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肝癌の腫瘍マーカー

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はじめに

本邦における肝細胞癌（HCC）による死亡者数は年間約34,000人余りにおよんでおり，その大半はウイルス性肝硬変（LC），主にC型を発生母地としている。そして，HCC早期診断を目的に定期的な腫瘍マーカーの測定が行われている。そして，HCCにおいては，腫瘍マーカーとして，アルファフェトプロテイン（AFP）とPIVKA（protein induced by vitamin K absence or antagonist）-Ⅱという有力な二つが存在する。さらに，近年AFPにおいてはそのHCCに対する特異性を改善したL3（フコシル化AFP）分画測定法が加わった。本稿ではHCC早期診断におけるこれら肝腫瘍マーカーの読み方について概説する。

A. 腫瘍マーカーの評価基準

〈表1〉にあげるように腫瘍マーカーの評価基準として，感度（陽性率），特異性，正診率があげられる。陽性率とはHCCを例に取ると，HCCで陽性を呈する率のことである。また，特異性とは対照となる疾患，すなわち慢性肝疾患，LCなどで上昇しない率のことで，1－偽陽性率ともいえる。その両者を加味した値が正診率である。一般に陽性率が重視されがちであるが，特異性にも優れていることが重要であり，結果として正診率に反映されることになる。

〈表1〉腫瘍マーカー評価における指標

感度 (sensitivity) = $\frac{\text{True positive}}{\text{True positive + False negative}}$

（肝細胞癌中の陽性率）

特異性 (specificity) = $\frac{\text{True negative}}{\text{True negative + False positive}}$

（肝硬変での陰性率）

正診率 = $\frac{\text{True positive + True negative}}{\text{True positive + False negative + True negative + False positive}}$

(overall accuracy)

B. AFP

1．AFP濃度によるHCC診断能

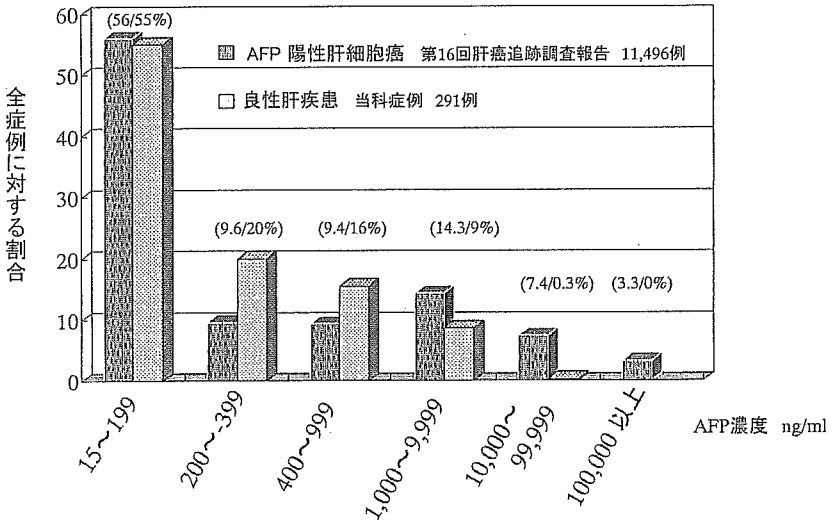
17,538例を集計した第16回全国原発性肝癌追跡調査報告（2000-2001の全国集計）によれば，AFPの陽性率（今回よりカットオフをAFP 15ng/mlに設定）は66%と報告され，いまだその診断的価値が高いことを示している[1]。〈図1〉はこれらの報告を各濃度域別に階層化したものであるが，AFP陽性を示した11,496例のHCCのうちの56%が200ng/ml未満のレンジで診断に供している。また，1,000ng/ml以上で診断されている割合はAFP産生HCCの中のわずか25%であった。

2．AFPの早期診断における意義

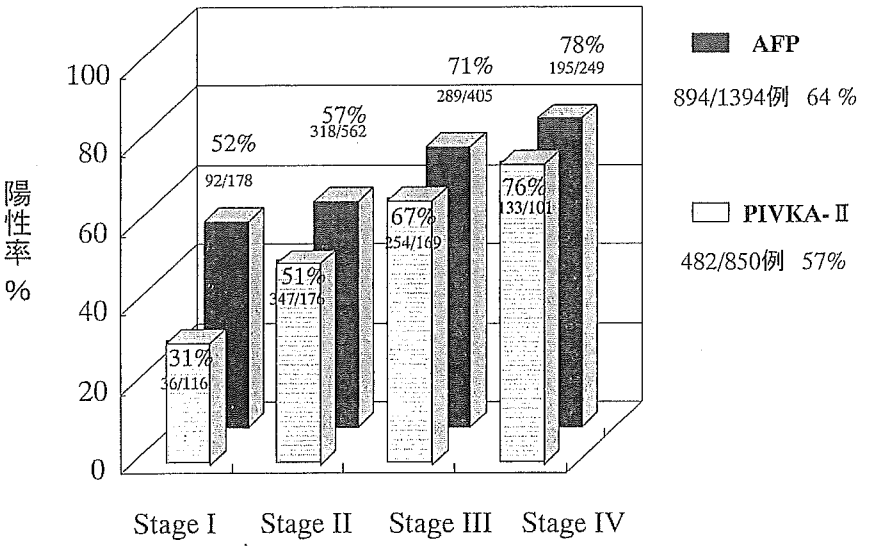
以前の報告では最大径2cm以下単発例でのAFP陽性率は60%程度とされるが，最近の報告では40～50%の報告が多い。〈図2〉は当科でのStage別AFP陽性率を示すが，早期のHCCである2cm以下のStage Ⅰの症例において178例中92例，52%が陽性を示している。すなわち，20ng/mlをカットオフ値とした場合の早期HCCでのAFP陽性率は40～60%の間にあると考えられる。

3．進行度別陽性率

AFP陽性率はStageの進行に伴って上昇し，〈図2〉に示すごとく，Stages Ⅰの52%よりⅡの57%，



〈図1〉



〈図2〉

Ⅲの71%と増加し，Stage IVでは249例中195例78%が陽性を示している。

4．高分化型HCCでのAFP陽性率

Stageとは異なる組織学的分化度による分類により定義される高分化型HCCにおけるAFP陽性率は30-40%と低い報告が多く，当科における高分化型HCCにおいても21ng/ml以上を示した割合はわずか42%であり，2cm以下単発の陽性率に比較して低い値を示している。すなわち，サイズなどのStageと組織分化度は異なる因子としてとらえる必要がある。

5．AFPの特異性（慢性肝疾患でのAFP上昇）

AFPのカッオフを20ng/mlとした場合，慢性肝疾患においてもしばしばその軽度な上昇を認める。当科の検討では，肝硬変（LC）で25%，慢性肝炎で17%が陽性を示している。〈図1〉に当科におけるHCCを否定した慢性肝疾患，主にLCでのAFP上昇を合わせて示す。この結果では55%が200ng/ml以下の間に分布し，1,000ng/ml以上の症例は9%であった。すなわち，第1項で述べたように，200ng/ml未満のレンジにAFP陽性のHCCの半数以上が分布し（第16回全国原発性肝癌追跡調査報告の結果），良性肝疾患の上昇域と重複することより，両疾患の鑑別が単回の濃度測定では困難であることを示している。

〈図3A〉は500ng/mlを上限として，当科でのHCCならびにLC，慢性肝炎，急性肝炎のAFP濃度をプロットしたものである。濃度でのHCCならびに良性肝疾患の識別は極めて困難であることが示されている。

