatocytes of SHRSP-ZF rats, but they were decreased by EGCG treatment (Fig. 4C). Furthermore, the increased levels of hepatic CYP2E1 and p-JNK proteins, both of which are critically important in HFD-induced NASH development by promoting oxidative stress and inflammation [34,35] in SHRSP-ZF rats were also decreased by EGCG treatment (Fig. 4D). On the other hand, the reduced expression levels of *GPx* and *CAT* mRNA, which encode antioxidant enzymes, in SHRSP-ZF rats ($P < 0.05$) were effectively restored by EGCG treatment (Fig. 4E; $P < 0.05$).

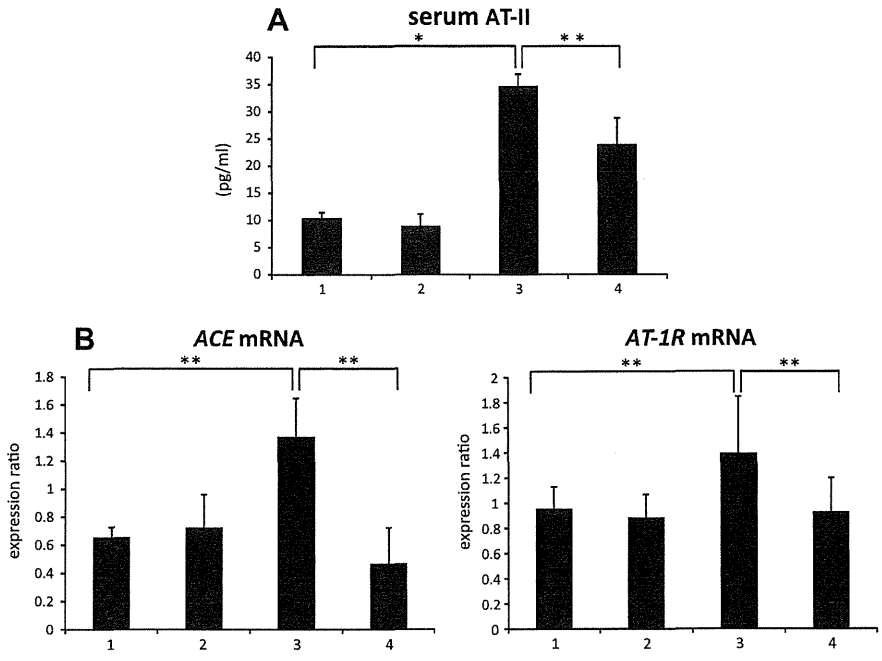

Fig. 3. Effects of EGCG on renin-angiotensin system in the experimental rats. (A) The serum concentrations of AT-II were measured using enzyme immunoassay. (B) The expression levels of ACE and AT-1R mRNA in the livers of the experimental rats were examined by quantitative real-time RT-PCR by using specific primers. The values are expressed as mean ± SD. $^{*}P < 0.001$, $^{**}P < 0.05$.

3.6. Effects of EGCG on serum levels of TNF-α and IL-6 and hepatic expression of TNF-α, IL-6, IL-1β, and MCP-1 mRNA in the experimental rats

Chronic inflammation plays a critical role in the progression of liver fibrosis and subsequent HCC development [5]. Therefore, the levels of inflammatory mediators, including TNF-α, IL-6, IL-1β, and MCP-1, were investigated. The serum levels of TNF-α and IL-6 in SHRSP-ZF rats were significantly elevated relative to those in WKY rats (Fig. 5A; $P < 0.05$). There was also a marked increase in the expression levels of *TNF-α*, *IL-6*, *IL-1β*, and *MCP-1* mRNA in the livers of SHRSP-ZF rats (Fig. 5B; $P < 0.05$). Although EGCG treatment did not significantly affect the serum levels of TNF-α and IL-6 in both SHRSP-ZF and WKY rats (Fig. 5A), the treatment significantly decreased the hepatic expression levels of *TNF-α*, *IL-6*, *IL-1β*, and *MCP-1* mRNA in SHRSP-ZF rats (Fig. 5B, $P < 0.05$).

