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Ⅳ. 研究成果の刊行物・別冊

Annual Review 糖尿病・代謝・内分泌 2011

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1. 糖尿病・肥満症患者における遺伝子発現変化

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key words　type 2 diabetes, obesity, insulin resistance, gene expression, mitochondria

動　向

近年，2型糖尿病・肥満症は，生体ストレス・炎症や過栄養，多臓器由来液性因子など多因子が体内において複雑なネットワークを形成して発症することが認識されてきた．なかでも肝臓は，糖・蛋白・脂質代謝の司令塔として，これらの多因子を感じとり，遺伝子発現をダイナミックに変化させることで生体の恒常性を維持している．肥満症，糖尿病，およびそれらの合併症である動脈硬化，癌などの，過栄養が関与する症候群に肝臓の機能破綻とそれに伴うインスリン抵抗性が大きく関与している可能性がある．

一方，包括的発現遺伝子解析をはじめとするGenomics技術が進歩したが，一般臨床の場における診断への応用は不十分である．末梢血単核球(PBMC) は全身を巡りながら，2型糖尿病に起因する生体ストレス・炎症，過栄養，多臓器由来液性因子などとともに培養され，発現遺伝子を変化させることで臓器間ネットワークの破綻を反映している可能性がある．

本稿では，糖尿病患者および肥満症患者の肝臓およびPBMCで生じている発現遺伝子プロファイルと代謝パスウェイの協調的発現変動を示し，糖尿病患者の肝臓がインスリン抵抗性を形成する可能性を考察したい．

A. 肝脂肪化がインスリン抵抗性を形成する可能性

2007年のOECD Health Data (http://www.swivel.com/graphs/show/28649976) によれば，BMI 30kg/m^{2}以上の人の割合を見ると，日本人は世界12位で，米国人の30.9%に対し，3.6%にすぎない．にもかかわらず，日本人では，比較的軽度な肥満域から動脈硬化につながる代謝異常が増大する[1,2]．日本人の代謝異常には肥満以外の要素も関わっていると考えた著者らは，メタボリックシンドロームの肝表現型である非アルコール性脂肪肝 (NAFLD) 患者の肝病理像と臨床像の関連を解析した結果，年齢，性，BMIで補正しても，肝の脂肪化，炎症，線維化はインスリン抵抗性指標と有意に関連しており，さらに互いの病理像を補正すると，肝脂肪化のみが独立してインスリン抵抗性を予知していた[3]．

B. 過栄養とヒト肝臓遺伝子発現プロファイル

過栄養によって生じた肝の脂肪化がインスリン抵抗性をはじめとする2型糖尿病の病態を形成する分子機構を解析するため，著者らは，Serial analy-

sis of gene expression (SAGE) およびDNA chip, Real-time PCR等の手法を駆使して，ヒト肝臓の包括的発現遺伝子情報を整備してきた[4]．

in-house cDNA microarrayやReal-time PCR法を用いた解析により，軽度の脂肪肝を有する2型糖尿病患者の肝臓では，糖・脂質代謝，蛋白代謝が大きく変動するとともに，個々の遺伝子に着目すると，TGF-βやBMPなどのTGFスーパーファミリーとその下流で活性化されるVEGFやPDGFなどの血管新生因子，そしてplasminogen activator inhibitor-1 (PAI-1) やコラーゲンなどの全身の動脈硬化症と肝臓の線維化に促進的に作用する生理活性物質やそれらの受容体の遺伝子発現が亢進していることがわかった[5]．動脈硬化関連分子と線維化関連分子はクロストークしており，血管新生因子は線維産生星細胞を活性化し，PAI-1は線溶化を阻害することで線維分解を阻害する．さらに，肥満が加わった患者の肝臓では，正常体重患者の肝臓に比し，インスリン抵抗性を惹起する炎症性サイトカインであるTNF-α，動脈硬化・線維化関連PAI-1の各遺伝子[6]，および肝線維化の準備状態をも作るレニン・アンギオテンシン系に関わる遺伝子[7] が高発現していた．肝臓におけるPAI-1遺伝子発現量が血漿PAI-1レベルと有意に相関する事実[7] は，血漿PAI-1レベルに肝臓が寄与する可能性を示唆する．ヒト正常肝細胞由来培養THLE-5b細胞を用いた検討により，TNF-αとアンギオテン系の経路は一部クロストークしてPAI-1発現を誘導すること[7]，ピオグリタゾンとスタチンがこのTNF-α誘導性PAI-1発現を抑制することがわかった[6]．これらの知見は，糖尿病および肥満状態の肝臓では，すでにインスリン抵抗性，動脈硬化症，肝線維化の病態形成に向けた準備状態にあることを示唆する．事実，肥満と糖尿病の存在は輸血後C型慢性肝炎患者の肝線維化進展[8] あるいは術後肝癌再発[9] の独立した予知因子であった．また，日本人NAFLD患者の臨床病理学的自然歴を追跡した著者らの検討では，厳格な血糖コントロールが，減量にも増して，NAFLD患者の肝線維化改善を予知していた[10]．

脂肪組織と肝臓のネットワークは代謝異常につながる臓器間ネットワークの中でもその中心的存在と考えられる．肥満患者における内臓脂肪組織由来の脂肪酸や生理活性分子は門脈を介して肝臓に注ぎ込む．肝臓は，インスリン感受性亢進作用，抗線維化作用を有する脂肪細胞由来アディポネクチンの重要な標的臓器である．インスリン抵抗性と脂肪肝を有する患者では，血清アディポネクチンレベルが低値であることに加えて，肝臓におけるアディポネクチン受容体の中でも，AdipoR2が選択的に発現低下していた[11]．

C. 2型糖尿病患者の肝臓で変動する代謝パスウェイ

上述の既知のキー分子に加え，未知の肝臓由来生理活性分子が2型糖尿病・肥満症の病態を形成している可能性がある．さらに，2型糖尿病や肥満症は多因子疾患であるため，個々の分子の発現変動のみで全病態を説明できる可能性はむしろ低く，細胞局在や機能などのontologyを考慮した代謝パスウェイの変動をも解析することが求められている．まず，2型糖尿病患者5名と健常人5名の肝発現遺伝子をSAGE法を用いて包括的に解析し，コード蛋白の細胞内局在別に分類したところ，2型糖尿病患者の肝臓では分泌蛋白にもまして，ミトコンドリア蛋白をコードする遺伝子群の発現が有意に亢進していた[12] (表1)．さらにDNA chip法を用いてより多数の糖尿病患者の肝発現遺伝子プロファイルを解析した結果，2型糖尿病患者の肝臓ではミトコンドリア遺伝子のなかでもATP産生を司る酸化的リン酸化 (OXPHOS) 関連遺伝子群が協調的に発現亢進していた[12] (図1)．