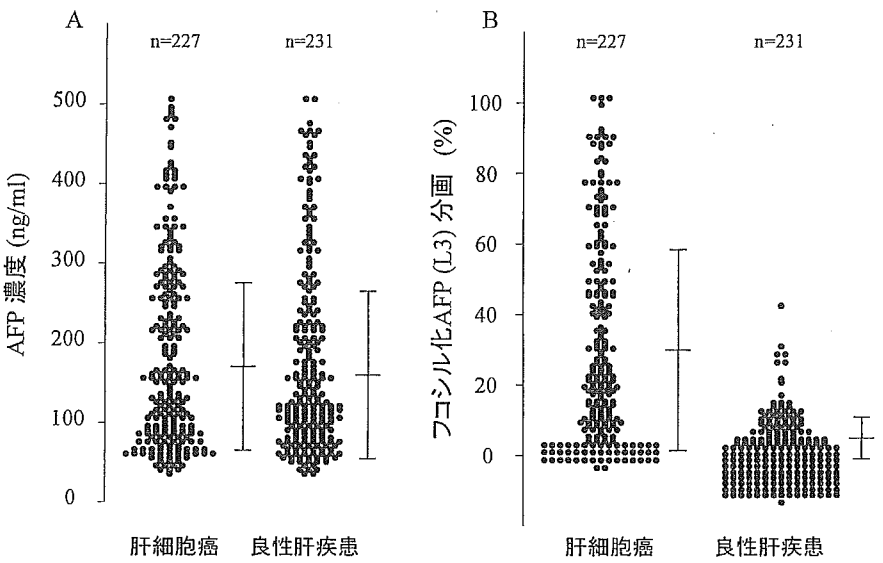
6．AFPの経時的推移

一般に，腫瘍の進展増大に伴いAFP濃度が上昇し，良性肝疾患との鑑別や治療効果判定の指標として用いることが出来る。しかしながら，早期のHCCにおいては，自然腫瘍壊死に伴い，無治療にもかかわらず低下を示すことがある。すなわち，自然低下が必ずしも腫瘍の存在を否定することには結びつかない点に注意すべきである[2]。

C．フコシル化AFP（L3）分画による鑑別

1．AFP糖鎖のフコシル化

著者らはAFPの疾患特異性の向上を目的とした，HCCならびに良性肝疾患由来AFPの分子識別を行ってきた。そして，HCC由来AFPでは，良性肝疾患由来AFPに比較してレンズマメレクチンに親和性を有する画分の増加を認め[3]，この分画のAFPにおいては二分岐複合型糖鎖の還元末端側のN-アセチルグルコサミンにα1-6のフコースが結合していることを見出した〈表2〉[4,5]。そして，総AFPに対するレンズマメレクチン結合性，すなわちフコシル化AFP分画のHCC早期診断における意義を報告してきた[2,6,7]。このフコシル化AFP分画はL3分画として保険収載されている[8]。



〈図3〉

〈表２〉AFP糖鎖構造とレンズマメレクチン結合生

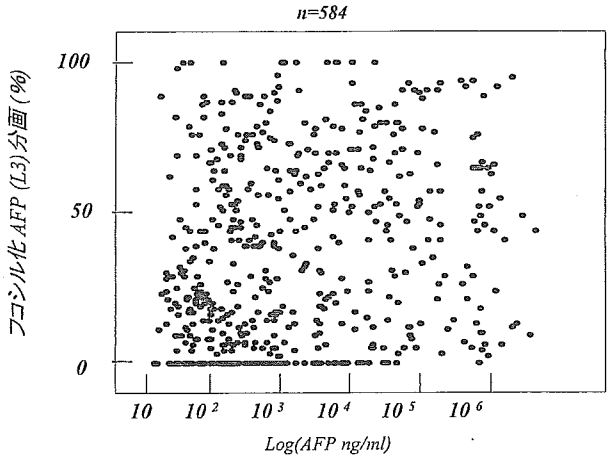
糖鎖構造		レンズマメレクチン結合性
二分岐型複合型	良性肝疾患	
Gal β 1-4GlcNAc β 1-2Man α 1-6 ＼ Gal β 1-4GlcNAc β 1-2Man α 1-3 ／ Man β 1-4GlcNAc β 1-4GlcNAc-Asn	L1	(−)
フコシル化 二分岐型複合型	肝細胞癌	
Gal β 1-4GlcNAc β 1-2Man α 1-6 ＼ Gal β 1-4GlcNAc β 1-2Man α 1-3 ／ Man β 1-4GlcNAc β 1-4GlcNAc-Asn	L3 (Fuc α 1-6)	(+)

2．L3分画による鑑別

〈図３B〉はAFP濃度では極めてオーバーラップが多かったHCC診断時のAFP値が500ng/ml以下の症例と，同じ500ng/ml以下の良性肝疾患のフコシル化AFP（L3）分画の割合をプロットしたものである。同症例でのL3分画の平均は，HCC，良性肝疾患で，それぞれ30±29，4±7%（mean±SD）で，有意なHCC群での上昇を認めた。すなわち，AFP濃度では困難な両疾患の鑑別が可能であることを示している。また，AFP濃度とL3は弱い相関関係を認めるのみで，互いに独立した因子としてみなすべきと考えられる〈図４〉。

3．L3の診断能

当科でのL3分画の診断能を検討すると（15%をカットオフ），陽性率（sensitivity）64.7%，LC，慢性肝炎を対照とした特異性（specificity）は95.8%で正診率（total accuracy）は74.4%であった。良性肝疾患でフコシル化分画20%以上を示した例は自己免疫性肝炎を含む重症型急性肝炎または慢性肝炎の急性増悪や活動性LCの例が多く，臨床的に鑑別可能例が大半であった[7]。



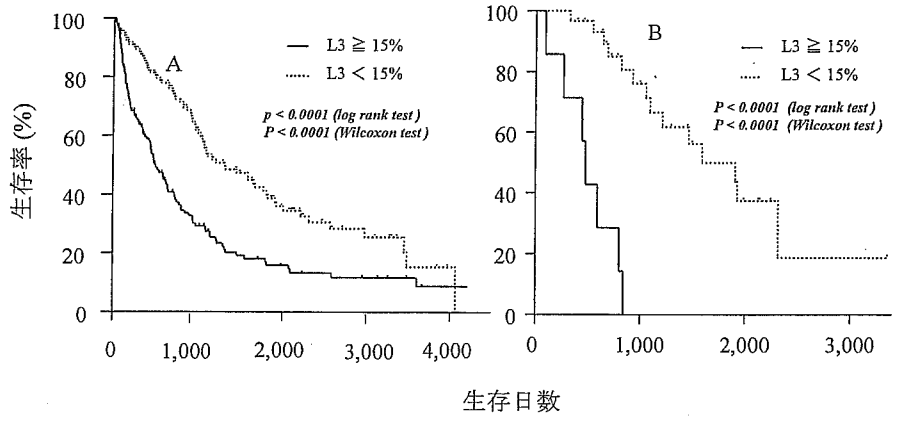
〈図４〉

4．L3分画解釈上の注意点

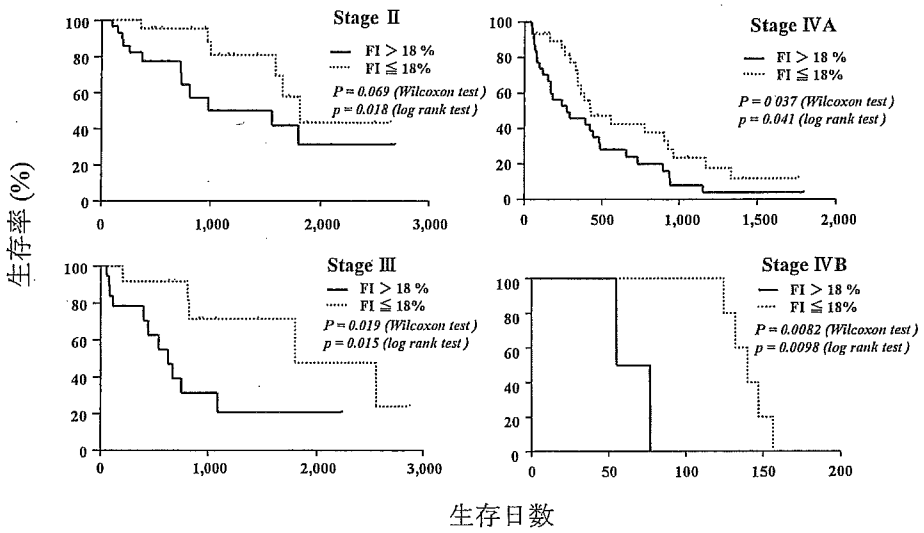
LCなどにおける本分画の上昇（15%以上）はHCCが存在することを前提に各種画像診断を行う必要がある。また，10%を越えた症例も注意深く経過観察する必要がある。また，重要なことは，低値がすなわちHCCの否定にはつながらないことに留意すべきである。当科のデータではHCCの約30%が本分画が10%以下を示している。