3.7. Effects of EGCG on serum parameters in the experimental rats

Irrespective of EGCG treatment, the serum levels of AST, ALT, total cholesterol, NEFA, and triglycerides in SHRSP-ZF rats were significantly higher than those in WKY rats (Table 2; $P < 0.05$). The serum levels of glucose and insulin increased significantly, while the value of QUICKI, a useful index of insulin sensitivity [36], decreased ($P < 0.05$). The serum levels of leptin in SHRSP-ZF rats were significantly elevated relative to those in WKY rats, but the levels of adiponectin were lower ($P < 0.05$). Among the parameters elevated in SHRSP-ZF rats, only the serum level of NEFA was significantly suppressed by EGCG treatment ($P < 0.05$). These findings suggest that, in comparison to the improvement of insulin resistance and adipokine imbalance, reduction of oxidative stress and attenuation of inflammation in the liver (Figs. 4 and 5) are more critical mechanisms of EGCG that prevented the early phase of NASH-related liver carcinogenesis in the present study.

4. Discussion

In order to develop an effective strategy for the prevention of NASH-related liver tumorigenesis, there is a critical need to establish an appropriate rodent model that displays the histopathological and pathophysiological characteristics of NASH. The present study provides the first evidence that SHRSP-ZF rats, which present with obesity, diabetes, and hypertension and thus mimic human metabolic syndrome [14,15], more readily develop hepatic preneoplastic lesions, GST-P^{+} foci, than non-obese and normotensive WKY rats when the rats were fed HFD and received CCl_4 injections. The results of the present study clearly indicate that early phase of hepatic tumorigenesis is associated with accelerated steatosis, liver fibrosis, chronic liver damage, presence of insulin resistance, imbalance of adipokines and induction of chronic inflammation and oxidative stress. Because these pathophysiological conditions are critically involved in the progression of NASH and its related liver tumorigenesis [1–5], we propose that our new model using SHRSP-ZF rats might be useful for analyzing the mechanisms of NASH-related liver tumorigenesis and evaluating the efficacy of specific agents that can prevent such tumorigenesis.

One of the limitations in the current study is that we did not observe hepatocellular neoplasms. This might be associated with the duration of the experiment (8 weeks), which was insufficient to develop hepatic tumors. Therefore, future study should recruit longer-term experiments to see that HFD- and CCl_4-treated SHRSP-ZF rats develop hepatocellular neoplasms. Long-term experiments are also useful for evaluating whether the alteration of hepatic gene expression occurred in the present short-term study contribute to the development of hepatocellular neoplasms practically. In addition, it remains unclear whether, not only obesity, but also hypertension actually plays a critical role in the early events of liver carcinogenesis. There are no previous studies that have evaluated the effect of HFD and CCl_4 treatment in hypertensive SHRSP rats as well as in obese ZF rats. Therefore, in order to dissect the effect of hypertension or obesity in liver carcinogenesis,