表1　2型糖尿病患者の肝臓において，肥満症合併の有無で，協調的差異的に発現変動するエネルギー代謝パスウェイ（文献13より改変）

Pathway description	No of genes	LS Permutation P	KS Permutation P
Glucose metabolism			
Citrate Cycle（TCA Cycle）	44	1.00E-05	1.00E-05
Pyruvate Metabolism	69	1.00E-05	1.00E-05
Pentose Phosphate Cycle	47	1.00E-05	1.00E-05
Lipid metabolism			
Fatty Acid Biosynthesis（path 2）	23	1.00E-05	0.00038
Ceramide Signaling Pathway	49	0.00238	1.00E-05
Mitcohondria			
Oxidative phosphorylation（OXPHOS）	144	1.00E-05	1.00E-05
ATP Synthesis	66	1.00E-05	1.00E-05
Protein metabolism			
Proteasome	30	9.00E-05	1.00E-05
Selenoamino Acid Metabolism	33	0.00043	0.22588

肥満合併および非合併2型糖尿病患者，各々10名，11名の肝発現遺伝子プロファイルAffymetrix 社のHuman Genome U133 Plus 2.0 Array を用いて包括的に解析し，差異的協調的に発現変動する代謝パスウェイを示す．2型糖尿病患者に肥満が加わると，上記のエネルギー代謝パスウェイが肝臓で協調的に発現亢進した．

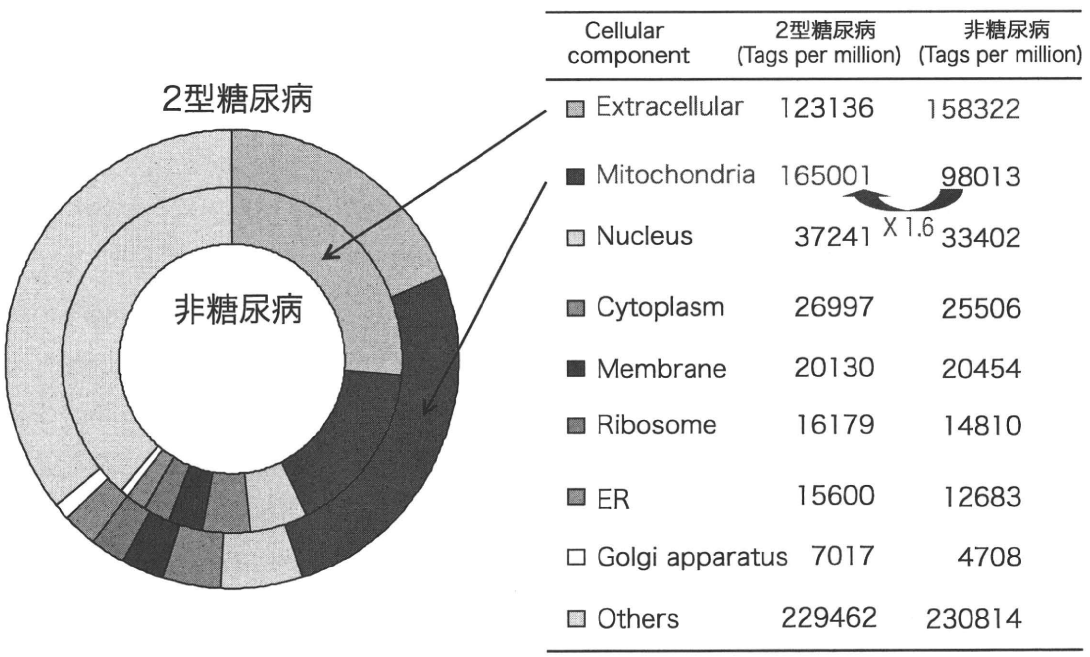

図1　2型糖尿病および健常人の肝臓に発現する遺伝子の細胞局在比較（文献12より改変）
2型糖尿病患者5名と健常人5名の肝発現遺伝子プロファイルをSAGE法を用いて包括的に解析し，発現遺伝子をコード蛋白の細胞内局在別に分類した．2型糖尿病患者の肝臓ではミトコンドリア蛋白をコードする遺伝子群の発現が有意に亢進していた．

OXPHOS関連114遺伝子の発現シグナルを標準化して求めたOXPHOS mean centroidは，各症例のFPGと有意に正相関し，さらにインスリン抵抗性指標であるグルコースクランプ法にて算出したMetabolic Clearance Rateと負に相関した[12]．さらに，OXPHOS遺伝子群は，肝糖新生酵素遺

伝子，エネルギー代謝関連転写因子遺伝子，および活性酸素関連遺伝子群と関連して協調的に発現変動した[13]．以上の結果より，2型糖尿病患者の肝臓におけるミトコンドリアOXPHOS遺伝子群の発現亢進は，ATPを過剰に産生し肝糖新生酵素に供給することで，さらに，活性酸素産生を介してインスリン抵抗性を生じて，肝糖新生の亢進を招く可能性がある．一方，2型糖尿病患者の骨格筋[14]，脂肪組織[15]や末梢血単核球[16]では，肝臓と逆に，OXPHOS遺伝子群は協調的に発現低下していることが報告された．肝特異的および骨格筋特異的AIF遺伝子ノックアウトマウスではOXPHOS遺伝子発現が協調的に減弱し，これらのマウスの肝臓，骨格筋におけるインスリン感受性はむしろ亢進することが報告された[17]．このことは，2型糖尿病患者の骨格筋で観察されたOXPHOS遺伝子発現の低下はむしろ代償機構であることが示唆され，肝臓で観察されたOXPHOS遺伝子の発現亢進がインスリン抵抗性の原因となる可能性が支持されつつあるが，まだ検討の余地がある．また，このような代謝パスウェイの臓器特異的な発現変動を制御する上流の因子が，糖尿病の治療標的となるかもしれない．

