診断病理の精度管理

分子病理診断の標準化と精度管理

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はじめに

タンパクや核酸分子の状態把握のために行われる免疫組織化学 immunohistochemistry (IHC) 法や *in situ* ハイブリダイゼーション *in situ* hybridization (ISH) 法は，ホルマリン固定パラフィン包埋検体 (FFPE検体) を対象とした検索において日常病理診断上欠くことのできない検査手法となっている．1990年頃より普及が進んだIHC法は，2000年以降の自動化の流れにより，多くの施設でルーチン検査[注1]として本格的に導入され，現在では腫瘍鑑別や悪性度評価，病原体の同定等の補助診断法として一般化している．また分子標的治療の登場を契機に，IHC検査やISH検査は治療対象患者の選別に用いられるようになった．こうした標的分子の有無や異常を検出する検査薬を治療薬と組み合わせて行う併用的診断はコンパニオン診断と呼ばれ，がん個別化医療の中心的役割を担っている[1]．さらに近年，遺伝子変異検査などの体細胞遺伝子検査[注2]においても，FFPE検体が利用されるようになり，対象材料 (パラフィンブロック) の選択，腫瘍細胞をエンリッチする場合の核酸抽出エリアの特定等に病理医が携わるようになった．最近では体細胞遺伝子検査を病理診断部門で実施，もしくは窓口となり外注するケースが増えつつある．こうしたFFPE検体を用いた検査が分子病理診断の柱をなす一方で，これら検査の標準化や精度管理は十分とはいえず，一部の検査項目を除き本格的な取り組みが必要となってきている．

本稿では分子病理診断で行われている検査のうちFFPE検体を用いる検査にフォーカスし，それら検査における標準化や精度管理の問題点について述べるとともに改善に向けた取り組みについて紹介する．

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Ⅰ．分子病理診断に用いられる検査の現状

一般に検査は，プレアナリシス段階 pre-analytic phase，アナリシス段階 analytic phase，ポストアナリシス段階 post-analytic phase の三段階に大別され，検査の標準化を含め精度管理を実践する上で重要な分類となっている[2-3]．これを踏まえFFPE検体を用いた分子病理診断として現在実施されている検査とそれらの各段階における作業を**表1**にまとめた．特にアナリシス段階において，組織切片上で反応を進めるIHC検査やISH検査は概ね手法が画一化されているのに対し，核酸抽出サンプルを用いて行う体細胞遺伝子検査では多くの手法が用いられており，状況が大きく異なる．これら検査の保険適用状況を**表2**に示す．分子病理診断において，コンパニオン診断を目的とした項目は，IHC検査では estrogen receptor (ER)，progesterone receptor (PgR)，HER2，EGFR，ISH検査ではHER2のみとごく一部ではあるものの，これら検査は全て臨床妥当性が確認され標準化された体外診断用医薬品 *in vitro* diagnostics (IVD) が使用されており，また診療報酬点数表では個別の項目と保険点数が設定されている．一方，体細胞遺伝子検査の多くはIVD未承認の研究用試薬で実施されており，点数表上は個

注1：平成20年の診療報酬改定において病理診断が第3部 検査から第13部へ新設移行されたことに伴い，診療報酬点数表上はIHC法やISH法を用いた検索は「染色」や「標本作製」の用語が適用されるようになったが，国際的にこれら検索には通常「test」や「testing」といった用語が用いられているため本稿では「検査」という用語を使用した．

注2：日本臨床検査標準協議会 (JCCLS)・専門家委員会では，これまで一般に用いられてきた「遺伝子検査」の総称を「遺伝子関連検査」とし，さらにこれを「病原体遺伝子検査」「ヒト体細胞遺伝子検査」「ヒト遺伝学的検査 (生殖細胞系列遺伝子検査)」へと分類・定義したため，これらの用語を使用した．

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表1　分子病理診断で用いられる検査とその段階別の作業

検査段階	〈組織タンパク関連検査〉 IHC検査	〈組織遺伝子関連検査〉 ISH検査	〈遺伝子関連検査〉 体細胞遺伝子検査
プレアナリシス段階 [作製作業]	検体採取 → 切り出し → ホルマリン固定パラフィン包埋ブロック作製		
	切片作製	切片作製	(切片作製 →) 核酸抽出
アナリシス段階 [解析作業]	IHC法 ・前処理(抗原賦活処理) ・検出(染色)	FISH法，BRISH法 ・前処理 ・検出(染色)	Direct sequence法 Scorpion-ARMS法 PNA-LNA PCR clamp法 PCR-invader法 Cycleave法 Luminex法 PCR-SSCP法　など
ポストアナリシス段階 [判定・診断作業]	定性的判定 半定量的判定 定量的(計数的)判定	定量的(計数的)判定	各基準に準じた判定

[ ]は分子病理診断の場合の主な作業.

表2　主な分子病理診断関連の検査項目と保険適用

カテゴリー	〈組織タンパク関連検査〉 IHC検査	〈組織遺伝子関連検査〉 ISH検査	〈遺伝子関連検査〉 体細胞遺伝子検査
コンパニオン診断項目	第13部 N002　1　720点 ER(乳癌) 第13部 N002　2　690点 PgR(乳癌) 第13部 N002　3　690点 HER2(乳癌・胃癌) 第13部 N002　4　690点 EGFR(大腸癌)	第13部 N005　2,500点 HER2(乳癌・胃癌)	第3部 D004-2　1　2,000点 悪性腫瘍遺伝子検査 ・肺癌(EGFR) ・大腸癌(KRAS) ・GIST(KIT，PDGFRA)
補助診断項目	第13部 N002　5　400点 その他 ※4種類以上の抗体を用いた場合は1,600点を加算	第3部 D004-2　1　2,000点 悪性腫瘍遺伝子検査 ・骨軟部腫瘍 ※脳腫瘍や悪性リンパ腫なども対象となる場合がある	第3部 D006-6　2,400点 免疫関連遺伝子再構成 ・悪性リンパ腫(IgH，TCR) 第3部 D004-2　1　2,000点 悪性腫瘍遺伝子検査 ・膵癌(KRAS)

別項目化されていない．補助診断項目においてはいずれの検査においてもIVD承認品が皆無に近い状況にあり，その多くはやはり研究用試薬で実施されている．特にIHC検査は，研究用試薬として市販されている特異抗体を入手すれば，検査として取り入れることが容易なことから，多くの施設で100項目以上の検索が日常行われており，そのため検査と研究の境界がわかりづらくなっている．

Ⅱ．IHC検査の標準化と精度管理

1．IHC検査の現状把握

本検査における標準化と精度管理については，前述のとおりコンパニオン診断項目と補助診断項目では状況が大きく異なる．ER，PgR，HER2などのコンパニオン診断項目は，体外診断用医薬品の使用や標準化プロトコールの遵守などが進んだことで標準化は浸透し，現在取り組みの中心は検査の精度管理を含め，診断精度向上へと移行している(次項参照)．これに対し補助診断項目はその大部分が研究用試薬を用いて行われている上，抗原賦活処理や染色といったアナリシス段階の作業が各施設の状況に合わせカスタマイズされたために多様化してしまっており，研究手法のような検査形態をとっている．それゆえ補助診断項目部分の標準化は大きな遅れをとっているが，各項目の検査実施件数やその施設間差などの実態が把握できていない状況にあり，取り組みへの足がかりが見出しにくくなっている．

表3 IHC検査実施件数の上位40項目

順位	IHC検査項目	平均年間実施件数	検査占有率(%)
1	Ki-67	357	6.5
2	p53	243	10.9
3	cytokeratin (pan)	227	15.0
4	D2-40	223	19.0
5	CD20	213	22.9
6	CD3	209	26.7
7	estrogen receptor	195	30.2
8	progesterone receptor	187	33.6
9	HER2	173	36.7
10	CD34	149	39.4
11	CD79a	140	42.0
12	CD10	134	44.4
13	α-SMA	134	46.8
14	S100	132	49.2
15	CD68 (KP1, PGM1)	106	51.2
16	cytokeratin 7	104	53.0
17	BCL2	97	54.8
18	CD56 (NCAM)	95	56.5
19	p63	90	58.1
20	CD5	87	59.7
21	cytokeratin 20	82	61.2
22	synaptophysin	77	62.6
23	vimentin	75	64.0
24	chromogranin A	75	65.3
25	TTF-1	74	66.6
26	desmin	70	67.9
27	CK-HMW (34βE12)	63	69.1
28	CD31	54	70.0
29	EMA (E29)	49	70.9
30	CD117/KIT	48	71.8
31	cyclin D1	47	72.7
32	CK-LMW (CAM5.2)	46	73.5
33	PIN cocktail	42	74.3
34	CD30/Ki-1	41	75.0
35	myeloperoxidase	37	75.7
36	CD4	37	76.3
37	CEA	37	77.0
38	cytokeratin 5/6	36	77.7
39	kappa chain	36	78.3
40	lambda chain	36	79.0