D．生物学的悪性度の指標としてのL3分画

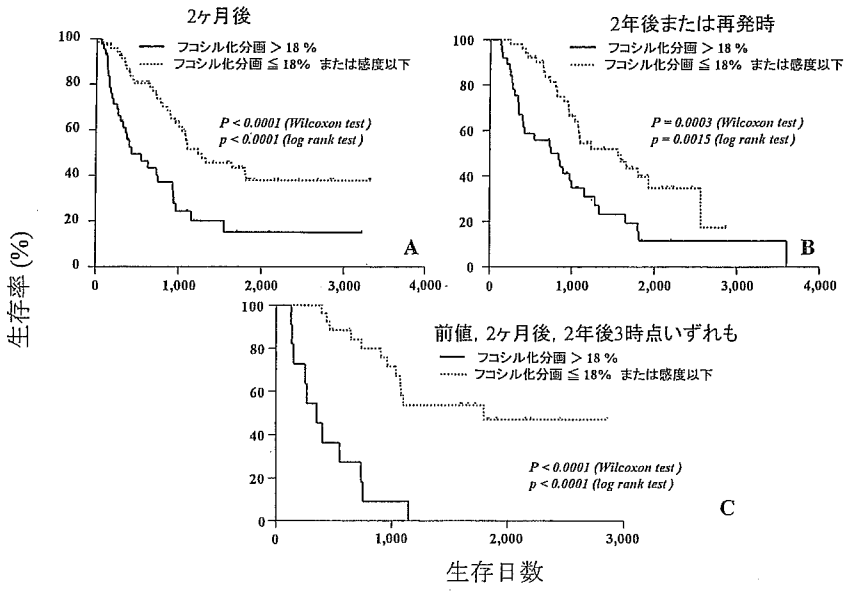
L3分画はHCCならびに良性肝疾患の鑑別以外にも，その生物学的悪性度を表すマーカーとしての意義が明らかになってきた[9,10]。〈図５A〉は当科で治療後経過観察を行ったHCC 299例での検討を示す。診断時のL3分画のカットオフを15%とすると，高値群で，低値群に比較し有意な生存率の低下を認めている。また，この生存率の低下は，腫瘍進展度（Tumor stage）とは関係がなかった〈図６〉。そして，この傾向はAFP濃度が極めて低い30ng/ml以下の症例においても認められた〈図５B〉。さらに，経過観察例で治療後L3分画が低下した群では低下しなかった群に比較して良好な予後を示した〈図７〉[11]。すなわち，L3分画は肝癌細胞に特異性が高い分子種であることより，本分画が残っている限り十分な治療でないことを理解して治療にあたる必要があることを示している。


A
B
L3 ≧ 15%
L3 < 15%
p < 0.0001 (log rank test)
P < 0.0001 (Wilcoxon test)
生存率 (%)
生存日数

〈図 5 〉


Stage II
FI > 18 %
FI ≦ 18%
P = 0.069 (Wilcoxon test)
p = 0.018 (log rank test)
Stage IVA
P = 0 037 (Wilcoxon test)
p = 0.041 (log rank test)
Stage III
P = 0.019 (Wilcoxon test)
p = 0.015 (log rank test)
Stage IVB
P = 0.0082 (Wilcoxon test)
p = 0.0098 (log rank test)
生存率 (%)
生存日数

〈図 6 〉


2ヶ月後
フコシル化分画 > 18 %
フコシル化分画 ≦ 18% または感度以下
P < 0.0001 (Wilcoxon test)
p < 0.0001 (log rank test)
A
2年後または再発時
P = 0.0003 (Wilcoxon test)
p = 0.0015 (log rank test)
B
前値，2ヶ月後，2年後3時点いずれも
P < 0.0001 (Wilcoxon test)
p < 0.0001 (log rank test)
C
生存率 (%)
生存日数

〈図 7 〉

E. L3分画測定法

現在L3分画測定は保険収載されており日常診療に用いることが出来る。現在行われている測定法には大きく分けてTaketaらの開発したLCA存在下の親和性電気泳動法[8]とイオン交換カラムを用いてLCA結合性AFPを分画するLBA（liquid-phase binding assay）法の二種類が存在する。LBA法においてはAFP上に存在する三種類の異なる抗原決定基に対するモノクローナル抗体を用いる。その中の一つは糖鎖結合部位近傍に存在する抗原決定基に対するモノクローナル抗体である。すなわち，糖鎖がフコシル化されている時は，あらかじめLCAを反応させることにより，立体障害的に糖鎖近傍認識モノクローナル抗体が結合出来ないという原理を利用した測定法である。この糖鎖近傍認識抗体を用いたフコシル化AFP測定については著者らが以前に報告しており[13,14]，LBAワコーL3はこれを実用化したものである。

F. PIVKA-II

PIVKA-IIはビタミンK欠乏やワーファリン（アンタゴニスト）存在下で生ずる凝固活性を有しないプロトロンビンのことである。近年，HCCの腫瘍マーカーとして有用であると報告されている[15-17]。

1．PIVKA-II陽性率

当科ならびに関連施設での検討ではPIVKA-II陽性（40mAU/ml以上）を示した症例は850例中482例で57 %であった〈図1〉。また，第16回全国原発性肝癌追跡調査報告の結果では，HCCで40mAU/ml以上を示した例は15,377例中9,469例，62%と報告されている。

2．進行度別陽性率

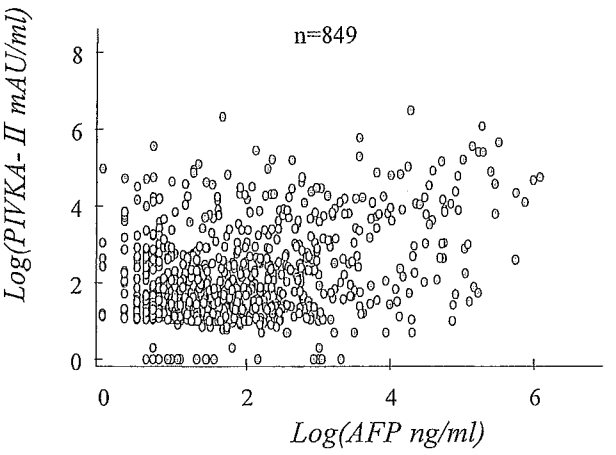
当科ならびに関連施設での検討ではPIVKA-II陽性率はAFP同様Stageの進行に伴って上昇し，Stage Iでは31%と低い値であるが，IIの51%，IIIの67%と増加し，Stage IVでは76%とAFPとほぼ同程度の陽性率を示している〈図2〉。

3．PIVKA-IIの特異性

PIVKA-IIの特性として，LCなどの良性肝疾患での擬陽性例はほとんど認められず，その特異性（HCC以外での陰性率）は高感度化後も90%以上と高い特徴を有している。注意点として，アルコール性LCにおいては軽度の上昇を認める場合がある。また，長期の黄疸例のように，ビタミンKの吸収障害が示唆される例や，ビタミンKサイクルを阻害すると考えられるMTZ（N-methyltetrazolethiol）基を有するセフェム系抗生剤の投与例などの場合に擬陽性を認めることがある。当然のことながらワーファリン投与症例では異常高値を示す。

G. AFP，PIVKA-IIの関係

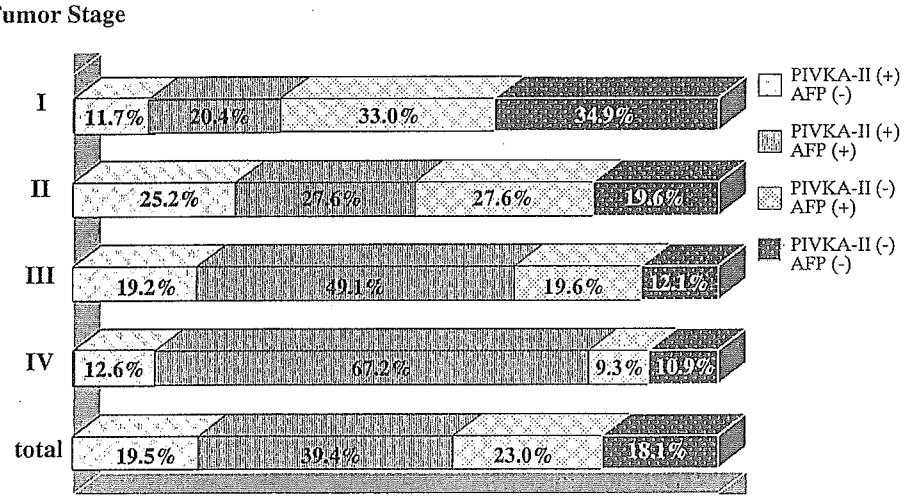
AFP，PIVKA-II濃度は互いに有意な関連は認められず，相補的で，両マーカーのcombination assayによりその診断能は向上すると考えられる〈図8〉。