D. 肥満症患者の肝臓で変動する代謝パスウェイ

OXPHOS遺伝子群の協調的発現亢進は肥満が加わることでさらに顕著になることがわかった．

そこで，2型糖尿病患者に肥満が加わることで肝臓において差異的協調的に発現変動するエネルギー代謝パスウェイをコードする発現遺伝子群を解析した[13]（表1）．

図2に示すごとく，2型糖尿病患者に肥満が加わると，肝臓において，解糖系および糖新生系を構成する遺伝子群が協調的に発現亢進した．解糖系から派生する経路として，脂肪酸とコレステロール合成に必要な還元力となるNADPHと水素イオンを供給するPentose-phosphate cycle，ATPを供給するTCA cycle，Dihydroxyacetone phosphateをbranch pointとしてglycerol-3-phosphate dehydrogenaseを律速段階酵素とする中性脂肪合成系，それに続きく脂肪酸合成・分解系を構成する遺伝子群が協調的に発現亢進した．これらの酵素遺伝子の発現が基質依存性に誘導されているとすれば，解糖系の代謝産物としてAcetyl CoA，脂肪酸酸化の代謝産物としてAcyl CoAがそれぞれOXPHOS経路の基質としてミトコンドリアに流入する．おそらくこのような基質の過剰流入を受けて，OXPHOSを構成する遺伝子群が協調的に発現誘導された（表1）可能性を考える．

近年，蛋白代謝がインスリン抵抗性やエイジングと関連する可能性を示す基礎研究が報告されている．蛋白合成系を制御するmTOR-S6 Kinase経路の活性化が長寿の阻害[18]やインスリン抵抗性[19]を引き起こすこと，カロリー制限やメトフォルミンがこの経路を抑制しうることが示された[19,20]．一方，著者らは，肥満症患者の肝臓で選択的蛋白分解系を担うプロテアソーム経路を構成する遺伝子が協調的に発現亢進することを見出し（表1），病態との意義を明らかにするために現在機能解析を進めているところである．

E. 2型糖尿病患者の病態を形成する新規肝臓由来分泌蛋白の探索

代謝の司令塔であり分泌蛋白の主要な産生源である肝臓には臓器間ネットワークを制御する未知のホルモン，すなわちヘパトカインが存在することが推定されてきたが，それらの同定と解析は未だ不十分である．そこで，SAGE，DNA chip解析から得た遺伝子情報から，シグナルペプチドの構造を有すると推測される遺伝子，あるいは

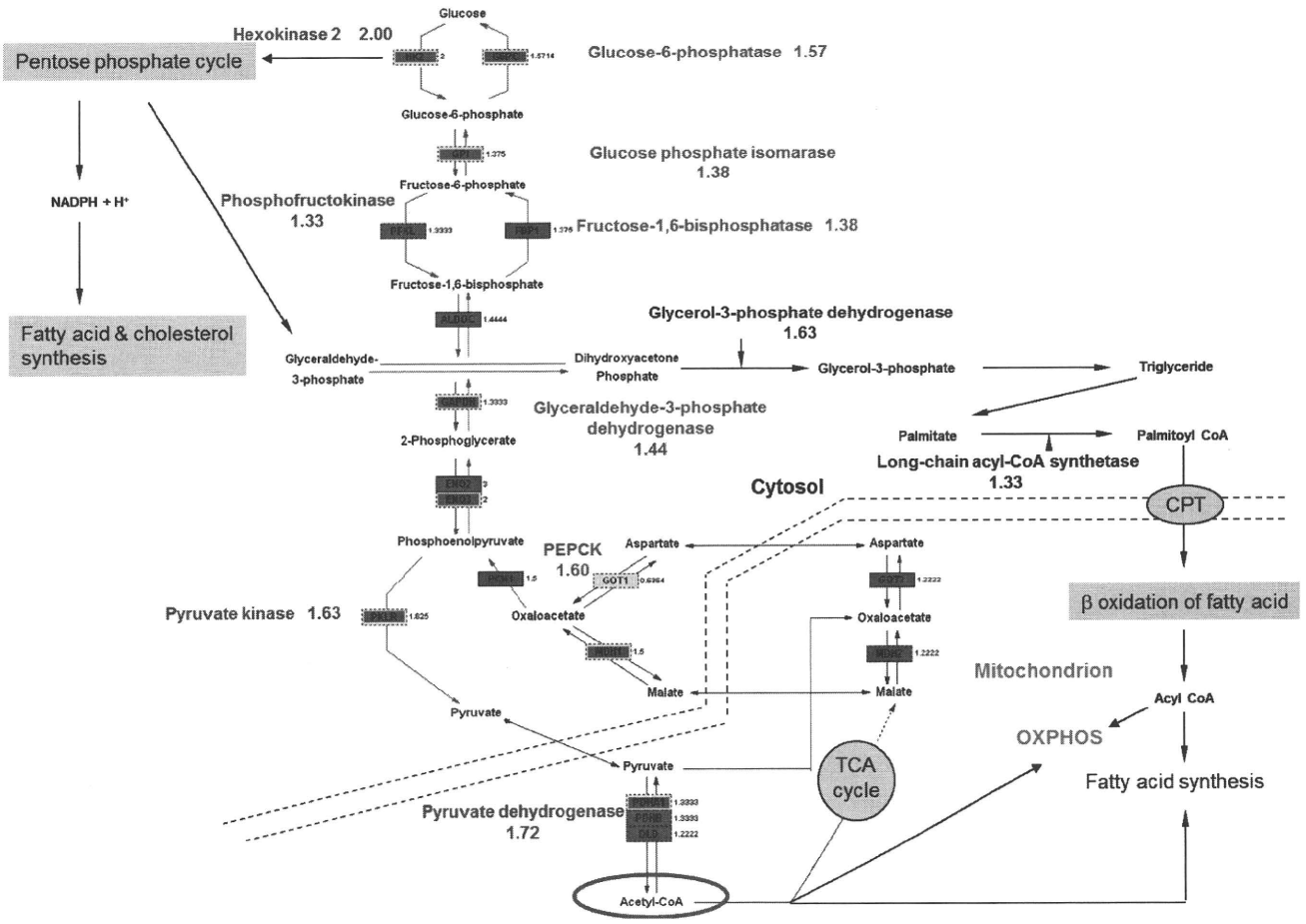

図2　2型糖尿病に肥満が加わることで発現変動する糖・脂質代謝経路構成遺伝子群（文献13より改変）
解糖系および糖新生経路を構成する遺伝子群がともに発現亢進した．解糖系から派生する経路として，脂肪酸とコレステロール合成に必要な還元力となるNADPHと水素イオンを供給するPentose-phosphate cycle，ATPを供給するTCA cycle，脂肪酸合成・分解系を構成する遺伝子群が協調的に発現亢進した．有意に（$P<0.05$）発現亢進する遺伝子を赤で示す．