こうした背景から，我々は厚生労働省がん研究助成金「がん診療を標準化するための病理診断基準の確立」に関する研究班（長谷川班）で平成19年度にパイロット調査を行い，さらにこれを踏まえ平成22年度に本調査を後継班であるがん研究開発費研究班（津田班）において実施した．この調査は国内24施設（このうちがん拠点病院は21施設）を対象にIHC検査項目別の実施件数に関する実態調査を行った．全対象施設から集計したIHC検査実施項目の総数は284項目（施設別の年間実施項目数では最少の施設は65項目，最多の施設は186項目）であった．284項目のうち上位40項目とその累積検査占有率を表3に示した．上位項目にはコンパニオン診断項目（ER，PgR，HER2）が含まれるほか，補助診断項目では悪性度評価・良悪性鑑別（Ki-67，p53など），脈管同定（podoplanin/D2-40，CD34など），悪性リンパ腫，神経内分泌腫瘍，消化管間質腫瘍や軟部腫瘍の鑑別，原発臓器推定等に関わるマーカーやパネルが含まれ，パネルについては平成22年の診療報酬改定で1,600点加算の対象となっている（「N002 免疫染色（免疫抗体法）病理組織標本作製」に関する通知）．24施設の平均年間IHC検査実施件数は約5,500件で，上位30項目で全体の約70%，上位40項目で全体の約80%，上位60項目で全体の約90%を占めており，少なくとも上位40〜60項目がルーチン検査上の必須項目とみなせるように思われた[4]．

2. IHC検査標準化への取り組み

IHC検査は長らく保険点数が低い状況が続き，さらにこの10年の自動化の浸透はさらに検査コストを上げることになり，検査現場ではコスト抑制のための技術的対応を余儀なくされていた．特に特異抗体試薬については，精度管理上予め最適化されたready-to-use（RTU）タイプの抗体試薬の使用が望ましいにもかかわらずコスト面から敬遠され，低コスト化が可能な精製抗体がこれまで好んで使用されてきた．つまり各施設での染色条件検討や希釈作業の実施，使用期限を越えた凍結保管など，試薬コスト抑制への努力が行われていた反面，これを人的コストでまかなうといった構造が続いていた．しかし平成22年の診療報酬改定でIHC検査の補助診断項目は400点への引き上げ，そして対象疾患が限定されているとはいえ4項目以上の場合は1,600点の加算とプラス改定が行われ，十分とはいえないものの，ようやく標準化の議論が可能な状況となった．今後はこれを契機に，コンパニオン診断用試薬と同様に，RTUタイプの抗体試薬の使用などが望まれる．またこうした試薬は，通常専用の抗原賦活処理試薬と検出試薬，そして完全自動化された免疫染色機との一体的使用が前提となっており，アナリシス段階の標準化では，これを念頭に置くことが肝要である．

一方規制面においても大きな問題が横たわっている．保険診療上実施される検査では体外診断用医薬品の使用が原則となっているが，RTU抗体を含め抗体試薬の多くは前述のとおり未承認のままとなっている．病理診断領域における先発品IVDの開発は，企業の研究者，対象疾患を専門とする病理医や臨床医，その他の医学研究者などにより進められ，治験・臨床

試験などにおける臨床有用性の確認（特コンパニオン診断薬の場合），臨床性能試験ならびに安定性試験の実施を経て薬事申請を行い，最終的に承認取得となる[5]．このとき承認品の添付文書には上記試験にて臨床妥当性が確認された検査手法が記載されることになり，遵守すべき標準化法となる．また後発品としてIVDが開発される場合も，先発品との相関性確認を行った後は概ね同様の流れとなる．しかしIVD承認された試薬がない項目ではこうしたプロセスを経ないため方法は標準化されないままとなり，未承認試薬間の性能差についても把握されないまま検査が実施されているのが実情である．

前述の上位項目のうち，補助診断項目でIVDとして承認が得られている項目は，CD20，CD3，pan-cytokeratin（CK），vimentin，S100，desmin，kappa/lambda chainなど10項目にも満たない状況であり，検査件数が多いKi-67やpodoplanin/D2-40をはじめ大部分の補助診断項目は今も研究用試薬として用いられている．現在市販されているRTU抗体試薬の多くは欧米で既に承認が得られたものが輸入されているため，染色性は一定レベル担保され実用上問題はないように思われるが，高額な規制関連コストや市場環境などの問題を抱える現状では，今後も本邦での承認申請は進まない状況が続くことが予想され憂慮すべき状況にある．

3．IHC検査精度管理への取り組み

一般に検査，特にアナリシス段階の精度保証quality assuranceは施設内で検査手法の管理を行う内部精度管理internal quality controlと検査データの施設間差の調査・管理を行う外部精度評価external quality assessmentに大別され[6]，両面での取り組みが不可欠となる．本邦のIHC検査においては，後者は十分に体制が整備されていないことから，精度管理は現状内部精度管理に頼らざるをえない状況となっている．アナリシス段階の内部精度管理には標準物質に相当する陽性コントロール組織の利用が重要となる．前述の津田班の調査研究の際に並行して行ったアンケート調査では，86％の施設がIHC検査時に陽性コントロール組織を使用しているものの，全てのIHC検査に対し行っている施設は14％にとどまっている．また陽性コントロール組織を使用する形態は，検体組織と同一スライド上に配置するかたちを採用している施設は50％，検体組織と陽性コントロール組織を各々染色するかたちを採用している施設は38％，両方を採用している施設は12％であった[4]．

当院では2000年頃より全てのIHC検査において，検体組織と同一スライド上に陽性コントロール組織を配置する内部精度管理を開始し，2010年より組織マイクロアレイ（TMA）の利用を開始した．また津田班における研究の一環として，IHC検査実施件数上位項目に対応できる陽性コントロールTMAのデザインについて検討を行っており，搭載するコア数や組織の組み合わせについて全身主要臓器からなる組織マイクロアレイを作製し，データの集積を現在進めている（図1）．

Ⅲ．組織ISH検査の標準化と精度管理

コンパニオン診断項目として実施されているHER2を除外すると，その他の項目は未だ研究的要素を残したまま検査が実施されており，標準化や精度管理は議論しにくい状況となっている．ISH検査のほとんど全ては現在FISH法で実施されている．自動化が中心となっているIHC検査とは異なり，アナリシス段階の多くの処理ステップは用手法で行われており，そのため，アナリシス・エラーを招来しやすい状況にはある．検査の成否はFISHシグナルの有無を検体組織中の内部コントロール細胞で確認することにより可能となることから，陽性コントロール組織は必ずしも必要とはならないが，検体組織でプレアナリシス・エラー（不適切な固定など）が起こっている場合には原因が特定できないことから，当院ではIHC検査同様，陽性コントロール組織を検体組織と同一スライド上に配置しFISH染色を行っている．

一方，ポストアナリシス段階においては，FISHシグナルのカウント作業は検査精度に大きな影響を与える要因の一つとなっている．ISH検査は遺伝子増幅の判定を行うほか，悪性リンパ腫や軟部腫瘍でみられる相互転座や脳腫瘍1p/19qのように染色体欠失を検出・判定する場合にも用いられる．判定方法にあたっては解析する腫瘍細胞数，陽性のカットオフ（異常細胞の割合），相互転座判定については分離シグナル間の距離など基準設定が重要となるが，特に相互転座や染色体欠失の判定においては，検査基準に関するコンセンサスはほとんど得られていない．またFISHの判定は暗視野で限られたエリアのみを対象に行い，さらに解析対象となる細胞核は観察者の主観で判定することから，観察者間誤差を生じやすい．特に胃癌のHER2遺伝子増幅など組織にheterogeneityがみられる場合，暗視野の判定ではばらつきを生じやすくな

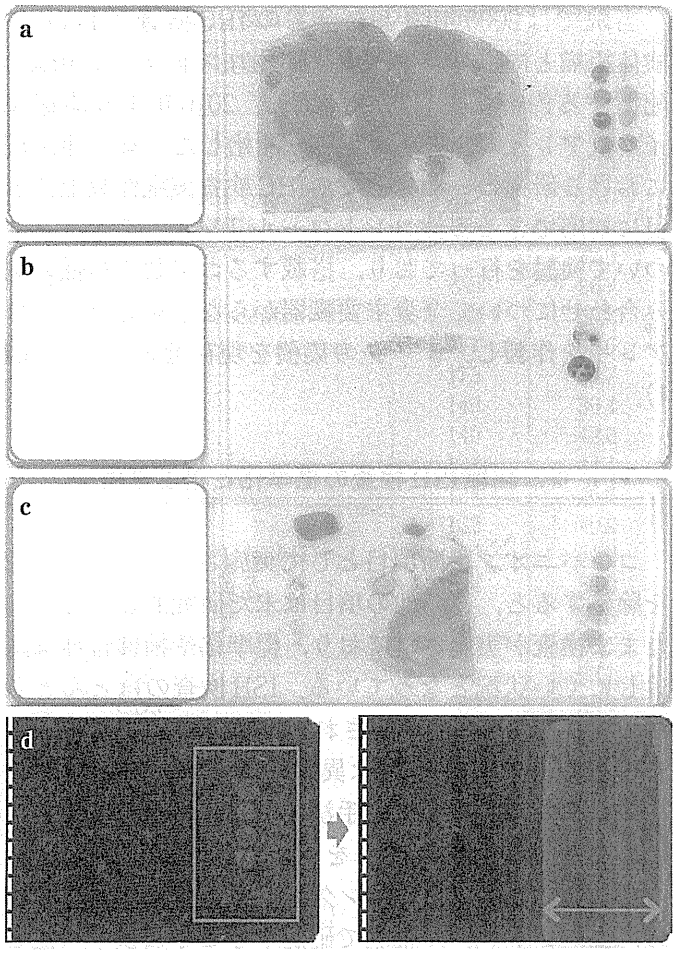

図1　内部精度管理用組織マイクロアレイ（TMA）
a, b：現在試験的に作製・使用している補助診断項目用 TMA．上位項目用は扁桃，虫垂，肝臓，膵臓を含めたΦ2 mmの8コア TMAで検討を進めている（a）．また使用頻度の高い悪性リンパ腫マーカー用には扁桃と虫垂からなるΦ3 mmの2コア TMAを使用している（b）．これらの TMAは1週間分に相当する枚数をまとめて薄切し，スライドグラスに TMAを事前に載せた状態で保管するようにし負担軽減を図っている．
c, d：コンパニオン診断用は HER2，ER，PgR，EGFRの4項目をカバーした4コア TMA（乳癌3コアと大腸癌1コア）を作製し使用している（c）．これらの項目ではより厳密な管理が求められるため，薄切後の切片にパラフィンコーティング（dの両矢印．写真ではカケンジェネックス社のパラメートを使用）を行い，酸化による劣化を防止し，また長期保存可能な状態にしている．