〈図8〉

H. AFP，PIVKA-II combination assayの意義

〈図9〉は当科ならびに関連施設でのAFP，PIVKA-IIのcombination assayの結果を示すが，いずれかのマーカーが陽性を示したものは747例中580例82%であった。すなわち，腫瘍マーカーが陰性を示した例はわずか18%であり診断的価値が高いことを示している。また，AFP，PIVKA-IIのTumor Stage別の陽性率では，早期のHCCであるStage IにおいてもAFP，PIVKA-IIの両者またはいずれかが陽性を示した割合は65%で早期診断に有用であると考えられる。当然のことながら両



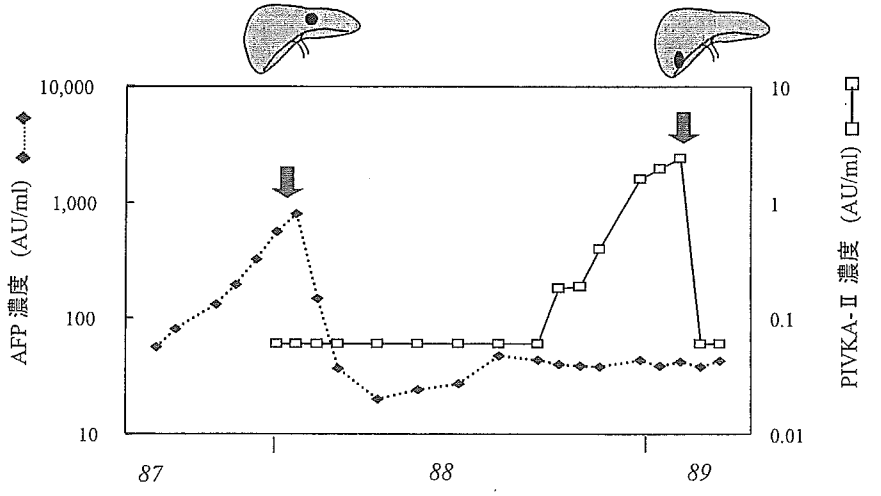
〈図９〉

者のcombination assay陽性率はStageの進行とともに上昇を示し，StageⅢやⅣでは約90%の症例で，いずれかのマーカーが陽性を示している。しかし，保険請求の問題より，両者の隔月の繰り返し測定が現実的な対応と考えられる。

I. AFPとPIVKA-IIの経時変化と乖離

基本的にHCC症例において腫瘍マーカーは，腫瘍の進展ならびに治療による縮小を反映し増減を示す。そして，AFPとPIVKA-Ⅱ，両者がともに陽性を示す際は，その濃度は互いに平行関係を示す場合が多いと考えられる。しかしながら，治療例においては，両腫瘍マーカーに乖離が認められることがあり，その解釈に注意を要する。

〈図10〉はC型LC合併HCCであるが，初発時においてはAFPの上昇を認めたが，再発時（異所性）にはAFPの上昇は認めずPIVKA-Ⅱの上昇のみを認めた。初発時上昇のAFPのみの測定では血清学的に再発を見逃すと思われる症例である。また，逆に，初発時にPIVKA-Ⅱが上昇を示し，再発時にはAFPの上昇に転換した症例も経験している。著者らが行ったHCC 146例のAFP，PIVKA-Ⅱ同時測定経過観察例の検討では，上記のような典型的な乖離だけでない乖離，すなわち，再発時に両マーカーが初発時とは異なる何らかの動き（乖離）を認めた症例は55例（38%）存在した[18]。異時性多発の際は明らかに腫瘍クローンの違いとして理解できるが，局所再発においても同様の乖離を経験している。すなわち，初発時のマーカーにとらわれることなく，両マーカー測定が必要と考えられる。



〈図10〉

おわりに

肝腫瘍マーカーはHCCの早期診断ならびに再発における意義のみならず，最近では予後規定因子や生物学的悪性度を評価する意義が次第に明らかになってきた[19]。今後，これらの新しい腫瘍マーカーの意義を含めた読み方がいっそう重要となると考えられる。

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図の説明

〈図１〉 良性肝疾患ならびにHCCにおけるAFP上昇の分布比較，第16回全国原発性肝癌追跡調査報告でAFP 15ng/ml以上を示した11,496例のAFP濃度分布と当科におけるHCCを否定し得た良性肝疾患291例の分布の両者を示す。

〈図２〉 当科でのHCCのSatge別AFP，PIVKA-Ⅱ陽性率

〈図３〉 HCCと良性肝疾患におけるAFP濃度とL3分画のプロット。A，HCC診断時のAFP値が500ng/ml以下のHCC症例227例と同じくAFP500ng/ml以下の良性肝疾患231例の血清AFP値のプロット。単なるAFPの絶対量では両疾患の鑑別は困難であることを示している。B，同症例でのL3分画プロット，L3測定で両疾患の鑑別が可能である事を示す。

〈図４〉 HCC584例における血清AFP濃度とL3のプロット，両者にはごく弱い相関関係を認めるのみで，互いに独立した因子と考えられる。

〈図５〉 L3分画高値，低値HCC群の生存率の比較，Aは全症例（299例）での検討。BはAFP濃度が30ng/ml以下の38例での結果。

〈図６〉 L3分画高低によるStage別検討。StageⅠでは有意差を認めなかったが，StagesⅡ，Ⅲ，ⅣA，ⅣBにおいては，同一Stage内においてもL3高値群は明らかに低値群に比較して予後不良であった。

〈図７〉 治療後のL3分画の高低による群別生存率の比較。A 治療後2ヶ月，B 治療2年後または再発時のフコシル化AFP（L3分画）が低値，または，高値群，C 治療前，治療後2ヶ月，治療2年後または再発時の3者がいずれも高値または低値群における生存率の差を示す。

〈図８〉 AFPとPIVKA-Ⅱ濃度との関連をプロット(269例)。両者は互いに独立で有意な相関関係は認められず相補的な関係にあるため，同時測定によりその診断能は上昇する。

〈図９〉 当科ならびに関連施設でのAFP，PIVKA-Ⅱのcombination assayの結果。

〈図10〉 AFPとPIVKA-Ⅱの乖離例における経時変化(矢印は内科治療を示す)

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Predisposition to mouse thymic lymphomas in response to ionizing radiation depends on variant alleles encoding metal-responsive transcription factor-1 (*Mtf-1*)

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Genetic predisposition to cancers is significant to public health because a high proportion of cancers probably arise in a susceptible human subpopulation. Using a mouse model of γ-ray-induced thymic lymphomas, we performed linkage analysis and haplotype mapping that suggested *Mtf-1*, metal-responsive transcription factor-1 (*Mtf-1*), as a candidate lymphoma susceptibility gene. Sequence analysis revealed a polymorphism of *Mtf-1* that alters the corresponding amino acid at position 424 in the proline-rich domain from a serine in susceptibility strains to proline in resistant strains. The transcriptional activity of *Mtf-1* encoding serine and proline was compared by transfecting the DNA to *Mtf-1*-null cells, and the change to proline conferred a higher metal responsiveness in transfections. Furthermore, the resistant congenic strains possessing the *Mtf-1* allele of proline type exhibited higher radiation inducibility of target genes than susceptible background strains having the *Mtf-1* allele of serine type. Since products of the targets such as metallothionein are able to suppress cellular stresses generated by irradiation, these results suggest that highly inducible strains having *Mtf-1* of proline type are refractory to radiation effects and hence are resistant to lymphoma development.
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Keywords: cancer susceptibility; lymphoma; positional cloning; γ-radiation; radical scavenger

Introduction

Genetic variation plays a key role in determining the range of individual susceptibility to cancers in human. Population-based epidemiological studies suggest that a high proportion of cancers arise in a susceptible subpopulation that carry low-penetrance variant alleles (Demant, 1992; Nadeau, 2001; Ponder, 2001; Balmain, 2002; Balmain *et al.*, 2003). Thus, predisposition by combinations of weak genetic variants may be of much greater significance to public health than marked individual risks seen in the inherited cancer syndromes (Ponder, 2001; Balmain *et al.*, 2003). One major model for polygenic predisposition is the common variant-common disease model, in which common variants that have arisen only once, early in the history of the population, underlie disease predisposition in humans and perhaps in mice (Wright and Hastie, 2001). The mapping and isolation of low-penetrance susceptibility genes in humans are complicated by the multiplicity of unlinked loci that mask clearcut familial clustering. On the other hand, mouse models of cancer susceptibility show that combinations of common alleles can exert a profound influence on tumor susceptibility. Accordingly, experimental strategies for fine mapping of complex traits are well established in mouse models (Darvasi, 1998).