Gene ontology cellular compartmentでextracellularに分類される遺伝子に絞り，発現量と臨床マーカーの関連を照合した．このようなアプローチで，インスリン抵抗性，肥満，あるいは血糖コントロールと関連する肝臓由来分泌蛋白コード遺伝子を絞り込んだ．

これらの中で，ヒト肝臓での発現がインスリン抵抗性および高血糖と関連する機能性ヘパトカインとしてセレノプロテインP selenoprotein P (SeP) を同定した[21]．SePは主に肝臓で産生される分子量約50キロダルトンの分泌蛋白で，必須微量元素であるセレンの輸送蛋白として機能することが知られていたが，糖代謝に与える影響は不明であった．in vitroおよびin vivoの検討から，SePは，少なくとも一部にAMPキナーゼの活性抑制を介して全身のインスリン抵抗性を引き起こし，糖代謝を障害することがわかった[21]．この結果は，ヒトの臨床サンプルを用いて臨床情報との関連をもとに絞り込む候補遺伝子アプローチが，新規機能性分泌蛋白のスクリーニングに有用であることを示唆する．

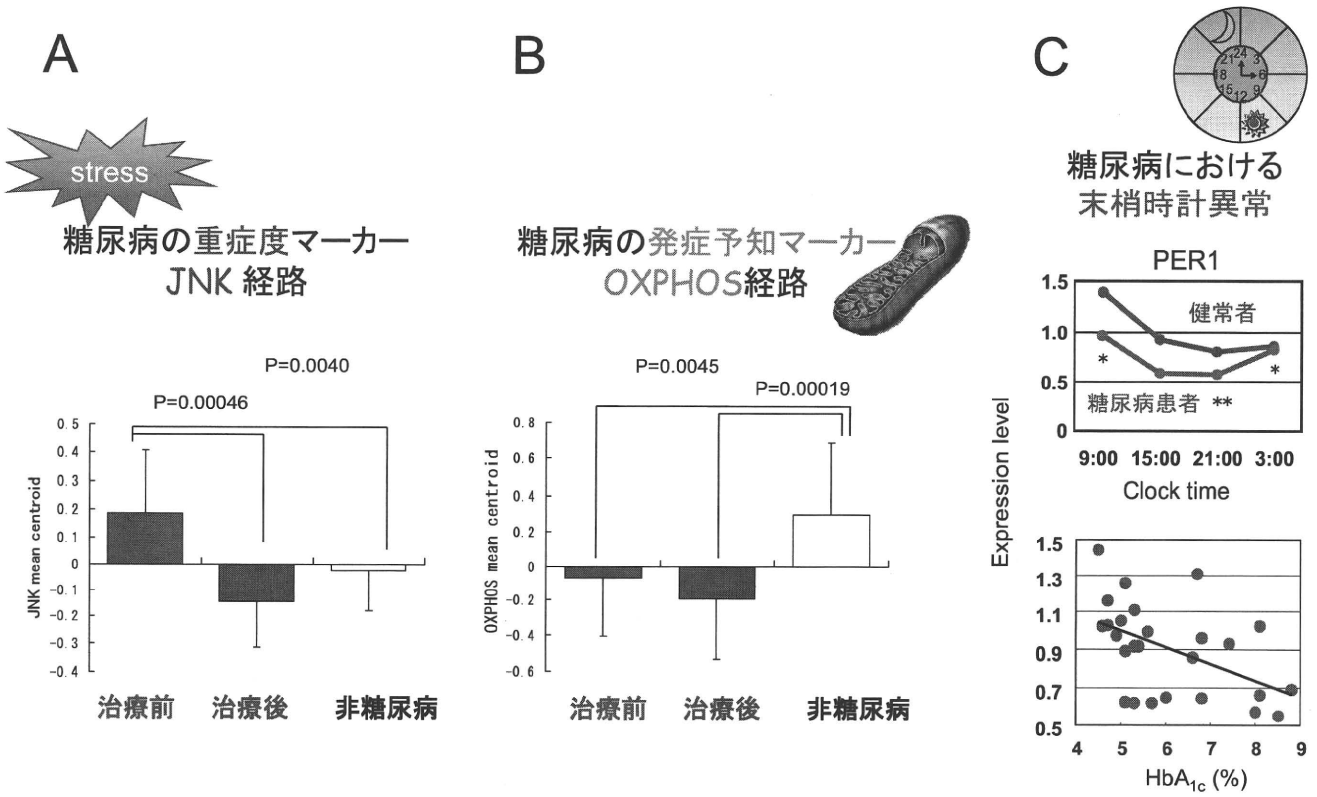

図3　末梢血単核球（PBMC）の遺伝子発現プロファイルは糖尿病患者の病態を予知する（文献16，25より改変）
A，B: JNK経路およびOXPHOS経路を構成する遺伝子群の協調的発現変動（平均±SD）
A: JNK mean centroidは糖尿病群で健常群に比し有意に高値で，血糖コントロールにより改善した．
B: OXPHOS mean centroidは糖尿病群で健常群に比し有意に低値で，血糖コントロールにより変化せず，血糖コントロール後も健常者に比し低値であった．
C: 糖尿病患者のPBMCではPER1をはじめとする時計遺伝子のリズム性発現が減弱しており（上），その程度は血糖コントロール依存性であった（下）．