る．最近は IHC法と同様にアナリシス段階の完全自動化とポストアナリシス段階での明視野観察が可能となった明視野 ISH（bright-field *in situ* hybridization：BRISH）が HER2など一部の項目で実用化され，本邦でも先ごろ承認された．ISH検査の標準化や精度管理上の利点の多い技術であることから今後の普及が見込まれる．

Ⅳ．体細胞遺伝子検査の標準化と精度管理

近年大腸癌 KRAS変異検査や肺癌 EGFR変異検査など体細胞遺伝子検査において FFPE検体を用いた検査が急増している．国内の体細胞遺伝子検査のアナリシス段階は，その大部分が大手検査センターにおいて集約的に実施されている状況にある．肺癌 EGFR変異検査など検査項目によっては各検査センターで採用されている検査手法が異なるため，検査センターごとに検査の標準化が行われ，またこれに則った精度管理も日常厳密に行われている．それゆえこの段階の検査精度は現在のところ一定水準を維持しているといえる．一方で核酸を対象とする検査はとりわけプレアナリシス段階の影響を受けやすいことから，FFPE検体の作製プロセスは検査の成否を決定する極めて重要な段階といえる．体細胞遺伝子検査では，抗体や核酸プローブとの反応性回復を目的とした賦活処理を行うことができないため，深刻な核酸断片化はそのままアナリシス段階の PCR反応へ影響を与え致命的となる．それゆえホルマリン固定などにより受ける影響は賦活処理が可能な IHC検査や ISH検査に比べ大きいといえ，今後の FFPE検体作製にあたっては遺伝子関連検査での使用も十分念頭に置き作業を行う必要がある．

こうした状況のなか，日本臨床検査標準協議会（JCCLS）・遺伝子関連検査標準化専門委員会では，検体の品質管理による検査精度確保を目的とし「遺伝子関連検査の検体品質管理マニュアル」を策定し2009年に公表した[7]．本マニュアルは2009年2月の厚生労働省の先進医療専門家委員会で先進医療申請において遵守すべき要件として採択されている．また現在，同委員会では産学からなるワーキンググループを設け，血液検体，喀痰検体，組織検体を柱とした遺伝子関連検査のプレアナリシスに関する情報収集と検証作業を行っており，本活動の一環として我々は組織検体に関する検討を進めている．この検証作業での課題の一つに，検体の品質に関する指標の設定が挙げられる．特に FFPE検体の作製時に行われるホルマリン固定は核酸断片化への影響が大きく，検体の品質を事前評価できることが検査上望ましい．

現段階での核酸の品質管理方法として，DNAを評価する場合は，DNA抽出サンプルをアガロース電気泳動で確認する方法や，既知 DNA領域の PCR増幅の成否をみる方法，リアルタイム PCR法を用いた増

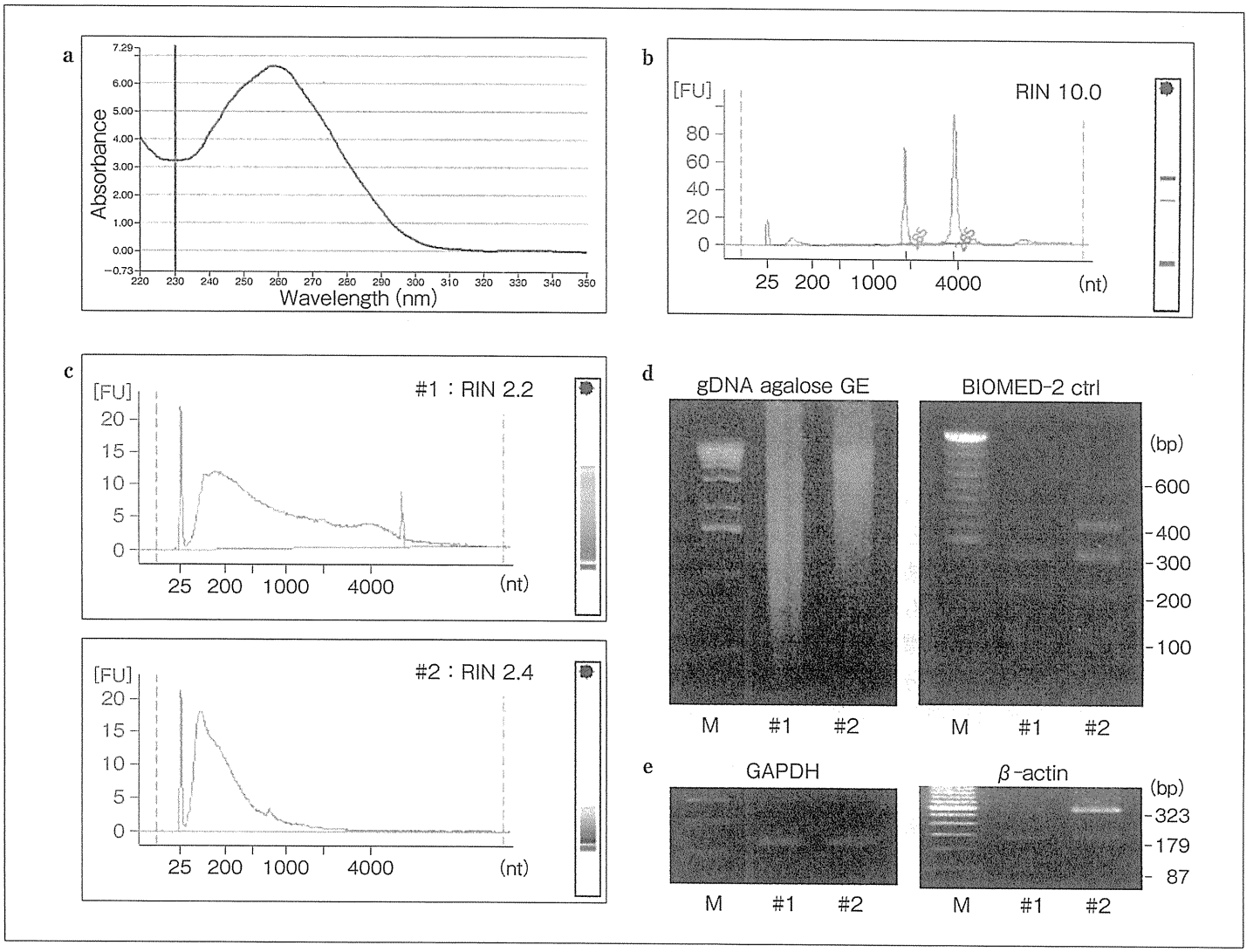

図2　核酸の品質評価　a：吸光度A260/A280比による核酸品質確認．カラム法を用いたFFPE検体からのDNA抽出液では比はおよそ1.9～2.1となる（JCCLS・遺伝子関連検査標準化専門委員会調査）．抽出に問題があった場合はA230の値が高値を示すためA260/A230比が低くなる場合があるため，この比の確認も必要である．b：マイクロチップ型電気泳動装置を用いたRIN値によるRNA品質確認．培養細胞などから良好にRNA抽出ができた場合などは高いRIN値を示す．c：RIN値を用いたFFPE検体等の品質確認．通常のFFPE検体ではしばしばRIN値は低値を示す（上段，#1）．核酸安定性が高いとされるアルコール系固定液（ここではキアゲン社のPAXgeneを使用）で固定したパラフィン検体でもRIN値は同程度となり（下段，#2），こうした検体の品質評価にRIN値は適していないようである．d：DNAの品質確認．従来から用いられている抽出ゲノムDNAのアガロースゲル電気泳動とBIOMED-2コントロール・プライマー・セットを用いたマルチプレックスPCRの結果．サンプル#1に比べ#2のほうがDNA断片化の程度が低く，特にPCRの結果では400bpのサイズの増幅で差が顕著である．e：ハウスキーピング遺伝子RT-PCRによるRNAの品質確認．GAPDH（108bp）とβ-actin（87，179，323bp）のうち，長いサイズ（β-actin 323bp）において#1と#2間の差が確認可能である．

幅速度の状態により評価する方法が挙げられる．一方RNAを評価する場合は，精製したRNAの純度を吸光度A260/A280比により確認する方法，rRNA，GAPDH，β-actinなどのハウスキーピング遺伝子の発現状態をRT-PCR法により確認する方法，28S/18S rRNAの比を確認する方法が知られている[6]．近年マイクロアレイ研究の普及に伴い，解析サンプルの核酸断片化をみる指標として，RNA integrity number（RIN）値の使用が研究領域で一般化しつつある[8]（図2）．RIN値はマイクロチップ型電気泳動装置（Agilent2100バイオアナライザ）を用いて得られる18Sや28S rRNAやその分解物のエレクトロフェログラムをデジタル解析しRNAの分解度をアルゴリズムにより算出した数値であり，RIN値は品質の低い1～高い10の10段階で表される．RIN値はしばしばFFPE検体の品質の確認に用いられるが，臨床材料で用いる場合では固定条件などが良く，保存状態が比較的良い場合であってもしばしば低値を示すことから，新鮮材料の