Thymic lymphomas are a major malignancy in the mouse and often develop in mice lacking a variety of tumor suppressor genes identified in human cancers (Ghebranious and Donehower, 1998). We previously carried out a genome-wide scan of susceptibility to thymic lymphomas that induced by γ-irradiation in backcross and congenic mice between a susceptible BALB/c strain and a resistant MSM strain (Saito *et al.*, 2001). Results indicated the existence of a major BALB/c susceptibility allele near *D4Mit12* on chromosome 4. We report here the fine mapping of the susceptibility locus using subcongenic mouse lines and the haplotype mapping of the deduced candidate region. These mapping and database search suggest the presence of *Mtf-1* (metal-responsive transcription factor-1) in the candidate region that encodes a transcription factor regulating responses to heavy metals and γ-irradiation and contributes to cell survival (Lichtlen and Schaffner, 2001). We also show an Mtf-1 protein

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polymorphism and its relevance for mouse thymic lymphoma susceptibility.

Results

Our previous association study of γ-ray-induced thymic lymphomas in backcross mice between BALB/c and MSM and in congenic mice demonstrated the presence of the susceptibility locus near *D4Mit12*; the BALB/c allele exhibited a dominance or codominance over the MSM allele (Saito *et al.*, 2001). This BALB/c allele also provided susceptibility to MNU-induced thymic lymphomas (Sato *et al.*, 2003). The congenic mice were of BALB/c background and carried a chromosomal region of 40 Mb between *D4Mit9* and *D4Mit203* on chromosome 4 that was derived from the MSM chromosome. They were mated with BALB/c mice to narrow down the congenic region, and four independent subcongenic lines were obtained by genotyping with Mit microsatellite markers. Genetic composition of the chromosome 4 in each line is depicted in Figure 1a. Note that the peak LOD score was in the vicinity of *D4Mit12* in the previous study. Mice of each line were mated with BALB/c and the hybrids were then crossed with MSM mice. Offspring obtained were subjected to fractionated γ-irradiation, followed by inspection 300 days thereafter. Of the experiment with line-3 mice, 49 of the 83 irradiated mice developed thymic lymphomas and 29 mice remained free of thymic lymphomas or other cancers for 300 days after irradiation. Four of the remaining five mice were diagnosed with systemic leukemias and one with a subcutaneous tumor, as determined by macroscopic inspection. The five mice were not included in the present study. Lymphoma incidence was separately calculated in mice of BALB/c(C)/MSM(M) genotype and of M/M genotype at each locus. Table 1 summarizes the lymphoma incidence of the line-3 mice and the other three lines analysed in a similar manner.

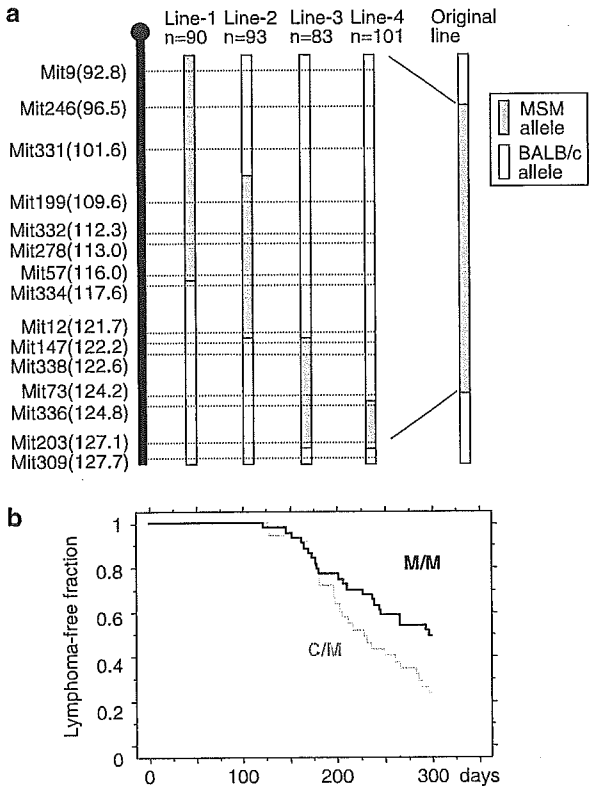

Figure 1 (a) Genetic constitution of subcongenic mouse lines of BALB/c background. Vertical bars represent a chromosomal region and shadowed portions carry an MSM-derived region. Numbers of mice subjected to irradiation are shown on the top. (b) The cumulative frequency distributions of γ-ray-induced thymic lymphoma in the congenic line-3 mice of BALB/c/MSM(C/M) and MSM/MSM(M/M) genotypes at *D4Mit73*

The linkage was evaluated by χ^2 test for goodness of fit against an expected 1:1 ratio of C/M and M/M genotypes. In lines 1 and 2, there was no association between tumor incidence and genotypes. On the other hand, the genotype of mice with lymphoma in line 3 showed excess C/M heterozygosity relative to M/M homozygosity at *D4Mit73* or *D4Mit336*. The incidences in mice of C/M and M/M genotypes are in agreement with those found in the previous analysis using the original congenic mouse line. The *P*-value at *D4Mit73* in the χ^2 test is 0.018, which is statistically significant, even given the concern about the use of multiple statistical tests (Lander and Kruglyak, 1995). Figure 1b shows the cumulative lymphoma incidences in mice heterozygous and homozygous at *D4Mit73*, demonstrating different incidence and similar latency. An unexpected result was obtained in line 4. The incidence was 82% in mice of M/M genotype, compared with 61% in mice of C/M genotype at *D4Mit336*, suggesting the presence of a resistance-conferring BALB/c allele in the vicinity. Compilation of these data suggests the existence of a susceptibility locus in the interval that is contained in the line-3 congenic region but not in the line-4 congenic region. The length of this interval between *D4Mit12* and *D4Mit336* is approximately 3 Mb.

Mouse inbred laboratory strains are known to have originated from a mixed but limited founder population, and recent haplotype analysis with single-nucleotide polymorphisms (SNPs) suggests that their genomes are mosaics with the vast majority of chromosomal segments derived from *domesticus* and *musculus* sources (Wade *et al.*, 2002). Determination of patterns of genetic variation in the interval between *D4Mit12* and *D4Mit336* in mouse strains may provide clues to aid interpretation of the susceptibility mapping, since it is likely that the susceptibility locus is due to ancestral differences common to several strains rather than a rare mutation specific to a single strain. Analysis of a particular strain of C57L/J or AKR may be important, because a recent report mapped a susceptibility/resistance locus near *D4Mit12* by analysis of MNU-induced thymic lymphomas using the C57L/J susceptible strain and the AKR/J-resistant strain (Angel and Richie, 2002). The available mouse genome database (Ensembl

Table 1 Association of markers with the development of γ-ray-induced thymic lymphoma

Sublines	*Marker*	*Lym (+)*		*Lym (−)*[a]		χ^2 *value*[c]	P-*value*[d]
		C/M	*M/M*	*C/M*	*M/M*[b]		
Line 1	D4Mit9	24	29	19	15	0.93	0.33
	D4Mit246	23	30	20	14	1.98	0.16
	D4Mit331	23	30	20	14	1.98	0.16
	D4Mit332	22	31	19	15	1.72	0.19
	D4Mit278	22	31	20	14	2.49	0.11
Line 2	D4Mit332	22	31	21	17	1.68	0.20
	D4Mit278	23	30	21	17	1.25	0.26
	D4Mit12	24	29	21	17	0.88	0.35
Line 3	D4Mit147	26	23	9	20	3.57	0.059
	D4Mit338	26	23	9	20	3.57	0.059
	D4Mit73	27	22	8	21	5.58	0.018
	D4Mit336	27	22	8	21	5.58	0.018
	D4Mit203	26	23	10	19	2.53	0.11
Line 4	D4Mit336	30	41	19	9	5.27	0.022
	D4Mit203	29	42	18	10	4.43	0.035
Original	D4Mit12	25	21	8	24	8.27	0.0037

[a]Lym(+), numbers of mice developing thymic lymphomas; Lym(−), numbers of mice free from lymphomas. [b]C/M, heterozygous for BALB/c and MSM alleles; M/M, homozygous for MSM alleles. [c,d]Calculated by χ^2 test