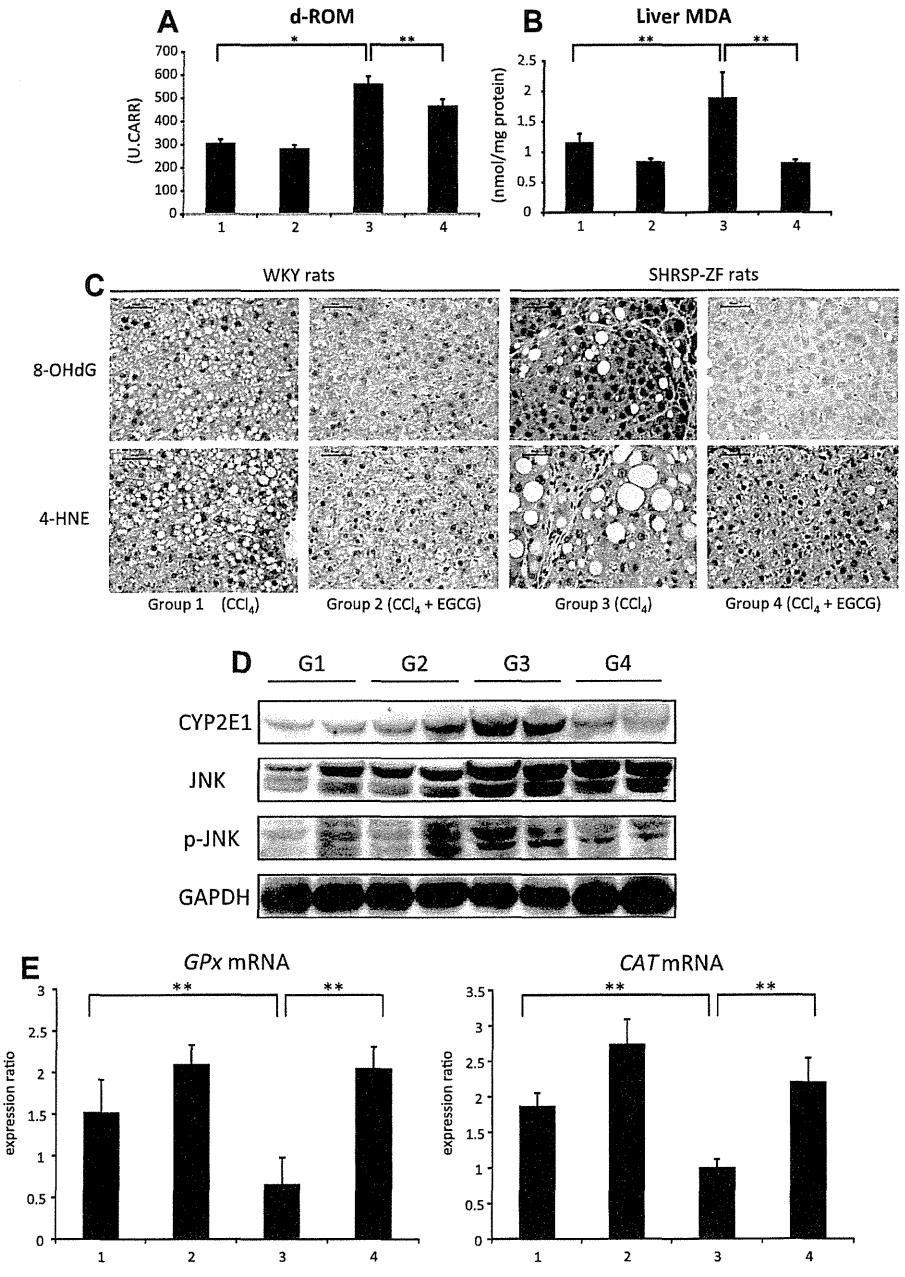

Fig. 4. Effects of EGCG on the serum levels of d-ROM, hepatic concentration of MDA, hepatic expression levels of 8-OHdG, 4-HNE, CYP2E1, JNK, and p-JNK proteins, and hepatic expression levels of GPx and CAT mRNA in the experimental rats. (A) Hydroperoxide levels in the serum were determined by the d-ROM test. (B) The hepatic concentration of MDA was measured by enzyme immunoassay. (C) The results of the immunohistochemical analyses of 8-OHdG and 4-HNE in the livers of the experimental rats. (D) Total proteins were extracted from the livers of the experimental rats and the expression levels of CYP2E1, JNK, and p-JNK proteins were examined by western blot analysis. GAPDH antibody served as the loading control. (E) Total RNA was isolated from the livers of experimental rats, and the expression levels of GPx and CAT mRNA were examined by quantitative real-time RT-PCR by using specific primers. Bars are 50 μm (C). The values are expressed as mean ± SD. $^{*}P < 0.001$, $^{**}P < 0.05$.