わずかな品質劣化を検出する場合とは異なり，相対的に品質が低いFFPE検体の場合はそのまま適用することは難しく，従来から用いられている方法のほうが品質の差を見極めやすい（**図2**）．欧州ではFFPE検体からの核酸抽出の多施設間比較に関する検討が，欧州委員会が支援する研究プロジェクトで行われている．この検討ではDNAの品質の確認に欧州共同研究プロジェクトBIOMED-2において検討されたBIOMED-2 control gene primer setが使用されており[9]，現在我々も検証に取り入れている．

おわりに

分子病理診断およびこれに用いられる検査は今後も拡大し重要性を増していくことは必至である．特に検査の件数はコンパニオン診断薬や診断に有用な新規試薬の開発・上市の急拡大とともに近年急速な伸びをみせている一方，それら検査の標準化や精度管理は大きな遅れをとっている．適切な分子病理診断はより正確な患者の層別化や治療の個別化を可能にし，これにより医療の質や効率化の向上，ひいては医療費削減などの医療経済面にも大きなインパクトを与える．こうした状況を踏まえ，分子病理診断で必要となる検査標準化・精度管理に関する体制づくりが急がれる．

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Survival and Prognostic Factors in Malignant Pleural Mesothelioma: A Retrospective Study of 314 patients in the West Part of Japan

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Objective: The objective in our study was to examine baseline and other characteristics associated with survival in patients with malignant pleural mesothelioma in Japan.
Methods: Three hundred and fourteen patients with an adjudicated diagnosis of mesothelioma were examined. Survival was evaluated by the Kaplan–Meier method with the log-rank test. The Cox model was used to estimate the hazard ratio for the possible prognostic factors.
Results: Of 314 patients, 223 (71%) died and only 40 (13%) were still alive at the end of the observation period starting from the day of diagnosis, while 51 (16%) were transferred to other hospitals or had the last health service contact before the end of the study period yielding the median survival of 308 days. In the multivariate analysis, age older than 70 years (hazard ratio = 2.17; 95% confidence interval, 1.36–3.46), non-epithelioid type (hazard ratio = 1.58; 95% confidence interval, 1.15–2.18), poor performance status (hazard ratio = 3.22; 95% confidence interval, 1.19–8.74), high white blood cell count (hazard ratio = 1.49; 95% confidence interval, 0.99–2.26) and high C-reactive protein level (hazard ratio = 1.80; 95% confidence interval, 1.06–3.06) were negatively associated with survival, after adjustment for other factors.
Conclusions: Some baseline conditions including old age, poor performance status, non-epithelioid type, high white blood cell count and high C-reactive protein level were determinants of poor survival of patients with malignant mesothelioma.

Key words: malignant mesothelioma – prognostic factor – retrospective study and survival

INTRODUCTION

Malignant pleural mesothelioma (MPM) is a rare neoplasm arising from the serosal surface and often diagnosed long after the exposure to asbestos. Once diagnosed as mesothelioma, the median survival is short or only 4–16 months (1–3). For example, in an epidemiological study using the Osaka Cancer Registry during the period 1966–2001, the median survival was 6 months for males and 5 months for females (4). Imports of asbestos, a well-known causative substance of mesothelioma, had peaked in 1974 in Japan and the incidence of mesothelioma has been increasing thereafter: during the period of 1975–1977, the age-standardized incidence rates in males and females were 0.8 and 0.3 but during 1999–2001, they were 12.5 and 3.0 per 1 000 000 person-years, respectively. The increase of the incidence made the Japanese government start a campaign entitled 'a comprehensive strategy against asbestos-related diseases' in 2006 (5).

Factors associated with poor prognosis in patients with MPM include old age, poor performance status (PS), advanced disease stage, thrombocytosis, chest pain, weight loss, asbestos exposure and long duration of symptoms (2,5–9). The epithelioid type is known to be a good prognostic factor whereas the mixed type has been associated with either poor (10) or good prognosis (7). In the present study, we examined survival and its association with clinicopathologic variables in Japanese patients with MPM with the study size relatively larger than the previous ones (11,12).

PATIENTS AND METHODS

Patients

Patients diagnosed with malignant mesothelioma between March 1996 and March 2006 in 26 participating hospitals in seven prefectures in the west part of Japan were evaluated. We followed up individual patients until death, the last health service contact, transfer to another hospital or the end of the study (31 March 2006), whichever came first. We used questionnaires to know gender, age at diagnosis, smoking history and baseline characteristics at diagnosis for PS as defined by the Eastern Cooperative Oncology Group (ECOG), and the clinical stage defined by the International Mesothelioma Interest Group (IMIG) (13). Information was also obtained on the clinical course, treatment (surgical excision, radiation therapy, pleurodesis and any chemotherapy) as well as hematological and other laboratory data [C-reactive protein (CRP), serum lactate dehydrogenase (LDH) and SPO_2 levels] at diagnosis of malignant mesothelioma.

Diagnosis of malignant mesothelioma made in the individual hospitals was evaluated by the mesothelioma Review Committee consisting of three respiratologists. The final diagnosis was made based on the results of the immunostaining of histological or cytological specimens, chest X-ray images, computer tomography scans and the clinical course of the patient. We carefully evaluated the date when the tissue used to establish diagnosis was taken because the starting point of observation was defined as the date of diagnosis of malignant mesothelioma in this study.

The study protocol was approved by the central ethics review committee (Public Health Research Foundation, Tokyo, Japan) in October 2006. The committee required strong confidentiality protection and a written informed consent from the patient being treated in the study hospital, but judged it was not practical to obtain the consent from those not being currently treated in the hospital and required to display a poster somewhere in each study hospital to announce the study during the study period.

Statistical Methods

In the crude analysis, the mortality rate was estimated by the person-year method, where the rate was calculated as the number of patients who died divided by corresponding person-years. We stratified patients by various factors and the rate of death was calculated for each level of the factor to estimate crude rate ratios (RRs). The following factors were examined: gender, age, histological type, ECOG PS (PS = 0 or 1, PS = 2 or 3, PS = 4), IMIG staging, smoking history, past asbestos exposure and pleural effusion at diagnosis. Patients were also stratified for the following laboratory data at diagnosis of malignant mesothelioma: hemoglobin, platelet level ($>350 \times 10^3$ and $<150 \times 10^3$ vs. $150–350 \times 10^3/mm^3$ as a reference), white blood cell (WBC) count (9000–10 000 and $>10\,000$ vs. $<9000/mm^3$ as a reference) and levels of CRP, LDH and SPO_2.

Survival probabilities were calculated by the Kaplan–Meier method with the log-rank test. The Cox proportional hazard regression model was used to estimate the hazard ratio (HR) and 95% confidence intervals (CI) for the following factors: age, gender, histological type, ECOG PS, disease stage (IMIG staging), smoking history and past asbestos exposure, as well as laboratory data (hemoglobin level, platelet count, WBC count and levels of CRP, LDH and SPO_2). Four therapeutic modalities (surgical excision, radiation therapy, pleurodesis and any chemotherapy) were incorporated into the model as a time-dependent variable. The method of generalized estimating equations was used in order to take into account that one patient might have a baseline period with no intervention and one or more periods of exposure to different combinations of interventions. For missing variables, the method of multiple imputation was employed (14,15). We applied SAS PROC MIANALYZE to estimate the parameter of interest using five complete data sets.

RESULTS

Of the 328, 314 patients were with an adjudicated diagnosis of MPM, 314 had pleural mesothelioma, 12 had peritoneal mesothelioma, 1 had mesothelioma of the pericardium and the remaining 1 had mesothelioma of the tunica vaginalis testis. We only included patients with MPM for our analysis. During the average of the observation period of 523 days, 223 (71%) had died but 40 (12%) were still alive at the end of the observation period while 35 (11%) had been transferred to other hospitals and 16 (5%) had had the last health service contact before the end of the study period in patients with MPM.

The profile of patients with MPM is summarized in Table 1. Patients in this study were predominantly males (87%) with a median age of 67 years. The histological type was epithelioid in 38% of patients, sarcomatoid in 25% and mixed type in 17%. More than half of the patients were in the late stages of disease (stage III or IV of the IMIG staging system), although the majority had a good PS (PS = 0 or 1). Of 314 patients with pleural mesothelioma, 263 (84%) had pleural effusion at diagnosis and of the remaining 51, 7

Table 1. Characteristics of study subjects with confirmed malignant pleural mesothelioma

Characteristics	No. (%)
Overall	314
Age, year	
<60	75 (24)
60–69	112 (36)
≥70	127 (40)
Median (range)	67 (36–92)
Gender	
Female	41 (13)
Male	273 (87)
Histological type	
Epithelioid	120 (38)
Sarcomatoid	77 (25)
Biphasic	52 (17)
Others	6 (1.9)
Unknown	59 (19)
PS	
PS 0	43 (14)
PS 1	174 (55)
PS 2	48 (15)
PS 3	18 (5.7)
PS 4	7 (2.2)
Unknown	24 (7.6)
Stage (IMIG)	
Stage I	59 (19)
Stage II	43 (14)
Stage III	82 (26)
Stage IV	92 (29)
Unknown	38 (12)
Smoking Status	
Smoker (current/ex)	214 (68)
Non-smoker	92 (29)
Unknown	8 (2.5)
Past asbestos exposure	
Asbestos exposure	197 (63)
No asbestos exposure	71 (23)
Unknown	46 (15)

PS, performance status; IMIG, International Mesothelioma Interest Group.

developed pleural effusion during the observation period after diagnosis. Treatments given to 314 patients with MPM are summarized in Table 2. Surgical excision of tumors was possible only for 21%, while more than half received some type of chemotherapy. Most patients who underwent surgical

Table 2. Treatments of patients with malignant pleural mesothelioma

Treatment	*n* (%)
Surgical excision	
Wide local excision	11 (3.5)
Pleurectomy	4 (1.3)
Extrapleural pneumonectomy	52 (17)
No surgical excision	247 (79)
Radiation therapy	49 (16)
No radiation therapy	265 (84)
Pleurodesis	103 (33)
Pleurodesis with OK-432	66 (21)
No pleurodesis	211 (67)
Any chemotherapy	177 (56)
No chemotherapy	137 (44)

excision had this as the first treatment (64 of 67 patients), whereas ~80% ($n = 139$) of 177 patients who had some type of chemotherapy had chemotherapy as the first therapy. On the other hand, this was the case only for a quarter (11 of the 49) of patients who received radiation therapy, indicating that this was usually selected in the late stage after the patient had undergone other types of therapies.