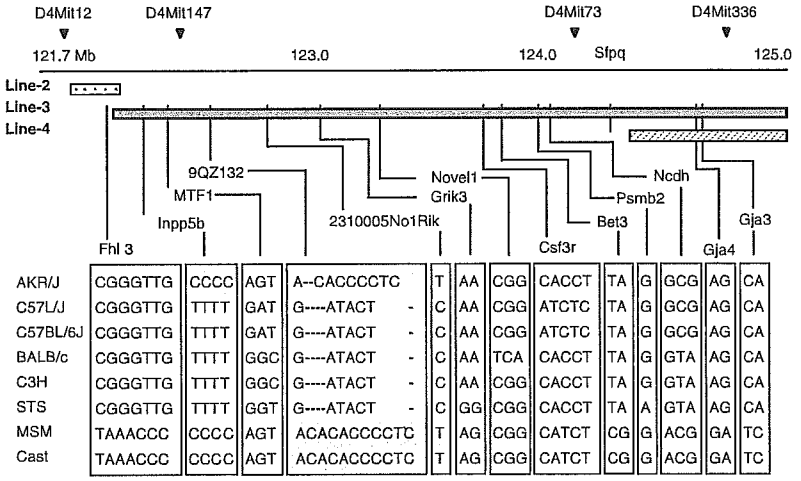

Figure 2 Genetic variation in mouse strains. Columns show SNPs discovered in 14 gene regions in the interval between *D4Mit12* and *D4Mit336*. Most laboratory strains, except for AKR, share a haplotype different from another haplotype seen in MSM that is derived from *Mus musculus molossinus*. AKR bears a mixture of the two haplotypes

mouse and human genome project: http://www.ensembl.org) revealed 15 known genes within the 3-Mb interval. For these gene loci, we identified a panel of SNPs by polymerase chain reaction (PCR) amplification and sequencing from six laboratory mouse strains (AKR/J, C57L/J, C57BL/6, BALB/c, C3H/HeJ and STS) and two wild-derived inbred strains (MSM and CAST/Ei). Analysis with 34 SNPs obtained revealed the presence of three different haplotype blocks (Figure 2), assuming that polymorphisms detected only in single strains have arisen recently after strains were inbred. The five laboratory mouse strains, except for the AKR/J strain, comprised the same haplotype, which is different from that of MSM and CAST/Ei. The AKR/J strain consisted of a mixed haplotype of the two, the centromeric half (*Inpp5b* to *Grik3*) being from the haplotype of the MSM strain and the telomeric half (*Csf3r* to *Gja3*) from the haplotype common to the five laboratory strains. It is more likely that the centromeric half carries the susceptibility/resistance gene, because the AKR and MSM strains may harbor the same resistant allele. This region is approximately 1 Mb long and according to the current mouse genome database contains three genes, *Inpp5b*, *Mtf-1* and *Grik3*, and the

corresponding region 1p34 of the human genome carries seven genes, *SF3A3*, *INPP5B*, *MTF1*, *HUMAAUANTIG*, *DNALI1*, *SNIP1* and *GRIK3*.

Gene expression in thymus or bone marrow may be a prerequisite for candidates of the susceptibility gene. Accordingly, RT–PCR was carried out on the 15 mouse genes located in the 3-Mb interval. Of the fifteen genes, 13 were expressed in the thymus or bone marrow (not shown), but only two known genes, *Inpp5b* and *Mtf-1*, were present in the centromeric half region. *Inpp5b* encodes a ubiquitously expressed type-II inositol polyphosphate 5-phosphatase (Hellsten *et al.*, 2001), and *Mtf-1* encodes a zinc-finger transcription factor that responds to heavy metal exposure and oxidative stress and plays a role in cellular stress responses (Lichtlen and Schaffner, 2001). Gene-knockout mice of *Inpp5b* do not show any prominent phenotype except for infertility in male mice (Hellsten *et al.*, 2001), whereas *Mtf-1*$^{-/-}$ mice die *in utero* due to acute degeneration of hepatocytes (Gunes *et al.*, 1998; Wang *et al.*, 2004). This embryonic lethality suggests *Mtf-1* as a plausible candidate because a polymorphism reducing gene function is likely to affect the phenotype. Expression of *Mtf-1* and other genes was compared between BALB/c and MSM mice, and no obvious difference was found in any of the genes (Figure 3a). Allele-specific expression was also determined by RT–PCR, and subsequently restriction fragment length polymorphism (RFLP) analysis of thymus from F_1 mice between BALB/c and MSM and thymic lymphomas developed in the F_1 mice. No difference in expression was detected between BALB/c and MSM alleles (Figure 3b). These results argue against any difference in promoter/expression level.

Polymorphism in the coding sequence could result in proteins with differing degrees of activity, which may account for the differing susceptibility. We therefore determined the coding sequence of *Mtf-1*. Polymorphisms conferring amino-acid substitutions in MSM were considered potentially important if they were also present in AKR/J but absent in the five laboratory strains. Eight SNPs were identified, one of which fitted the genetic screening criteria; a T to C change was identified in the coding sequence of MSM and AKR that alters the corresponding amino acid at position 424 from a serine to proline. This polymorphism might be relevant to the different susceptibility, because mouse MTF-1 possessing serine displays a reduced metal response than human MTF-1 having proline at the corresponding position in transient transfections (Radtke *et al.*, 1995). To test this possibility directly, we replaced the DNA fragment encoding serine with that encoding proline and assayed their transcriptional activity by transfecting plasmid DNA to *Mtf-1*-null cells. Transcriptional activity was determined by the luciferase activity in a reporter gene with and without zinc treatment. The change to proline conferred a higher metal responsiveness in transfections (Figure 4a), indicating that this amino acid is important for high inducibility by metals and possibly other stress conditions. Radiation effect on MTF-1 activity was also examined in *Mtf-1*-null cells after transfection. However, the effect was unclear, since the results varied from experiment to experiment probably due to apoptosis conferred by irradiation (data not shown). This failure of detection in cultured cells is consistent with the previous result (Koropatnick *et al.*, 1989).

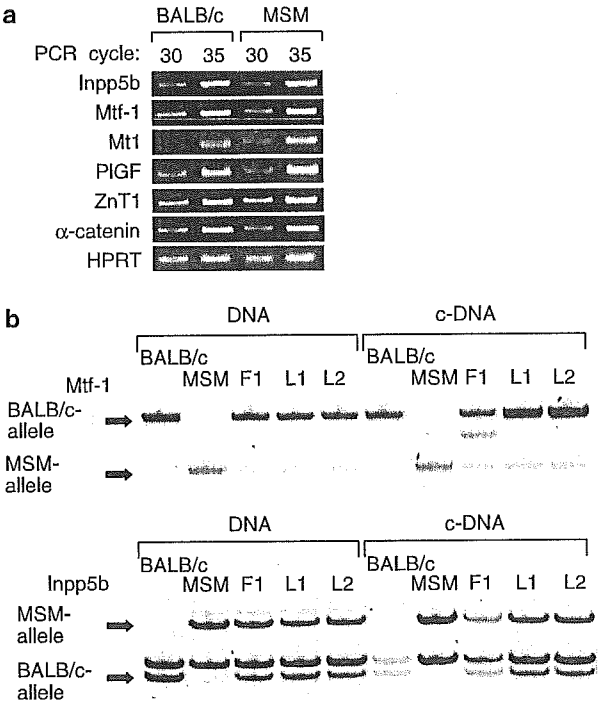

Figure 3 Comparison of mRNA expression between BALB/c and MSM. (a) Staining of gels displays amounts of RT–PCR products of indicated genes. The number of PCR cycles was 30 and 35. (b) Allele-specific expression of *Mtf-1* and *Inpp5b*. DNA and RNA from BALB/c, MSM, their F_1 mouse and lymphomas (L1 and L2) developed in F_1 were subjected to PCR or RT–PCR, and the products were digested with with *Mbo*I and *Nla*III, respectively. The ratio of BALB/c and MSM in RNA is similar to that in DNA