F. 末梢血単核球の遺伝子発現プロファイルが2型糖尿病患者の病態を反映する可能性

先述のごとく，2型糖尿病は，生体ストレス・炎症や過栄養，多臓器由来液性因子など多因子が複雑なネットワークを形成して発症する．著者らは，末梢血単核球（PBMC）が，こうした多彩な因子にさらされて，遺伝子発現をダイナミックに変化させることで病態を反映している可能性に着目した．そこで，糖尿病の有無，および高血糖を反映するPBMC発現遺伝子パスウェイを抽出し，それらの意義を検証した[16]．2型糖尿病患者および若年健常者よりPBMCを単離し，DNAチップを用いて差異のある発現遺伝子およびパスウェイを抽出した．糖尿病の有無でP＜0.0005と大きく発現変動していたパスウェイの中で，血糖コントロールによって改善したMAPキナーゼ経路の一つであるc-Jun N-terminal kinase（JNK）経路を同定した．一方，糖尿病の有無で有意に変動し，血糖コントロールによって変動しないミトコンドリア酸化的リン酸化経路（OXPHOS）を同定した．JNKとOXPHOS両経路を構成する，各々99，77遺伝子の発現レベルを標準化し，症例ごとに平均化したmean centroidを求めた．JNK mean centroidは糖尿病群で健常群に比し有意に高値で，血糖コントロールにより改善した（図3A）．OXPHOS mean centroidは糖尿病群で健常群に

比し有意に低値で，血糖コントロールにより変化せず，血糖コントロール後も健常者に比し低値であった（図3B）．次に，各々の経路の協調的遺伝子発現と関連する糖尿病の病態を解析した．JNK mean centroidは，血糖コントロールの指標である空腹時血糖値およびHbA1c値と有意な正相関を示した．一方，OXPHOS mean centroidは肥満，血糖コントロール，いずれの病態とも関連は低かった[16]．各々の遺伝子群からP値の低い10遺伝子を選択してアルゴリズムを作成したところ，JNK径路は血糖コントロール前後を，OXPHOS経路は糖尿病の有無を，80～90%の高い確率で，いずれも有意に診断し得た．以上の結果は，末梢血単核球における発現遺伝子プロファイルが2型糖尿病患者の病態を映し出す可能性を示唆する．JNK遺伝子群の協調的発現亢進は高血糖による酸化ストレスを反映しうる．一方，OXPHOS遺伝子群の協調的発現低下は2型糖尿病患者にもとより内在し，発症予知マーカーとなる可能性がある．

一方，現代人は生活や睡眠のリズムが乱れており，肥満の要因として寄与していることが示唆されている．生体には，細胞レベルでも個体レベルでも時計遺伝子がリズムを刻んでいることで恒常性を維持していることがわかってきた．筆者らは，肥満2型糖尿病モデル動物では肝臓や内臓脂肪組織における時計遺伝子のリズム性発現が減弱し，アディポサイトカイン遺伝子発現リズムの消失を認めることを見出し[22-24]，2型糖尿病の病態に時計遺伝子（生体リズム）異常が密接に関連する可能性を示した．ところが，このような生体リズム異常をヒトで診断する技術は存在しなかった．最近，筆者らは，発現遺伝子解析により，ヒト末梢血細胞に概日リズムがあること，糖尿病患者では時計遺伝子機能が減弱していること，さらに時計遺伝子のリズム性発現減弱が血糖コントロール依存性であることを示した[25]（図3C）．今後，ヒトにおいても，生体リズム異常がインスリン抵抗性と代謝異常の成因として関与し，治療の標的となりうるかを検証していく必要がある．

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Characteristics of Patients With Nonalcoholic Steatohepatitis Who Develop Hepatocellular Carcinoma

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BACKGROUND & AIMS: Nonalcoholic steatohepatitis (NASH) can progress to hepatocellular carcinoma (HCC). We aimed to characterize the clinical features of NASH patients with HCC. **METHODS:** In a cross-sectional multicenter study in Japan, we examined 87 patients (median age, 72 years; 62% male) with histologically proven NASH who developed HCC. The clinical data were collected at the time HCC was diagnosed. **RESULTS:** Obesity (body mass index ≥25 kg/m^2), diabetes, dyslipidemia, and hypertension were present in 54 (62%), 51 (59%), 24 (28%), and 47 (55%) patients, respectively. In nontumor liver tissues, the degree of fibrosis was stage 1 in 10 patients (11%), stage 2 in 15 (17%), stage 3 in 18 (21%), and stage 4 (ie, liver cirrhosis) in 44 (51%). The prevalence of cirrhosis was significantly lower among male patients (21 of 54, 39%) compared with female patients (23 of 33, 70%) (P = .008). **CONCLUSIONS: Most patients with NASH who develop HCC are men; the patients have high rates of obesity, diabetes, and hypertension. Male patients appear to develop HCC at a less advanced stage of liver fibrosis than female patients.**

Keywords: Liver Cancer; Incidence; Sex; Retrospective Study.

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and the third leading cause of cancer mortality.[1] HCC mostly occurs within an established background of chronic liver disease and cirrhosis. Although the risk factors for HCC, including infection with hepatitis B and C viruses as well as alcohol consumption, are well-defined, 5%–30% of patients with HCC lack a readily identifiable risk factor for their cancer. It has been suggested that a more severe form of nonalcoholic fatty liver disease (NAFLD), namely nonalcoholic steatohepatitis (NASH), might account for a substantial portion of cryptogenic cirrhosis and HCC cases.[2]

NAFLD is one of the most common causes of chronic liver disease in the world.[3,4] NAFLD is associated with obesity, diabetes, dyslipidemia, and insulin resistance and is recognized as a hepatic manifestation of metabolic syndrome. The spectrum of NAFLD ranges from a relatively benign accumulation of lipid (simple steatosis) to progressive NASH associated with fibrosis, necrosis, and inflammation. Despite its common occurrence and potentially serious nature, relatively little is known about the natural history or prognostic significance of NAFLD. Although prospective studies on the natural history of NAFLD and NASH with a larger cohort are awaited, these studies might be limited by the long and asymptomatic clinical course of these diseases, by their high prevalence in the general population, and by the lack of serologic markers for NASH. The evidence suggesting that NASH can progress to HCC comes from (1) case reports and case series,[5–8] (2) retrospective studies,[9–12] and (3) prospective studies.[13–17] These studies generally examined limited numbers of cases and follow-ups; therefore, the incidence of HCC and risk factors for HCC in NASH patients remain unclear.