Median survival [interquartile range (IQR)] was 308 days (IQR, 281–368 days) in the 314 study patients. Figure 1 shows Kaplan–Meier survival curves subclassified for six selected variables. Table 3 shows the results of crude and multivariate analyses for 18 possible prognostic factors. In both crude and multivariate analyses, survival was significantly poor for old age, non-epithelioid type and poor PS. For example, patients with epithelioid, mixed and sarcomatoid types had median survival of 427, 319 and 183 days, respectively. Similarly, poorer PS was associated with poorer survival. Gender, smoking status and past asbestos exposure were not associated with survival in the crude and Cox regression analysis.

Low hemoglobin level (<12.0 g/dl) was an unfavorable prognostic factor in the crude analysis. Both thrombocytosis and thrombocytopenia were associated with a poor prognosis in the crude analysis. High WBC count (>10 000/mm^3) was associated with poor survival (Table 3 and Fig. 1).

As shown in Table 3, an elevated LDH and lower SPO_2 were negatively associated with the prognosis in the crude analysis. In both the crude and multivariate analyses, an elevated CRP level was associated with shorter survival, with the median survival at 569, 314 and 201 days, for <0.3, 0.3–4.0 and >4.0 mg/dl CRP, respectively.

Radiation therapy had negative and surgical excision had positive effects on survival. For instance, the median survivals for patients treated with and without surgical excision were 710 days and 288 days, respectively. However, pleurodesis was not associated with survival either in the crude or

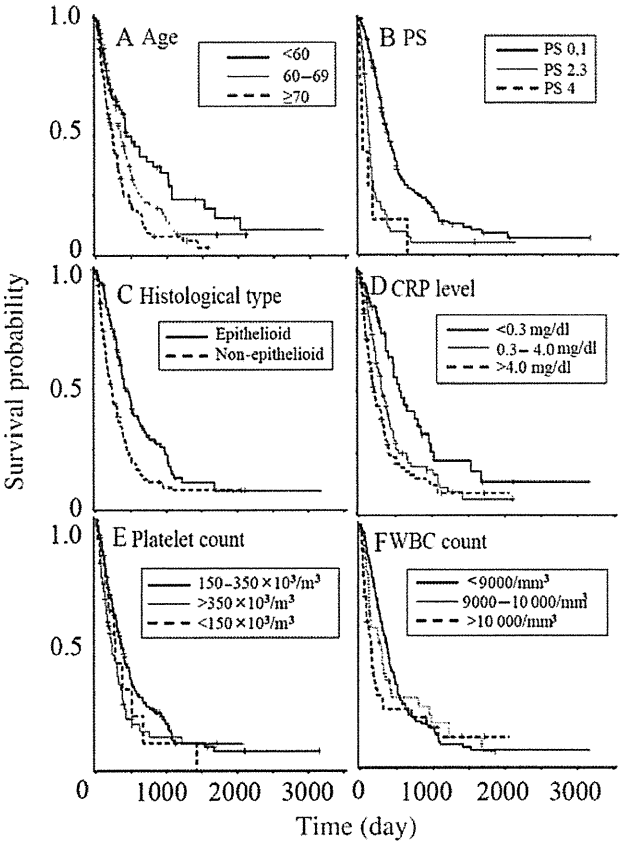

Figure 1. Kaplan–Meier survival curves for prognostic factors (log-rank test). (A) Three age groups ($P < 0.001$), (B) three groups for performance status ($P < 0.001$), (C) two groups for histological type ($P = 0.001$), (D) three groups for C-reactive protein (CRP) level ($P < 0.001$), (E) three groups for platelet count ($P = 0.051$) and (F) three groups for white blood cell (WBC) count ($P = 0.074$).

the multivariate analysis. In the crude analysis, 'any chemotherapy' was associated with poor prognosis, although this was weakened when adjusted for other variables. We also examined survival for patients given radiation and/or chemotherapy after surgical excision and compared with that for patients with surgical excision only in the 64 patients who had surgical excision as the first treatment. The crude RR was 2.39 while the HR in the Cox regression model was 2.72 for patients with radiation and/or chemotherapy after surgical excision when compared with those with surgical excision only (Table 3).

DISCUSSION

In the current study, we analyzed survival in a retrospective cohort study of 314 patients with MPM in Japan over a 10-year period. Factors associated with poor survival identified in previous studies, including old age, advanced stage of disease, poor PS, and non-epithelioid type and several laboratory results, such as low hemoglobin, high platelet and high WBC levels, were found as factors associated with poor prognosis in the crude and/or multivariate analysis in this study. In addition, this study revealed that a high CRP level was also an important prognostic factor. In our study, the median survival was 308 days (IQR, 281–368 days) or 10.1 from the day of diagnosis of mesothelioma for 314 patients with confirmed MPM. These results are in line with previous studies where the median survival was reported to be 4–16 months after diagnosis (1–3).

Old age has been recognized as a negative prognostic factor (9,10,16), being consistent with our results. The results of previous studies showed a significant association between male gender and poor survival (8,17). Gender was, however, not identified as a factor affecting survival in our study, probably in part due to the small proportion of female patients. Our study revealed longer survival for patients with the epithelioid type, being compatible with some (9,18) but not other (2,19) previous studies. We confirmed that an elevated platelet count ($>350 \times 10^3/mm^3$) is associated with a poor prognosis in the crude analysis, although the association was not remarkable in the multivariate analysis. This is in agreement with the study by Ruffie et al., where 42% of patients with a high platelet count ($>400 \times 10^3/mm^3$) had a poor prognosis. In our study, MPM patients with a high WBC count had worse survival, consistent with the EORTC study (8). We also observed that a high CRP level was associated with poor prognosis. A high CRP level is known to be associated with a poor prognosis in patients with malignancy in general (20,21).

In the present study, survival benefits attributed to different treatments must be interpreted with caution because patients had often received various therapies with a variety of combinations for different durations before and after the diagnosis of MPM was established. However, some aspects of the information may be worth mentioning. First, pleurodesis has been recommended for the patients with intractable pleural effusion. Because of inaccessibility to talc in Japan, picibanil (OK-432), a preparation of *Streptococcus pyogenes*, has been used as an antitumor immunomodulator for malignant pleural effusion (22). However, information for its safety in patients with mesothelioma has been insufficient. OK-432 was administered to 66 (64%) of 103 patients treated with pleurodesis in our study but the RRs for pleurodesis in the crude and multiple analyses were both near 1.0. Therefore, it is unlikely that pleurodesis (with OK-432) has a major impact on survival.

The benefits of surgical treatment for MPM remain controversial. The previous studies showed that surgical procedures for pleurectomy or extrapleural pneumonectomy did not prolong survival (1,9,13), whereas some studies showed that the survival rate in patients with surgical treatment was superior to patients without surgery (23,24). In our study, the RR for surgical excision was 0.37 in the crude analysis and the benefit of surgical excision was demonstrated even after adjustment of the baseline information including clinical conditions, such as stages and PS status, likely to represent

Table 3. Association between clinicopathologic variables and survival

Prognostic factors	Crude analysis						Multivariate analysis[a]	
	n	Death	Person-years	Rate/year	Rate-ratio	(95% CI)	HR	(95% CI)
Gender								
Female	41	28	36.4	0.77				
Male	273	195	285.2	0.68	0.89	(0.60–1.32)	1.27	(0.79–2.04)
Age (year)								
<60	75	44	104.2	0.42				
60–69	112	85	131.0	0.65	1.54	(1.07–2.21)	1.49	(0.99–2.24)
≥70	127	94	86.4	1.09	2.57	(1.80–3.68)	2.17	(1.36–3.46)
Histologic type								
Epithelioid	120	77	146.1	0.53				
Non-epithelioid	135	103	113.5	0.91	1.72	(1.28–2.31)	1.58	(1.15–2.18)
PS								
0/1	217	147	255.6	0.58				
2/3	66	55	36.6	1.50	2.61	(1.91–3.56)	2.17	(1.51–3.12)
4	7	7	2.8	2.47	4.30	(2.02–9.18)	3.22	(1.19–8.74)
Stage (IMIG)								
I/II	102	67	139.3	0.48				
III	82	55	83.8	0.66	1.37	(0.96–1.95)	1.26	(0.87–1.84)
IV	92	75	66.6	1.13	2.34	(1.68–3.25)	1.41	(0.91–2.07)
Smoking history								
No smoking	92	61	101.5	0.60				
Smoking	214	157	210.8	0.74	1.24	(0.92–1.67)	1.02	(0.97–1.15)
Past asbestos exposure								
No exposure	71	51	87.3	0.58				
Exposure	197	141	193.2	0.73	1.25	(0.91–1.72)	1.02	(0.98–1.07)
Hemoglobin level, g/dl								
≥13.5	145	104	175.0	0.59				
12.0–13.4	68	46	75.1	0.61	1.03	(0.73–1.46)	0.76	(0.53–1.08)
<12.0	86	62	49.9	1.24	2.09	(1.53–2.86)	0.88	(0.58–1.34)
Platelet count, /mm^3								
150–350 × 10^3	210	147	228.3	0.64				
>350 × 10^3	78	55	60.6	0.91	1.41	(1.03–1.92)	1.30	(0.89–2.82)
<150 × 10^3	11	10	11.0	0.91	1.41	(0.74–2.68)	1.28	(0.58–1.89)
WBC count, /mm^3								
<9000	223	151	225.1	0.67				
9000–10 000	36	29	40.3	0.72	1.07	(0.72–1.60)	0.75	(0.43–1.32)
>10 000	40	32	34.5	0.93	1.38	(0.94–2.02)	1.49	(0.99–2.26)
CRP level, mg/dl								
<0.3	53	33	84.2	0.39				
0.3–4.0	121	85	117.2	0.73	1.85	(1.24–2.77)	1.23	(0.77–1.95)
>4.0	116	85	89.9	0.95	2.41	(1.61–3.60)	1.80	(1.06–3.06)