additional studies that examine the effects of HFD and CCl_4 treatment in SHRSP rats and ZF rats should be conducted. On the other hand, this study aimed to compare the development of fibrogenesis and preneoplastic lesions (GST-P^{+} foci) between the SHRSP-ZF and WKY rats in order to establish NASH-associated liver carcinogenesis. Because GST-P^{+} foci are generally accepted as precursor or preneoplastic lesions for HCC in rodents [28,30,37], our findings suggest high susceptibility of the obese and hypertensive SHRSP-ZF rats to hapetocarcinogeneis.

What key mechanism accelerates liver fibrosis and tumorigenesis in SHRSP-ZF rats? We presume that activation of RAS caused by obesity and hypertension is critically involved in such disorders in SHRSP-ZF rats because RAS appears to play a major role in liver fibrosis [38]. AT-II induces the fibrotic effect in activated HSCs by stimulating TGF-β1 expression and increasing collagen synthesis in the liver through the activation of its receptor, AT-1R [8,9,38]. Activated HSCs, which highly express AT-1R, are capable of generating AT-II, suggesting that AT-II can act in an autocrine/paracrine manner in HSCs when liver fibrosis progresses [8]. On the other hand, blocking the generation of AT-II and/or its binding to AT-1R attenuates fibrosis development in experimental rodent models of chronic liver injury [39]. Moreover, the potential beneficial abil-

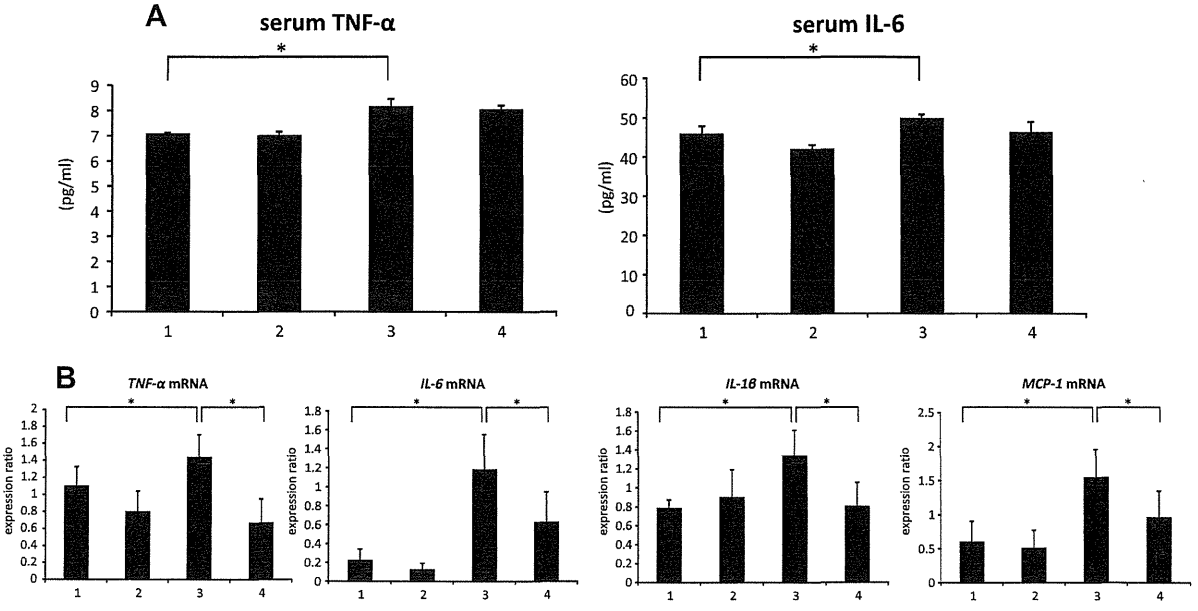

Fig. 5. Effects of EGCG on the serum levels of TNF-α and IL-6 and the expression levels of TNF-α, IL-6, IL-1β, and MCP-1 mRNA in the livers of the experimental rats. (A) The serum concentrations of TNF-α and IL-6 were measured by enzyme immunoassay. (B) Total RNA was isolated from the livers of experimental rats, and the expression levels of TNF-α, IL-6, IL-1β, and MCP-1 mRNA were determined by quantitative real-time RT-PCR by using specific primers. The values are expressed as mean ± SD. $^{*}P < 0.05$.