The Japan NASH Study Group (representative, Takeshi Okanoue)[18] was established in 2008 by the Ministry of Health, Labour and Welfare of Japan to address unmet research needs in the area of liver diseases. As a part of this mandate, the study group conducted a cross-sectional multicenter study to characterize the clinical features of histologically proven NASH patients who developed HCC.

Methods

Patients

We retrospectively identified and reviewed 87 Japanese patients with NASH, who developed HCC between 1993 and 2010, at 15 hepatology centers that belong to the Japan NASH Study Group[18] and their affiliated hospitals in Japan. The di-

Abbreviations used in this paper: AFP, α-fetoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; CT, computed tomography; DCP, des-γ-carboxy prothrombin; γ-GTP, γ-glutamyl transpeptidase; HCC, hepatocellular carcinoma; HDL, high-density lipoprotein; MRI, magnetic resonance imaging; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis.

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agnosis of NASH was based on (1) the histologic features of steatohepatitis, (2) negligible alcohol consumption, and (3) exclusion of liver diseases of other etiology. To determine alcohol consumption as accurately as possible, we reviewed medical records in our institutions, and when patients had been transferred from other institutions, we also reviewed a summary of medical records from those institutions. According to the medical records, alcohol consumption was assessed on the basis of a detailed history that was obtained by physicians and by interviewing family members. Exclusion criteria included consumption of more than 20 g of alcohol per day, positivity for hepatitis B virus surface antigen, positivity for anti-hepatitis C virus antibody, the presence of other types of liver diseases (eg, primary biliary cirrhosis, autoimmune hepatitis, Wilson's disease, or hemochromatosis), previous treatment with drugs known to produce hepatic steatosis, and a history of gastrointestinal bypass surgery. The sections of nontumor liver tissues were reanalyzed by experienced hepatopathologists (T.O., E.H.) who were blinded to the laboratory parameters and clinical data. We excluded patients whose histologic diagnosis of NASH was not confirmed by central review and patients with insufficient or inconclusive information concerning alcohol consumption, body mass index (BMI), and laboratory data including fasting glucose and lipid.

Of the 87 patients, 14 patients had been previously diagnosed as NAFLD or NASH and had been followed at our institutions; 73 patients had been transferred from other institutions to our institutions for investigation and treatment of HCC. Most patients had been identified as having HCC during screening, which included ultrasound and/or computed tomography (CT) of the liver and alpha-fetoprotein (AFP) testing.

The diagnosis of HCC was based on liver histology and, in the absence of histology, on typical features of HCC as assessed by dynamic CT or magnetic resonance imaging (MRI) (ie, hypervascular with washout in the portal/venous phase).[19] Of the 87 patients, 49 patients were diagnosed as HCC after hepatic resection, 21 patients were diagnosed after ultrasound-guided tumor biopsy, and 17 patients were diagnosed by dynamic CT or MRI.

The Ethics Committees of each participating center approved this study. Informed consent was obtained from each patient in accordance with the Declaration of Helsinki.

Clinical Assessment and Laboratory Tests

The clinical and laboratory data were collected at the time HCC was diagnosed. BMI was calculated by using the following formula: weight in kilograms/(height in meters)^2. Obesity was defined as BMI $\geq$25 kg/m^2 according to the criteria of the Japan Society for the Study of Obesity.[20] Diabetes was defined as fasting plasma glucose concentration of $\geq$126 mg/dL or 2-hour plasma glucose concentration of $\geq$200 mg/dL during an oral glucose (75 g) tolerance test or by the use of insulin or oral hypoglycemic agents to control blood glucose.[21] Hypertension was defined as systolic blood pressure $\geq$130 mm Hg or diastolic blood pressure $\geq$85 mm Hg or by the use of antihypertensive agents.[22] Dyslipidemia was defined as serum concentrations of triglycerides $\geq$150 mg/dL or high-density lipoprotein (HDL) cholesterol $<$40 mg/dL and $<$50 mg/dL for men and women, respectively, or by the use of specific medication.[22]

Venous blood samples were taken in the morning after 12-hour overnight fast. The laboratory evaluations included blood cell count and measurement of serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyl transpeptidase (γ-GTP), fasting plasma glucose, HbA1c, total cholesterol, HDL cholesterol, triglyceride, ferritin, hyaluronic acid, AFP, and des-γ-carboxy prothrombin (DCP). These parameters were measured by using standard clinical chemistry techniques.

Histopathologic Examination

Nontumor liver tissues were obtained from all 87 patients to diagnose the background liver tissue at the time HCC was diagnosed. In 49 patients who underwent hepatic resection for HCC, we examined nontumor liver tissues that were surgically resected. In 21 patients who underwent ultrasound-guided tumor biopsy, nontumor liver tissues far from HCC tumors were biopsied separately. In 17 patients who were diagnosed as HCC by dynamic CT or MRI and did not undergo either hepatic resection or tumor biopsy, only nontumor liver tissues far from HCC tumors were obtained by ultrasound-guided biopsy.

The specimens were fixed in formalin, embedded in paraffin, and stained with hematoxylin-eosin, with Masson trichrome, and by silver impregnation. NASH was defined as steatosis with lobular inflammation, hepatocellular ballooning, and Mallory's hyaline (Mallory's body) or fibrosis.[23-25] The necroinflammatory grade and the degree of fibrosis were evaluated and scored according to the criteria proposed by Brunt et al.[26]

Statistical Analysis

Results are presented as numbers with percentages in parentheses for qualitative data or as the medians and ranges (25th–75th percentiles) for quantitative data. Comparisons were made by using a χ^2 test for qualitative factors or a Mann-Whitney U test on ranks for quantitative factors with non-equal variance. P values less than .05 from two-sided tests were considered to be significant. All statistical analyses were performed by using SPSS 15.0 software (SPSS Inc, Chicago, IL).