Continued

Table 3. *Continued*

Prognostic factors	Crude analysis						Multivariate analysis[a]	
	n	Death	Person-years	Rate/year	Rate-ratio	(95% CI)	HR	(95% CI)
LDH level, IU/l								
≤229	239	169	238.2	0.71				
>229	46	38	39.5	0.96	1.36	(0.95–1.93)	0.91	(0.59–1.41)
SPO_2 level, %								
≥95	210	147	215.1	0.68				
<95	33	26	21.1	1.23	1.80	(1.19–2.73)	1.16	(0.80–1.91)
Pleurodesis								
No	211	145	220.3	0.66				
Yes	103	78	101.2	0.77	1.17	(0.89–1.54)	1.07	(0.76–1.50)
Radiation therapy								
No	265	184	288.8	0.64				
Yes	49	39	32.7	1.19	1.87	(1.32–2.64)	2.34	(1.57–1.78)
Any chemotherapy								
No	137	91	173.1	0.53				
Yes	177	132	148.5	0.89	1.69	(1.29–2.21)	1.26	(0.36–3.49)
Surgical excision								
No	247	190	219.2	0.87				
Yes	67	33	102.4	0.32	0.37	(0.26–0.54)	0.57	(0.89–0.92)
Surgical excision given as the first treatment								
Surgical excision only[b]	31	10	51.4	0.19				
Radiation and/or chemotherapy after surgery	33	22	47.3	0.47	2.39	(1.13–5.05)	2.72	(1.22–6.08)

CR, confidence interval; HR, hazard ratio; WBC, white blood cell; CRP, C-reactive protein; LDH, lactate dehydrogenase.
[a]Cox regression model.
Subjects with missing value were excluded from the crude analysis, while the method of multiple imputation was employed for the missing values in the Cox regression model.
[b]HR was estimated, after adjusting for the following covariates; gender, age, histological type, PS, stage (IMIG), smoking history, past asbestos exposure, hemoglobin level, platelet count, WBC count, CRP level, LDH level and SPO_2 level.

the conditions when the patient had this therapy (because 64 of 67 patients had it as the first therapy, normally soon after diagnosis of MPM). Therefore, the better prognosis in the multivariate analysis may be attributed to surgical excision itself rather than the early clinical stage. The best survival was observed in the patients who had surgical excision only. On the other hand, the RR larger than unity (RR = 1.69) in the crude analysis of 'any chemotherapy' approached unity in the multivariate analysis (HR = 1.26). Because the majority of patients (139 of 177) who received some type of chemotherapy had it as the first therapy, the crude RR higher than unity might indicate that chemotherapy was given to patients with relatively poor prognosis at diagnosis while chemotherapy itself had no major impact on the survival of patients with MPM. Radiation therapy was shown to be associated with a poor prognosis in the crude analysis (RR = 1.87), which became larger by multivariate analysis (HR = 2.34). As only a minority of patients (11 of 49) received radiation therapy as the first treatment, the association between radiation therapy and a poor prognosis might merely indicate that radiation was given relatively late during the clinical course and radiation might be viewed as a marker for poor overall condition when the patient had this therapy late in the clinical course in this study. Some investigators have suggested that radiation therapy has little impact on survival of patients with MPM (2,25).

In the present study, all of the eligible patients were identified in the study hospitals and a diagnosis of mesothelioma was confirmed by the review committee using immunohistochemistry data of sufficient quality and quantity for most patients. However, for prognostic factors, some information on the study patients was lacking due to the retrospective nature of the current study. For example, some of the previous studies (2,6,8) reported that information on clinical

manifestations such as chest pain and dyspnea was important for predicting survival of MPM patients. Though those data were not available in our study, it was unlikely that the estimates for other factors were seriously affected by the lack of those clinical manifestations. A total of 51(16%) patients were transferred to other hospitals or had the last health service contact before the end of the study period. The distribution of prognostic factors was, however, similar between those 51 patients and others. In addition, when those 51 were removed, the median survival was estimated to be 288 days, which is a little shorter than but fairly close to the 308 days estimated from the data of all of the 314 patients with MPM.

In conclusion, the baseline characteristics of MPM patients, such as old age, poor PS, non-epithelioid type, high WBC count and high CRP level at diagnosis had independent influence on the poor survival of patients with MPM in the current study, even though a variety of treatments were given to patients thereafter. Surgical excision was likely to have improved the prognosis of patients with MPM, while pleurodesis and chemotherapy seemed to have no major impact on survival. The median survival was 308 days in our study, which indicates that the course of MPM remains aggressive and unfavorable. Our study may provide the information important in evaluating the effect of new interventions in the future.

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Conflict of interest statement

None declared.

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Reduction of CXC Chemokine Receptor 3 in an *In Vitro* Model of Continuous Exposure to Asbestos in a Human T-Cell Line, MT-2

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Because patients with silicosis who are chronically exposed to silica particles develop not only pulmonary fibrosis, but also complications involving autoimmune diseases such as rheumatoid arthritis and systemic sclerosis, exposure to asbestos may affect the human immune system. This immunologic effect may impair antitumor immune function because cancer complications such as lung cancer and malignant mesothelioma are found in patients exposed to asbestos. To elucidate the antitumor immune status caused by $CD4^+$ T cells exposed to asbestos, an *in vitro* T-cell model of long-term and low-level exposure to chrysotile asbestos was established from a human adult T-cell leukemia virus–1–immortalized human polyclonal T cell line, MT-2, and the resulting six sublines showed resistance to asbestos-induced apoptosis after more than 8 months of continuous exposure. The results of DNA microarray analysis showed that the expression of 139 genes was altered by long-term and low-level exposure to asbestos, and the profile was almost similar among the six sublines when compared with the original MT-2 cells that had never been exposed to asbestos. Pathway and network analysis indicated a down-regulation of IFN-γ signaling and expression of CXC chemokine receptor 3 (CXCR3) in the sublines, whereas ELISA and flow cytometry analysis demonstrated a reduction in Th1-related IFN-γ production and cell-surface CXCR3 expression. These findings suggest that chronic exposure to asbestos may reduce antitumor immune status in $CD4^+$ T cells, and that an *in vitro* T-cell model may be useful in identifying molecules related to the impairment of antitumor immune function.

Keywords: asbestos; malignant mesothelioma; CXCR3; IFN-γ

CLINICAL RELEVANCE

We show that long-term and low-level exposures to asbestos decrease the expression of Th1-related molecules (CXCR3, IFN-γ, and CXCL10/IP10) in the $CD4^+$ T-cell line, MT-2, suggesting that exposure to asbestos may induce an impairment of antitumor immune responses. Therefore, our findings may be of use in detecting patients exposed to asbestos, identifying prognostic factors, and designing therapeutic devices to prevent the reduction of antitumor immune function found in immunocompetent cells exposed to asbestos.

Exposure to asbestos (i.e., chrysotile, crocidolite, or amosite) leads to the development of asbestos-related diseases such as asbestos-related pleural plaque (PP) and malignant mesothelioma (MM) (1–3). Both diseases arise from exposure to asbestos, but MM has a poor prognosis, whereas PP is benign. Given that asbestos-related MM possesses a latency period ranging from 20–50 years, the peak of annual deaths from these diseases is predicted to occur around 2030 in Japan (4). Many investigations sought to elucidate the mechanisms underlying these diseases, and reports show that asbestos induces DNA damage and apoptosis in alveolar epithelial and mesothelial cells through a process mediated by reactive oxygen and nitrogen species of the mitochondrial dysfunction pathway (5–10).

MM is caused by exposure to asbestos, including conditions of long-term and low-level exposure. We reported that exposure to asbestos decreases the cytotoxicity of human natural killer (NK) cells, and that the cytotoxicity of NK cells is impaired in patients with MM (11, 12), suggesting that long-term and low-level exposures to asbestos may lead to a reduction of antitumor immune function. On the other hand, we showed that high-level exposures to asbestos induced the apoptosis of $CD4^+$ T cells in peripheral blood mononuclear cells *in vitro* because of activation-induced cell death (13, 14). Therefore, in an effort to determine whether long-term and low-level exposures of human immune cells to asbestos can induce a reduction in antitumor immune function, we developed an *in vitro* experimental model of chronic exposure to asbestos (chrysotile), using a human T-cell leukemia virus type–1 (HTLV-1)–immortalized human polyclonal T-cell line, MT-2 (15, 16), and we successfully established an asbestos-induced, apoptosis-resistant subline (MT-2Rst) (17). Because the original MT-2 cells (MT-2Org) constitute an HTLV-1–immortalized cell line, this line can continue to divide for many generations. In previous studies, we showed that long-term and low-level exposures to chrysotile induced an up-regulation of Src-family kinase–mediated IL-10 production, with a subsequent activation of the signal transducer and activator of transcription 3 (STAT3), and an overexpression of the antiapoptotic protein Bcl-2, located downstream from STAT3 (17). In addition, short-term and high-level exposures to chrysotile promoted the production of reactive oxygen species (ROS) and triggered apoptosis via a caspase-dependent mitochondrial pathway in the original MT-2 cells (MT-2Org) (18). These mechanisms are summarized in Figure 1.