Expression of the two MTF-1-target genes, *metallothionein-1 (Mt1)* and *placental growth factor (PlGF)* (Andrews, 2000; Lichtlen *et al.*, 2001), was examined in thymocytes before and after 4 Gy γ-irradiation of mice by using multiple PCR with *α-catenin* gene as an internal control (Figure 4b). The level of metallothionein expression was very low before irradiation but increased 16 h after irradiation in most mouse strains. The increase was stronger in AKR and congenic mice of BALB/c background having the MSM-derived *Mtf-1* region (i.e. proline) than in BALB/c (serine). *PlGF* also showed a similar result but the level of radiation inducibility was less than that of *Mt1* (Figure 4c). This may be due to different promoter structures and/or differences in the transcription cofactors or coactivators involved (Andrews *et al.*, 2001; Zhang *et al.*, 2003). On the other hand, regulation of *Mtf-1* itself did not display any difference in expression before and after irradiation. We could not determine the inducibility of MSM because this particular strain displayed such a high

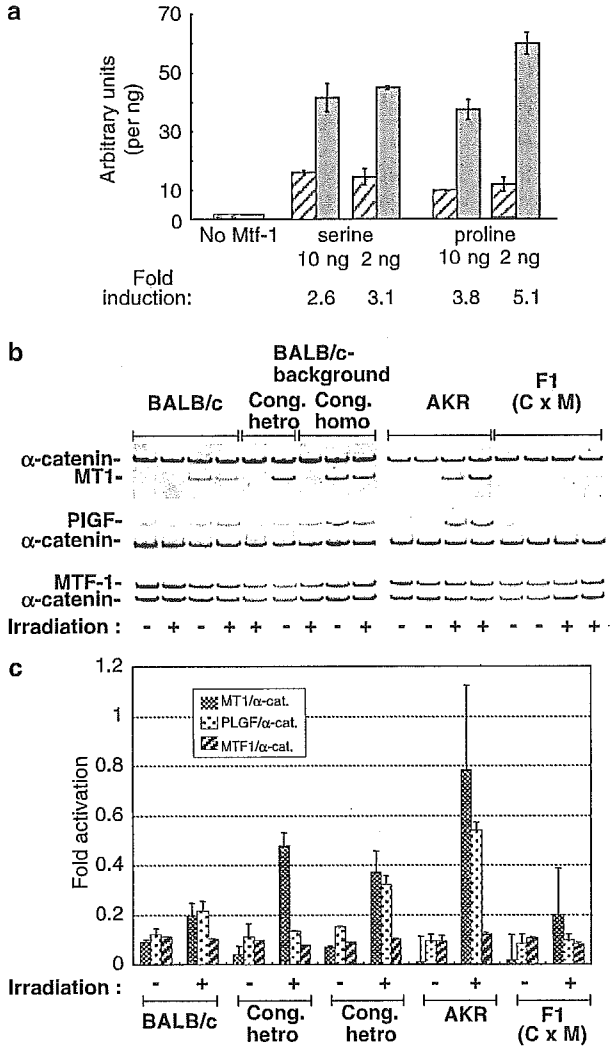

Figure 4 *Mtf-1* polymorphism and difference in transcriptional activity. (a) Comparison of metal inducibility between the two different *Mtf-1* of serine and proline types. Normalized luciferase activities (arbitrary units) are displayed per ng plasmid DNA from uninduced (shaded bars) and zinc-induced (gray bars) for comparison. (b) Accumulation of mRNA of indicated genes detected by RT–PCR in mouse thymocytes before and after γ-irradiation. *α-catenin* was included as a reference. (c) Display of relative expression levels of mRNAs. The *Mtf-1* levels are drawn to a scale of one-tenth. Three independent experiments were summarized and the amounts of indicated mRNAs were normalized to that of *α-catenin*

radiosensitivity that the cell number of thymocytes was reduced to one-tenth of unirradiated ones. These results, together with the transfection results described above, strongly suggest that the polymorphism of *Mtf-1* is responsible for different radiation inducibility.

Discussion

We report here the genetic and haplotype mapping of the lymphoma susceptibility locus on mouse chromosome 4. The candidate region was localized within an interval of approximately 1.0 Mb where at least eight genes were assigned including the *Mtf-1* gene. This mapping is based on the assumption that AKR and MSM strains carry the same susceptibility allele, although the susceptibility of AKR was not tested in this study. However, we found a base substitution in the coding sequence of *Mtf-1* in the resistant strains (MSM and AKR) that alters the corresponding amino acid at position 424 from a serine to proline. The resistant strains possessing the *Mtf-1* allele of proline type exhibited higher radiation inducibility of target genes, such as *Mt1* and *PlGF*, than susceptible strains having the *Mtf-1* allele of serine type. Products of the targets are able to suppress cellular stresses generated by irradiation and reduce the probability of DNA damage and apoptosis in the cells. It is therefore probable that highly inducible strains can be refractory to radiation effects, and thereby are more resistant to cancer

development. These results strongly suggest that *Mtf-1* is a candidate lymphoma susceptibility gene. Several cancer-susceptibility loci have been mapped in mice, but a few have been cloned so far, including *Mom-1*, *Ptprj* and *Stk6/STK15* (Macphee *et al.*, 1995; Cormier *et al.*, 1997; Gould and Dove, 1998; Ruivenkamp *et al.*, 2002; Ewart-Toland *et al.*, 2003). Furthermore, the latter two are weak tumor suppressor genes rather than susceptibility genes because of the presence of frequent allelic losses in tumors. In contrast, the *Mtf-1* locus did not show frequent allelic loss in thymic lymphomas, although certain tumor suppressor genes did (Matsumoto *et al.*, 1998; Okano *et al.*, 1999; Wakabayashi *et al.*, 2003a, b).

Exposure of cells to ionizing radiation causes DNA damages in the nucleus and also activates cytoplasmic signaling pathways through the generation of reactive oxygen species (Little, 2000; Criswell *et al.*, 2003). Any improperly repaired DNA can result in mutations or chromosomal damage, some of which may lead to cell death, and others to the initiation of the process that leads to neoplasia. The radiation-induced signaling pathway modulates apoptosis and the repair process and overlaps that activated by heavy metals such as zinc, which involves MTF-1. Activated MTF-1 in the cytoplasm enters the nucleus, binds to metal responsive elements and activates transcription of genes having the elements in the promoter (Smirnova *et al.*, 2000; Saydam *et al.*, 2001). The MTF-1 polymorphism affects the efficiency of the transcription activation, although the activation requires cofactors or coactivators to execute its full transcriptional activity (Andrews *et al.*, 2001; Lichtlen and Schaffner, 2001; Zhang *et al.*, 2003). Several other radiation-responsive transcription factors have been identified, including NF-κB, p53, c-Jun and c-Fos (Criswell *et al.*, 2003). These signaling pathways represent both pro- and antiproliferative signals, and their relative balance can determine cell fate.

The MTF-1 transcription factor plays a key role in stress response and contributes to cell survival. Its target genes so far identified are *MT1*, *α-fetoprotein*, *γ-GCS*, *Znt1* and *PlGF* (Andrews, 2000; Langmade *et al.*, 2000; Lichtlen *et al.*, 2001), and at least some of them are effector molecules involved in cellular stress response. MT1, α-fetoprotein and glutathione whose synthesis is regulated by a key enzyme, γ-GCS, are induced by stresses such as heavy metals, toxic compounds and reactive oxygen species. For instance, MTs are efficient scavengers of hydroxyl radicals, and yeast and mammalian MTs can functionally substitute for superoxide dismutase in protecting yeast from oxidative stress (Tamai *et al.*, 1993). PlGF is a survival factor for macrophages and vascular cells and inhibits apoptosis *in vitro* (Adini *et al.*, 2002). It is also possible that there are other unidentified targets that respond to and protect from stresses. Analysis of primary mouse embryo fibroblasts prepared from *Mtf-1*$^{-/-}$ mice suggests the existence of a threshold for the accumulation of harmful agents depending on an amount of MTF-1 proteins within the cell. *Mtf-1*$^{-/-}$ cells are more susceptible to cadmium or H_2O_2 treatment than *Mtf-1*$^{+/+}$ cells, and the susceptibility of *Mtf-1*$^{+/-}$ cells is intermediate, indicating that the defense activity is dose-dependent (Gunes *et al.*, 1998). This may account for the codominance of BALB/c susceptibility- and MSM resistance-giving alleles in lymphoma development. Congenic mice heterozygous for *Mtf-1* alleles of proline and serine types showed an approximately twofold higher incidence of lymphomas than those of homozygous *Mtf-1* alleles of proline type.