Results

The characteristics of the 87 NASH patients who developed HCC are summarized in Table 1. The median age was 72 years (25th percentile, 69; 75th percentile, 75); the mean age (standard deviation) was 71.2 (6.7) years. There were 54 male patients (62%) and 33 female patients (38%); the male:female ratio was 1.6:1. The median BMI was 26.0 kg/m^2, and 54 patients (62%) were obese (BMI $\geq$25 kg/m^2). Diabetes, dyslipidemia, and hypertension were present in 51 (59%), 24 (28%), and 47 (55%) patients, respectively. T1

The diagnosis of NASH was proved by histologic examination of nontumor liver tissues at the time HCC was diagnosed. The degree of steatosis was grade 1 (5%–33%) in 60 patients (69%), grade 2 (34%–66%) in 19 (22%), and grade 3 (>66%) in 7 (8%). One patient who showed less than 5% steatosis was diagnosed as "burn-out" NASH, because a previous liver biopsy that was performed before development of HCC had demonstrated typical histologic features of NASH. The necroinflammatory grade was mild (grade 1) in 31 patients (35%), moderate (grade 2) in 45 (52%), and severe (grade 3) in 11 (13%). The degree of

Table 1. Patient Characteristics

Characteristic	Total (n = 87)	Male (n = 54)	Female (n = 33)	*P* value[a]
Age (y)	72 (69–75)	72 (69–75)	72 (68–75)	.52
BMI (kg/m^2)	26.0 (23.8–28.3)	26.0 (23.8–28.8)	26.2 (23.9–27.7)	.54
Obesity	54 (62%)	35 (65%)	19 (58%)	.50
Diabetes	51 (59%)	31 (57%)	20 (61%)	.77
Dyslipidemia	24 (28%)	13 (24%)	11 (33%)	.35
Hypertension	47 (54%)	22 (41%)	25 (76%)	.001
Platelet count (×10^4/μL)	13.9 (10.1–18.0)	14.5 (11.7–18.0)	10.9 (7.8–18.0)	.05
AST (IU/L)	47 (30–59)	46 (27–60)	47 (35–58)	.45
ALT (IU/L)	36 (26–55)	43 (26–69)	34 (26–42)	.11
γ-GTP (IU/L)	75 (40–115)	68 (36–177)	75 (40–115)	.90
Fasting glucose (mg/dL)	114 (99–145)	112 (99–144)	120 (97–152)	.59
HbA1c (%)	6.1 (5.4–7.1)	5.9 (5.4–7.0)	6.3 (5.2–7.1)	.78
Total cholesterol (mg/dL)	169 (147–202)	169 (147–202)	169 (147–202)	.62
HDL cholesterol (mg/dL)	50 (41–60)	45 (41–58)	55 (50–73)	.03
Triglyceride (mg/dL)	100 (76–138)	118 (80–147)	96 (74–116)	.06
Ferritin (ng/dL)[b]	197 (74–401)	273 (154–703)	98 (23–172)	.005
Hyaluronic acid (ng/mL)[c]	166 (67–241)	151 (69–244)	174 (61–332)	.85
AFP (ng/mL)	7.1 (5.0–18.0)	6.0 (4.0–14.7)	10.8 (5.9–18.0)	.02
DCP (mAU/mL)	66 (22–298)	48 (22–243)	81 (21–942)	.42
HCC tumor size (cm)	3.0 (2.0–4.0)	3.1 (2.2–4.5)	2.6 (1.9–4.0)	.18
Number of HCC tumors				.78
1	65 (75%)	39 (72%)	26 (79%)	
2 or 3	16 (18%)	11 (20%)	5 (15%)	
≥4	6 (7%)	4 (8%)	2 (6%)	
Background liver tissue				
Steatosis grade				.64
0: <5%	1 (1%)	1 (2%)	0 (0%)	
1: 5%–33%	60 (69%)	36 (67%)	24 (73%)	
2: 34%–66%	19 (22%)	11 (20%)	8 (24%)	
3: >66%	7 (8%)	6 (11%)	1 (3%)	
Necroinflammatory grade[d]				.22
1: mild	31 (35%)	22 (41%)	9 (27%)	
2: moderate	45 (52%)	26 (48%)	19 (58%)	
3: severe	11 (13%)	6 (11%)	5 (15%)	
Fibrosis stage[d]				.003
1	10 (11%)	10 (18%)	0 (0%)	
2	15 (17%)	10 (18%)	5 (13%)	
3	18 (21%)	13 (25%)	5 (15%)	
4	44 (51%)	21 (39%)	23 (70%)	

Values are medians (25th–75th percentiles) or numbers (%). Where no other unit is specified, values refer to number of patients.
[a]Chi-square test or Mann-Whitney *U* test.
[b]Missing data for 27 patients.
[c]Missing data for 29 patients.
[d]According to reference 26.

fibrosis was stage 1 in 10 patients (11%), stage 2 in 15 (17%), stage 3 in 18 (21%), and stage 4 (ie, liver cirrhosis) in 44 (51%).

The median diameter of HCC tumors was 3.0 cm (25th percentile, 2.0; 75th percentile, 4.0). A single HCC lesion was present in 65 of 87 patients (75%).

Data were stratified according to sex (Table 1). Compared with female patients, male patients had significantly less hypertension, lower HDL cholesterol and AFP, higher ferritin, and a less advanced stage of fibrosis. The prevalence of cirrhosis was significantly lower in male patients (21 of 54, 39%) than in female patients (23 of 33, 70%) (P = .008).

Discussion

In this cross-sectional multicenter study in Japan, we showed the clinical features of a relatively large number (n = 87) of NASH patients with HCC. The male:female ratio was 1.6:1. Men have higher HCC rates than women in almost all populations, with male:female ratios usually averaging between 2:1 and 4:1.[2] In the latest nationwide survey of HCC in Japan,[27] this ratio was 2.5:1. The reasons underlying higher rates of HCC in men might relate to sex-specific differences in exposure to risk factors. Men are more likely to be infected with hepatitis B and C viruses, consume alcohol, smoke cigarettes, and have increased iron stores.[2] Moreover, androgens are considered to influence the development of HCC. With regard to the male:female ratio of HCC associated with NASH, a male:female ratio of 1.3:1 was reported in a summary of 16 published cases of HCC associated with NASH.[28] Ratios of 2.8:1 and 0.67:1 were reported in 2 retrospective studies of HCC arising from cryptogenic cirrhosis in Italy (n = 44)[10] and the United States (n =

30),[9] respectively, and a ratio of 1.6:1 was reported for 36 cases of NASH-associated HCC from a single center in Japan.[15] Overall, NASH patients with HCC are more often men. However, these male:female ratios might be lower than the ratios for HCC of other etiologies, including viral hepatitis and alcohol consumption.