We found that patients with MM manifest a high expression of *Bcl-2* in peripheral $CD4^+$ T cells (17), a high level of IL-10 and transforming growth factor (TGF)-β1 in plasma, and the multiple overrepresentation of the T-cell receptor Vβ in peripheral $CD3^+$ T cells (19, 20). Therefore, an analysis of the immunologic effects

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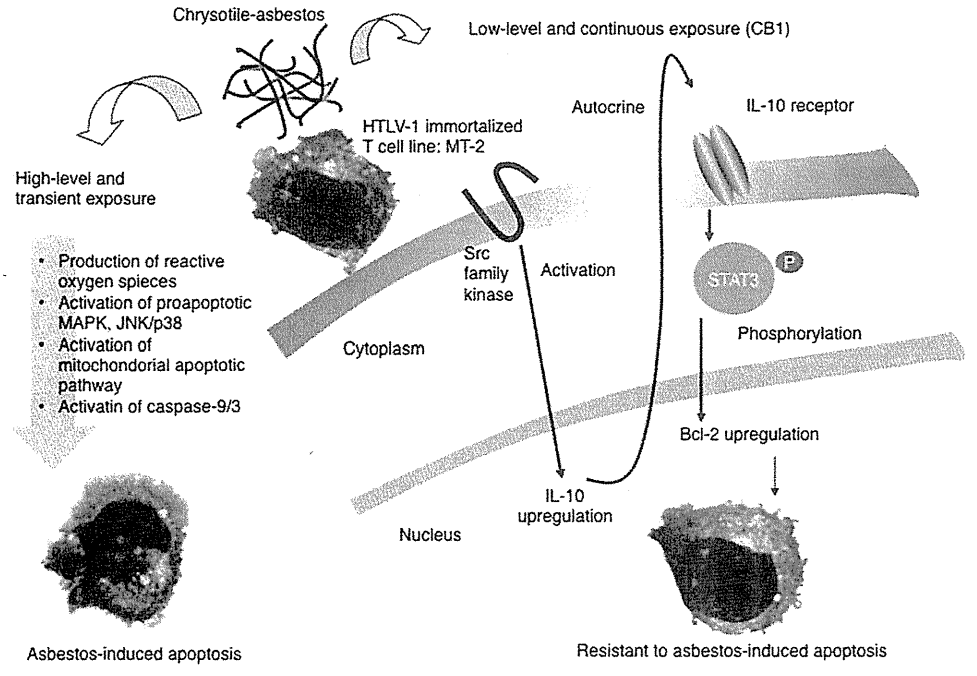

Figure 1. Schematic representation of molecular mechanism leading to resistance of MT-2Rst cells to asbestos-induced apoptosis. MT-2Rst cells continuously exposed to low-level chrysotile-B (CB) for more than 8 months showed resistance against asbestos-induced apoptosis, accompanied by the up-regulation of Src-family kinases, IL-10, signal transducer and activator of transcription 3 (STAT3), and Bcl-2, as previously reported (17, 18). HTLV-1, human T-cell leukemia virus type–1; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase.

of asbestos on experimental T-cell models may help explain the reduced antitumor immune function in patients with MM.

Here, we investigated differences in gene expression between MT-2Org and six independent asbestos-induced, apoptosis-resistant sublines (MT-2Rsts) in an effort to identify genes altered by long-term and low-dose exposures to asbestos in T cells. Because three of the sublines were exposed to chrysotile-A (CA) and the other three were exposed to chrysotile-B (CB), they are designated as MT-2CA1–3 and MT-2CB1–3 (the initial MT-2Rst was CB1), respectively. Using these *in vitro* models of low-level and continuous exposure to asbestos, we found a down-regulation of Th1-type molecules such as CXC chemokine receptor 3 (CXCR3), chemokine (C-X-C motif) ligand 10 (CXCL10)/IFN-γ–induced protein 10 kD (IP10), and IFN-γ in MT-2Rsts cells.

These findings may useful in detecting patients exposed to asbestos, in identifying prognostic factors, and in designing therapeutic devices to prevent the reduction of antitumor immune function found in immunocompetent cells exposed to asbestos.

MATERIALS AND METHODS

Cell Lines and Asbestos

MT-2Org and MT-2Rsts cells were passaged many times in RPMI-1640 medium supplemented with 10% FBS, streptomycin, and penicillin at 37°C, and maintained in a humidified atmosphere of 5% CO_2. The International Union against Cancer standard CA and CB were kindly provided by the Department of Occupational Health at the National Institute for Occupational Health of South Africa (21). Chrysotile asbestos is composed of $Mg_3Si_2O_5(OH)_4$. CA from Zimbabwe contains 2% fibrous anthophyllite, although CB from Canada does not contain any fibrous impurities.

Real-Time RT-PCR

Real-time RT-PCR was performed using the SYBER Green method (TaKaRa, Shiga, Japan) with the Mx3000P QPCR System (Agilent Technologies, Inc., Santa Clara, CA), as previously described (17), to amplify CXCR3, chemokine (C-C motif) ligand 4 (CCL4)/macrophage inflammatory protein-1β (Mip-1β), and CC chemokine receptor 5 (CCR5). We used the primers CXCR3 forward (ACACCTTCCTG CTCCACCTA), CXCR3 reverse (GTTCAGGTAGCGGTCAAAGC), CCL4/MIP-1β forward (GAAAACCTCTTTGCCACCAA), CCL4/MIP-1β reverse (TCACTGGGATCAGCACAGAC), CCR5 forward (TAGTCATCTTGGGGCTGGTC), and CCR5 reverse (TGTAGGGA GCCCAGAAGAGA).

Flow Cytometry

Cells were stained with fluorescent conjugated antibodies for 30 minutes at 4°C. After washing with PBS, cells were analyzed on a flow cytometer (FACSCalibur; BD Biosciences, Franklin Lakes, NJ). The antibody used in this study was CXCR3-PE (clone 1C6; BD Biosciences Pharmingen, San Diego, CA).

ELISA

MT-2Org and MT-2Rsts cells (1×10^5/ml) were cultured in 24-well plates for 72 hours. The culture supernatants were then collected and assessed for the production of IFN-γ and CXCL10/IP10 by immunoassay, using Quantikine ELISA kits (R&D Systems, Minneapolis, MN).

DNA Microarray Analysis

Total RNA was isolated using the RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany), and the quality of the RNA was assessed by examining the integrity of ribosomal RNA peaks, using an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc.). Purified RNA (0.5 μg) was reverse transcribed using moloney murine leukemia virus reverse transcriptase (Agilent Technologies, Inc.) and a T7-oligo(-dT) promoter primer. After synthesis of the cDNA second strand, this product was employed to generate labeled complementary RNA (cRNA), using T7 RNA polymerase with cyanine 3–cytidine triphosphate (Low RNA Input Fluorescent Linear Amplification Kit; Agilent Technologies, Inc.). Labeled cRNA (1.5 μg) was then fragmented and hybridized to a 60-mer oligonucleotide microarray containing approximately 41,000 human genes (Human Whole Genome Oligo Microarray; Agilent Technologies, Inc.) for 17 hours at 65°C. After washing, the array was scanned using an Agilent DNA microarray scanner.

Data analysis was performed using Genespring (Agilent Technologies, Inc.). For statistical evaluation, expression profiles were normalized for the MT-2Org cell expression ratio to unity. After the removal of saturated and low-signal genes, genes that were twofold up-regulated or down-regulated in MT-2Rsts cells compared with MT-2Org cells were listed. The resultant signal information was analyzed using the Student *t* test ($P < 0.05$), and was clustered based on correlation coefficients. The resulting sets of differentially expressed genes were examined by pathway and network analysis, using the MetaCore Analytical Suite (http://www.genego.com; GeneGo, St. Joseph, MI).