Table 2
Serum parameters in the experimental rats.

	Group 1	Group 2	Group 3	Group 4
AST (IU/l)	166.8 ± 16.9[a]	140.5 ± 23.6	325.3 ± 45.5[b]	293.0 ± 46.9[c]
ALT (IU/l)	35.5 ± 2.1	36.5 ± 5.4	183.8 ± 42.2[b]	219.3 ± 41.7[c]
Glucose (mg/dl)	106.7 ± 7.2	105.3 ± 4.6	135.1 ± 3.8[b]	127.8 ± 5.3[c]
Insulin (μIU/ml)	25.5 ± 5.4	50.6 ± 8.8	183.4 ± 61.3[b]	223.1 ± 37.5[c]
QUICKI	0.292 ± 0.009	0.269 ± 0.004	0.226 ± 0.008[b]	0.225 ± 0.002[c]
Adiponectin (ng/ml)	52.9 ± 1.9	52.2 ± 0.4	35.2 ± 5.8[b]	35.4 ± 4.0[c]
Leptin (pg/ml)	47.4 ± 4.7	48.4 ± 3.2	400.4 ± 7.3[b]	398.3 ± 5.8[c]
Total cholesterol (mg/dl)	98.2 ± 6.7	93.2 ± 5.0	151.8 ± 9.6[b]	149.8 ± 5.1[c]
NEFA (mEq/L)	0.311 ± 0.038	0.267 ± 0.035	0.698 ± 0.059[b]	0.577 ± 0.046[c,d]
Triglyceride (mg/dl)	53.7 ± 8.1	46.7 ± 8.5	139.9 ± 10.1[b]	128.8 ± 10.9[c]

[a] Mean ± SD.
[b] Significantly different from group 1 by Tukey–Kramer multiple comparison test ($P < 0.05$).
[c] Significantly different from group 2 by Tukey–Kramer multiple comparison test ($P < 0.05$).
[d] Significantly different from group 3 by Tukey–Kramer multiple comparison test ($P < 0.05$).

ity of RAS inhibitors in the attenuation of liver fibrosis in patients with NASH has been shown in clinical trials [40]. Therefore, in the present study, activation of RAS plays a pivotal role in the progression of liver fibrosis in obese and hypertensive SHRSP-ZF rats. EGCG inhibits this fibrogenesis, at least in part, by targeting RAS activation because this agent decreases serum levels of AT-II and suppresses the expression of *ACE* and *AT-1R* mRNA in the liver of these rats. The inhibition of liver fibrosis is significant when considering the chemoprevention of HCC because the risk of liver carcinogenesis increases along with the progression of liver fibrosis [41].