Although it is well-known that male gender is a risk factor for HCC in patients infected with hepatitis B and C viruses,[2] it remains unclear whether male gender is a factor associated with the development of HCC in NASH patients. It is now suspected that there is an even distribution of NASH among men and women.[29] In another study by our group,[30] the male:female ratio was 0.85:1 in 342 NASH patients without cirrhosis and HCC. The male:female ratio (1.6:1) of NASH patients with HCC in the present study is higher than this ratio. In agreement with our observations, a case-control study showed that the male: female ratio was 1.6:1 in 34 NASH patients with HCC, whereas the ratio was 0.69:1 in 348 NASH patients without HCC.[15] A recent prospective study indicated that older age and alcohol consumption were independent risk factors for the development of HCC in patients with NASH-cirrhosis and that male gender tended to be associated with the development of HCC, although this trend did not reach statistical significance.[17]

The median age of our patients was 72 years. There was no significant difference in age between men and women. Although the global age distribution of HCC varies by geographic region, sex, and etiology, in almost all areas the peak female age group in HCC patients is 5 years older than in male HCC patients.[2] In a nationwide survey of HCC in Japan,[27] the mean ages were 65.5 years for men and 69.4 years for women. The male patients in the present study are slightly older than the mean ages reported in these previous studies.

Consistent with the literature,[9-12] more than half of our patients displayed obesity, diabetes, and hypertension. Obesity constitutes a significant risk factor for cancer mortality in general and is an increasingly recognized risk factor for HCC in particular.[31,32] In the present study, body weight was measured at the time HCC was diagnosed. Because advanced HCC might cause weight loss, it is likely that our patients were obese before the development of HCC. Diabetes has also been proposed as a risk factor for HCC.[2] Thus, HCC shares 2 major risk factors, obesity and diabetes, with NASH.

Once cirrhosis and HCC are established, it is difficult to identify pathologic features of NASH. As NASH progresses to cirrhosis, steatosis tends to disappear, so-called burn-out NASH.[5] As expected, the grade of steatosis was mild in most of our cases. It was possible to diagnose 1 case without steatosis as burn-out NASH, because a previous liver biopsy specimen (liver biopsy was performed 25 years prior) was preserved and available. It is likely that many cases of NASH-associated HCC might have been missed because of loss of the telltale sign of steatosis.

Most HCC arises on a background of cirrhosis. It is less clear whether cirrhosis is a necessary predisposition for the development of HCC in patients with NASH. Case reports of HCC arising from NAFLD and NASH patients without fibrosis or cirrhosis have been accumulating.[33-36] Cirrhosis (fibrosis stage 4) was present in 51% of cases, and advanced stages of fibrosis (stage 3 or 4) were found in 72% of cases in the present study. Indeed, cirrhosis or advanced fibrosis appeared to be the predominant risk factors for HCC development. However, in the remaining 28% of cases, HCC developed in patients with less fibrosis (stage 1 or 2). Interestingly, male patients developed HCC at a less advanced stage of fibrosis than female patients, and the prevalence of cirrhosis was significantly lower in men (39%) than in women (70%). Although the reason for the sex differences is unclear, these findings indicate that screening for HCC is needed not only in NASH patients with advanced fibrosis but also in those with less fibrosis, particularly if they are men. Further studies are needed to confirm this potentially important observation. Paradis et al[37] reported that in patients whose only risk factors for chronic liver disease are features of metabolic syndrome, HCC usually occurs in the absence of significant liver fibrosis. In addition, they found that some of these HCCs developed on preexisting liver cell adenomas. However, no preexisting adenomas were observed in the present cases.

Compared with female patients, male patients had significantly higher serum ferritin value. The normal value for ferritin varies according to the age and gender of the individual. Adult men have serum ferritin values averaging approximately 100 ng/mL (range, 75-250), whereas adult women have levels averaging approximately 30 ng/mL (range, 20-75).[38] Thus, normal men have higher ferritin levels than women. Elevation of ferritin levels is associated with NASH.[39] Because we excluded patients with alcohol consumption as rigorously as possible, we believe that alcohol consumption did not contribute to the elevation of ferritin levels in our patients.

The median diameter of the HCCs in the present study was 3.0 cm, which is equal to or smaller than the size of previously reported HCCs.[9,10,12,28,37] This is probably because most of our patients had been identified as having HCC during screening. A single HCC lesion was present in 75% of patients. For early detection of NASH-associated HCC, vigilant screening is important,[9] and the development of serologic markers for NASH is necessary.

The mechanisms of carcinogenesis in NASH remain to be elucidated. Possible mechanisms include hyperinsulinemia caused by insulin resistance in NASH, increased levels of insulin-like growth factor that promotes tumor growth, increased susceptibility of the steatotic liver to lipid peroxidation, production of reactive oxygen species and subsequent DNA mutations, disordered energy and hormonal regulation in obesity, and aberrations in regenerative processes occurring in cirrhosis.[25]

Certain limitations should be considered in the interpretation of our findings. First, the cross-sectional study design hinders the ability to draw inferences regarding the causality of NASH in HCC. Second, the study did not include a control group of HCC patients with other liver diseases. Third, there might be a bias in patient selection, because patients were retrospectively identified as having NASH-associated HCC. Finally, although our patients were negative for hepatitis B virus surface antigen, it is still possible that occult hepatitis B virus infection might be associated with the development of HCC in some of our cases.

In summary, we showed the clinical features of NASH patients with HCC. NASH patients with HCC were more often men and frequently displayed obesity, diabetes, and hypertension. Our results suggest that male patients might develop HCC at a less advanced stage of fibrosis than female patients. Further prospective studies with a longer follow-up time and larger cohorts are needed to determine the causal association of NASH with HCC and to identify risk factors for the development of HCC in NASH patients.

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Reprint requests AQ:1

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Conflicts of Interest AQ:2

The authors disclose no conflicts.

Funding AQ:3

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