In conclusion, our data strongly suggest that the *Mtf-1* polymorphism accounts for the different susceptibility to γ-ray-induced mouse thymic lymphomas at a certain level. This observation raises the possible involvement of human *MTF-1* polymorphisms in the modulation of radiation-induced malignancies. Although examination of 50 human individuals for the polymorphism at the position 425 (corresponding the position 424 in the mouse) showed that all of them were of proline type, database search reveals other polymorphisms in human *Mtf-1* and flanking regions. Analysis of relevance of these polymorphisms to the modulation is of special importance when we consider that the cumulative risk of developing cancer conferred by diagnostic X-rays is estimated as high as approximately 1% (Berrington de Gonzalez and Darby, 2004). Conversely, chemical inhibition of MTF-1 may enhance sensitivity, leading to apoptosis of tumor cells that have been challenged with ionizing radiation therapy or DNA damage-inducing drugs, leading to increased apoptosis.

Materials and methods

Mice, irradiation and lymphoma induction

A congenic mouse line for *D4Mit12* on chromosome 4 was obtained previously (Saito *et al.*, 2001), which carried an MSM-derived chromosomal region spanning *D4Mit9* and *D4Mit203* (40 Mb). The original congenic mice were crossed with BALB/c mice and four subcongenic lines were obtained after genotyping with 15 microsatellite markers within this region. Genetic composition of the chromosome 4 in each line is depicted in Figure 1a. Lines 2 and 3 overlapped only a short interval in the vicinity of *Fhl3* (Figure 2). Heterozygous female mice of the subcongenic lines were crossed with MSM male mice, and progeny were subjected to fractionated γ-ray-irradiation, 2.5 Gy four times at a week interval, when they were at age of 4 weeks. Development of thymic lymphoma was diagnosed by the inspection of labored breathing up to 300 days. Existence of tumors was confirmed upon autopsy of the mice, and some lymphomas were histologically and immunologically examined. Comparison of the lymphoma incidences was made in parallel between mice of two different genotypes under the same conditions in each group because the incidence may vary from experiment to experiment. The genotyping was carried out after diagnosis of lymphoma development.

For RNA expression analysis, mice were once treated with 4 Gy of whole-body irradiation and killed after 16 h.

Genotyping and statistics

Genotyping was carried out using microsatellite markers as described previously (Saito *et al.*, 2001). χ^2 and *P*-values for association of markers with the development of lymphomas

were obtained by χ^2 test and Mantel–Cox test with StatView-J 5.0 software on a Macintosh personal computer. Evaluation of linkage followed the criteria of Lander and Kruglyak (1995). The statistical threshold of each recombinant lines were corrected for multiple comparisons using the formula, $\mu(T)=[C+2\rho GT^2]$ $\alpha(T)$: C(number of chromosomes) = 1, ρ(to account for corrected results among linked loci: for backcross with 1 df) = 1, G(genome size in Morgans) = 0.25, T(for the χ^2 statistic with 1 df) = 3.84 and $\mu(T)=0.05$. The genome size of lines 3 and 4 is calculated to be 0.030 and 0.014, respectively, assuming that one centiMorgan corresponds to 1.6 Mb. Linkage was taken as significant when P-value was less than 0.026 and 0.035 in lines 3 and 4, respectively.

SNPs and haplotype mapping

Genomic DNA was isolated from tail or liver of mice and lymphomas by a standard protocol. PCR and separation of PCR products by gel electrophoresis were performed as described previously (Saito *et al*., 2001). SNPs were determined on both the forward and reverse strands of DNA by sequencing PCR products that were amplified with primers of genes listed below. These primers were also used for haplotype mapping as follows: *Fhl3* – ACCACAGCTGC TTTTCCTGT, GCGCCAAAGTCTTGAACTCT; *Inpp5b* – AAGAACTGCCTTAAGCTGGT, ATGCATGAACATGTT TGCGG; *Mtf-1* – TCTTCCTGTGAGCACAGCAG, GGAT GTTCCTGGGATACGAG; *Q9Z132* – TTCCAGAACTG GAGCATCTG, ACGTGTACCACGGATGTGTA; *231000 05No1Rik* – CCGGCACAGAATGAATGTCT, CGATCAG ATGTGTCATCAGG; *Grik3* – CTCCTATGATGGTCAA GACG, GCTGGCCTTCACAGCTTCAG; *Novel1* – GCCA CTGAAGAACTGCTCAT, CTCATCATTGAGTGGACT CA; *Csf3r* – TCCGCTGAAATCTACTGGCC, TTTCTCTG TATAGCTCTGGC; *Bet3* – GTAAGCATCTCTAGGACT TG, TGGCAGACTACAGTGTCACT; *Psmb2* – ATCTGCC GACCTTCAGTGTC, ATCTATCAAGAGTGAACGTC; *Ncdh* – GGCCATTGACATCACAGTTC, CAGAAAGG TAGTTCCTGCTG; *Gja4* – ATGTCACTCAACAGAGCT GC, AGCAGCCTGCATCAGTGCTT; *Gja3* – ATGGTA CACAGGGCTTCTAC, TATGTCAAGGATATACGCCG. CAGTGCTGCCAAGAA, ACGTAGGACGCCTTCACTGT; *Mt1* – CTTCACCAGATCTCGGAATGG, TGTATAGTTCT GTCCCCGAG; *PlGF* – CCAGCTCACGTATTTATTACCG, TGCCTTCAATGCAGCCGG; *Znt1* – TGACAATCTGGAA GCGGAAGACAAC, GGAAGCGGGGTCCTCACATTTTA TG; *Hprt* – GCTGGTGAAAAGGACCTCT, TCATTATAGT CAAGGGCATATCCA. PCR–RFLP was performed for PCR products of *Inpp5b* and *Mtf-1* by digesting with *Nla*III and by *Mbo*I, respectively. The forward primer of *Mtf-1* was a degenerated primer: 5′-AAATTGGAAGGATGAAGAGA and the reverse primer was the same used for the haplotype mapping. The primers used for *Inpp5b* were the same as above.

RT–polymerase chain reaction

Total RNA was prepared from isolated thymocytes using the RNA Easy Mini kit (Quiagen) according to the protocol recommended by the manufacturer. cDNA was synthesized from 1–5 μg of total RNA with an oligo(dT)$_{12}$ primer using SuperScript Preamplification System (Gibco) and an aliquot ($\frac{1}{2}$ of cDNA products) was used for PCR using primers listed below. Multiplex PCR was carried out similarly where α-catenin primers were always included as a reference. PCR products were separated by electrophoresis in 8% polyacrylamide gel and visualized by staining with ethidium bromide or Syber Green. Quantification was carried out with Molecular Imager (BioRad Inc.) as follows: *α-catenin* – GTGAT-

Transfection assay

The mouse MTF-1 expression vectors coding serine and proline at amino-acid position 424 were derived from the plasmid pC-mMTF-1-VSV (Radtke *et al*., 1993). As for the MTF-1 vector having the serine residue, DNA fragments were synthesized with PCR using a set of primers (F: TTAGATCTCGCC CCGTTACATA; and R: TTAGATCTCGCCCCGTTACATA) and pC-mMTF-1-VSV as a template. Products were cloned into *Bgl*II and *Eco*RV sites of pcDNA3.1 vector after digestion of the PCR products with *Bgl*II. DNA fragments coding the proline residue were synthesized with PCR using mutagenic primers (mF: CTCGTACTGCAGCCTGGCATCTCC; and mR: AGATGCCAGGCTGCAGTACG) according to the method of splicing by overlap extension (Kammann *et al*., 1989), and then cloned into pcDNA3.1 vector in a similar manner. Inserts were sequenced to verify the desired mutation and no other alterations introduced. The SV40-transformed *Mtf-1*$^{-/-}$ embryonic stem cells designated as DKO7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Cells were transfected by the *Mtf-1* clone of the serine or proline type, together with reporter and reference genes, using the FuGENE kit (Roche Molecular Biochemicals, IN, USA). The reporter gene consisted of the firefly luciferase coding sequence driven by a synthetic 4xMRE/TATA box promoter (Radtke *et al*., 1995), and the reference was a β-galactosidase (CMV-LacZ) gene under the control of the ubiquitously active CMV promoter. At 24 h after transfection, cells in the dishes were washed twice with DMEM and incubated 24 h in DMEM containing 2% Chelex-treated FBS. A half of the dishes were treated with $ZnCl_2$ at a final concentration of 200 μM for 4 h and then harvested. Cells with and without zinc treatment were analysed by measuring luciferase activities, and the activity units were normalized to β-galactosidase values.

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