In the liver, RAS is also involved in chronic inflammation and oxidative stress, both of which play a critical role in the progression of fibrosis and subsequent carcinogenesis [8,33]. Administration of AT-II to rats induces HSCs activation, hepatic inflammation, oxidative stress, and lipid peroxidation [42]. Increased systemic AT-II also augments hepatic fibrosis and promotes inflammation and oxidative stress in rats undergoing biliary fibrosis [43]. AT-II stimulates the secretion of inflammatory cytokines such as TNF-α and MCP-1 [44], both of which are involved in the progression of NASH [2], suggesting that targeting RAS might be an effective way to attenuate chronic inflammation and reduce oxidative stress in NASH. AT-1R blockade suppresses HSCs activation, inhibits TNF-α expression, and reduces oxidative stress in rats fed a methionine-choline-deficient diet [39]. The specific delivery of an AT-1R blocker to activated HSCs also reduces inflammation and advanced liver fibrosis in rats [45]. Therefore, consistent with these reports [39,45], EGCG might also prevent liver fibrosis and subsequent tumorigenesis in obese and hypertensive rats by reducing chronic inflammation, systemic oxidative stress, and liver peroxidation, which were induced by RAS activation in the present study. In particular, the effects of EGCG on suppression of the elevated CYP2E1 protein in SHRSP-ZF rats is significant because CYP2E1, which is increased by HFD feeding, is critical in NASH development by promoting oxidative stress, lipid peroxidation, and inflammation [34,35].

Numerous clinical trials have been conducted to develop a therapy that is of proven benefit for NASH; however, no optimal treatment for this disease has yet been found. One of the most practical approaches to treat NASH is targeting insulin resistance and oxidative stress, both of which are implicated as key factors contributing to hepatic injury in patients with NASH [2]. A meta-analysis has

shown that thiazolidinediones, insulin sensitizers regulating glucose metabolism, improve steatosis and serum ALT levels in these patients [46]. In a recent randomized trial with NASH patients, treatment with vitamin E, an antioxidant, also reduced steatosis, lobular inflammation, and serum ALT and AST levels [47]. In the present study, EGCG significantly prevented NASH-related liver fibrosis and tumorigenesis, at least in part, by reducing oxidative stress. Moreover, EGCG also suppresses obesity-related liver and colorectal carcinogenesis by improving hyperinsulinemia [21,23]. The effects of GTCs, whereby they suppress metabolic syndrome, have also been investigated in laboratory animal, epidemiological, and intervention studies [17–19]. These reports [18,19,21,23], together with our findings described here, strongly suggest that GTCs may be useful for preventing the progression of NASH-related liver tumorigenesis, which is associated with oxidative stress and insulin resistance.

Finally, it should be mentioned that the beneficial effects of GTCs have been reported in clinical trials. Supplementation with GTCs can significantly prevent the development of both colorectal adenomas and prostate cancers without causing adverse effects [48,49]. These findings are significant because there are risks associated with medications that are expected to improve NASH, such as weight gain with thiazolidinediones and cardiovascular events and hemorrhagic stokes with vitamin E [46,47]. In summary, our data showed for the first time that liver fibrosis and the development of hepatocellular preneoplastic lesions (GST-P$^+$ foci) are significantly enhanced in obese and hypertensive SHRSP-ZF rats treated with HFD and CCl_4, which have characteristics similar to human NASH. Administration of EGCG effectively prevents liver fibrosis and early stage of hepatocarcinogenesis in these rats by targeting RAS activation and the subsequent inflammation and oxidative stress. Previous rodents studies have shown that GTCs prevent hypertension and target organ damage induced by AT-II through the reduction of oxidative stress [50,51]. GTCs also have a significant inhibitory effect on the activity of ACE and this might be associated with the suppression of high blood pressure in a clinical trial [52]. Although we did not measure the blood pressure of experimental rats in the present study, the results from both experimental and clinical studies [50–52], together with those of present study, strongly indicate the possibility of GTCs, including EGCG, to inhibit RAS activation and to decrease blood pressure subsequently.

In conclusion, our model could be a good option, allowing researchers to study not only the mechanisms involved in NASH-associated hepatocarcinogenesis and the early events involved in tumor formation, but also approaches to HCC prevention in NASH patients focusing on the molecular regulators of the disease. In addition, use of EGCG can improve the NAS score, reduce oxidative stress, and also attenuate chronic inflammation. EGCG therapy represents a potential new strategy for preventing the development of hepatic fibrosis and neoplasm in NASH patients.

5. Conflict of Interest

None declared.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.canlet.2013.08.031.

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