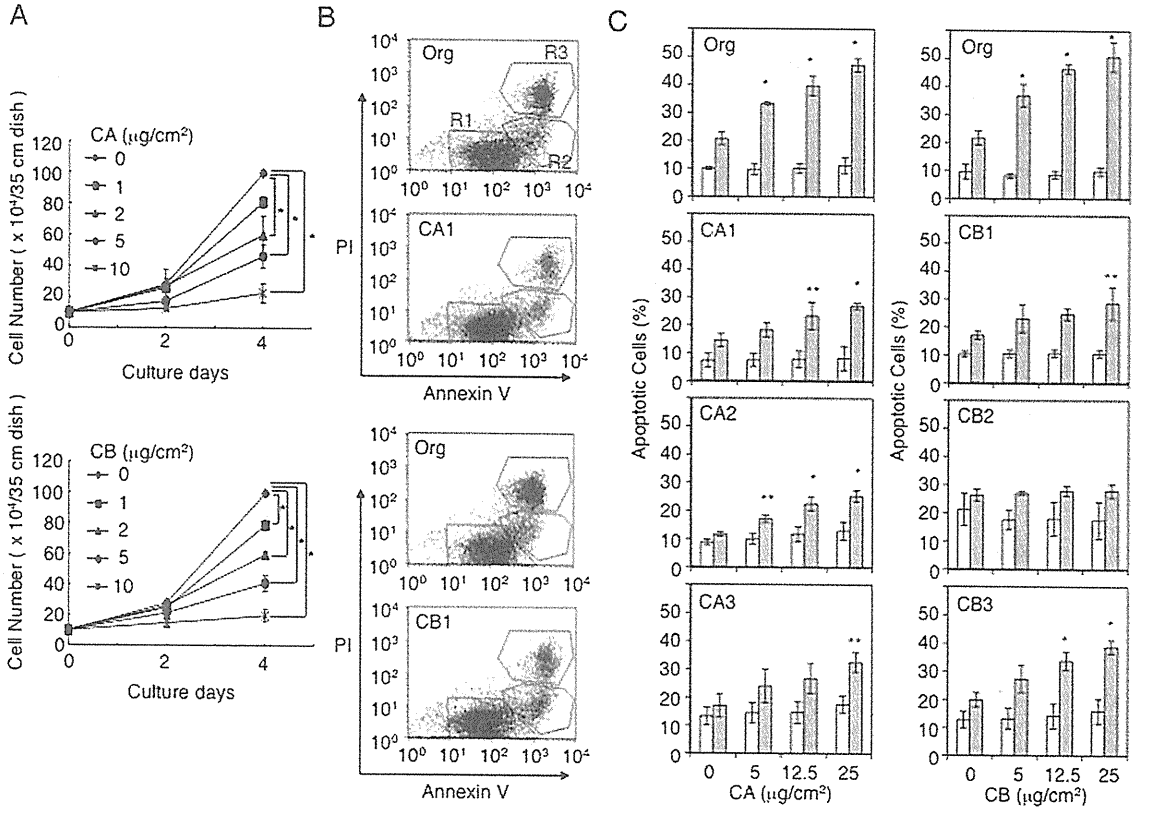

Figure 2. Original MT-2 (MT-2Org) cells acquire resistance to chrysotile-induced apoptosis by long-term and low-level exposures to chrysotile. (*A*) MT-2Org (Org) cells (1×10^5/2 ml) were cultured in the absence or presence of 1, 2, 5, or 10 μg/cm² chrysotile-A (CA) (*top*) or CB (*bottom*) in a 35-mm dish for 2 or 4 days. The number of viable cells was determined using the trypan blue dye exclusion test. (*B, C*) MT-2Org and MT-2Rsts (CA1, CA2, CA3, CB1, CB2, and CB3) cells (1×10^5/ml) were cultured in the absence or presence of 5, 12.5, or 25 μg/cm² CA or CB in 24-well plates. After 24 hours, apoptotic cells were detected by staining with Annexin V–FITC and propidium iodide, and stained cells were analyzed using FACS (FACS profiles shown in *B*). Region 1 (R1) represents viable cells (Annexin V-/PI-). Region 2 (R2) contains early apoptotic cells (Annexin V+/PI-). Region 3 (R3) includes late apoptotic cells (Annexin V+/PI+). (*C*) Percentages of apoptotic cells. *Open bars* and *gray bars* show (R2/(R1 + R2 + R3)) and ((R2 + R3)/(R1 + R2 + R3)), respectively. Data shown are the mean ± SD of three independent experiments. *P* values were obtained using Dunnett's test. $*P < 0.01$. $**P < 0.05$.

Statistical Analysis

Dunnett's test was performed to determine statistical differences between each experimental group and the control group.

RESULTS

Establishment of Six MT-2Rsts Cells (CA1–3 and CB1–3)

As we reported previously (17, 18), we initially established CB1 cells. Therefore, the other five independent MT-2Rsts cells continuously exposed to CA or CB were established according to a similar method. As shown in Figure 2A, the growth of MT-2Org cells was inhibited in a dose-dependent manner by culturing with CA or CB. This growth inhibition was confirmed by the appearance of apoptosis, as reported previously (17, 18), and as demonstrated in Figures 2B and 2C. All cultures for the establishment of MT-2Rsts cells were initiated in the presence of 2 μg/cm² CA or CB, at which stage the proliferation of MT-2Org cells was inhibited by half (Figure 2A). After 8–12 months of culture with CA or CB, MT-2Rsts cells began to exhibit a reduced apoptotic fraction when these cells were cultured with various concentrations of CA or CB (Figure 2C). Thus, we determined that six MT-2Rsts cells representing the acquisition of resistance to asbestos-induced apoptosis had been established, and these cells were designated CA1–3 and CB1–3. In this study, to identify those genes involved in the reduction of antitumor immune functions induced by exposure to asbestos, we used these six MT-2Rsts cells for DNA microarray analysis.

Gene Expression in MT-2Rsts Cells Altered by Chronic Exposure to Chrysotile

To examine alterations in gene expression by chronic exposure to chrysotile, DNA microarray analysis was performed with MT-2Org and MT-2Rsts cells. As listed in Table 1, the expression of 139 genes was altered (84 were up-regulated, and 55 were down-regulated) significantly (greater than twofold changes), and most were categorized in cellular components, biological processes, and molecular function groups by gene ontology analysis (data not shown). As shown in Figure 3, clustering analysis using these 139 genes revealed that the gene expression pattern was obviously different between MT-2Org and MT-2Rsts cells, and gene expression patterns were similar among all six MT-2Rsts cells, although small differences were evident. These results indicated that the changes in gene expression of MT-2Org cells are similarly induced by chronic exposure to CA and CB, suggesting that MT-2Rsts cells would be useful in further analyzing the immunologic effects of chrysotile asbestos.

Pathway and Network Analysis Using the MetaCore System

In an effort to identify genes related to the suppression of antitumor immunity among the 139 genes identified, expression

TABLE 1. GENES WITH AT LEAST A TWOFOLD DIFFERENCE BETWEEN MT-2ORG AND MT-2RSTS AT $P < 0.05$

Description	Genes	Accession Numbers
Down-regulated in MT-2Rsts compared with MT-2Org		
Solute carrier family 15, member 3	*SLC15A3*	NM_016582
Stomatin	*STOM*	NM_198194
Nedd4 family interacting protein 1	*NDFIP1*	NM_030571
Creatine kinase, brain	*CKB*	NM_001823
Tripartite motif–containing 22	*TRIM22*	NM_006074
Apolipoprotein C–I	*APOC1*	NM_001645
Forkhead box O1	*FOXO1*	NM_002015
Chromosome 1 open reading frame 218	*C1orf218*	NM_019049
Solute carrier family 6 (neurotransmitter transporter, taurine), member 6	*SLC6A6*	AB209172
c-mer proto-oncogene tyrosine kinase	*MERTK*	NM_006343
Interferon regulatory factor 9	*IRF9*	NM_006084
Asparaginase-like–1	*ASRGL1*	BC021295
Ankyrin repeat and death domain–containing 1A	*ANKDD1A*	AK075298
Protein phosphatase 1, regulatory (inhibitor) subunit 16B	*PPP1R16B*	NM_015568
Secreted protein, acidic, cysteine-rich (osteonectin)	*SPARC*	NM_003118
Chromosome 5 open reading frame 30	*C5orf30*	NM_033211
Stromal antigen 3	*STAG3*	NM_012447
Apolipoprotein L, 6	*APOL6*	AK074645
Chromosome 5 open reading frame 40	*C5orf40*	NM_001001343
CXXC finger 5	*CXXC5*	NM_016463
RNase, RNase A family, 1 (pancreatic)	*RNASE1*	NM_198232
Eukaryotic translation initiation factor 4 γ, 3	*EIF4G3*	NM_003760
Leukemia inhibitory factor (cholinergic differentiation factor)	*LIF*	NM_002309
Radial spoke head 1 homologue (*Chlamydomonas*)	*RSPH1*	NM_080860
cDNA DKFZp564D0472	*TOMM22*	AL110179
X-linked Kx blood group (McLeod syndrome)	*XK*	NM_021083
Caspase 2 and receptor-interacting serine-threonine kinase 1 domain containing adaptor with death domain	*CRADD*	NM_003805
Chromosome 13 open reading frame 15	*C13orf15*	NM_014059
An acute myeloid leukemia protein (486 bp)	*aml1*	X90980
Serine propidium iodide Kazal type 5–like 3	*SPINK5L3*	AK001520
Transmembrane and coiled-coil domain family 2	*TMCC2*	NM_014858
Von Willebrand factor	*VWF*	NM_000552
Acid phosphatase–like 2	*ACPL2*	NM_152282
Interferon-induced protein with tetratricopeptide repeats 2	*IFIT2*	NM_001547
Chemokine (C-C motif) ligand 4	*CCL4*	NM_002984
Napsin A aspartic peptidase	*NAPSA*	NM_004851
Hypothetical gene supported by AK125122	*FLJ13137*	AK125122
G-protein–coupled receptor 56	*GPR56*	NM_201525
Zinc finger CCCH-type containing 12D	*ZC3H12D*	AK127932
Similar to ciliary rootlet coiled-coil, rootletin	*LOC285188*	XM_209505
Membrane-associated ring finger (C3HC4) 3	*MARCH3*	NM_178450
Sequence 155 from Patent WO0220754	*AX721195*	AX721195
Protein kinase C, β1	*PRKCB1*	NM_002738
Interleukin 28A (interferon, λ2)	*IL28A*	NM_172138
DKFZP564O0823 protein	*DKFZP564O0823*	NM_015393
AF032119 hCASK {*Homo sapiens*;}, partial (13%)	*THC2443571*	THC2443571
Chromosome 19 open reading frame 38	*C19orf38*	XM_172995
Chemokine (C-X-C motif) receptor 3	*CXCR3*	NM_001504
Sema domain, transmembrane domain (TM), and cytoplasmic domain, (semaphorin) 6A	*SEMA6A*	NM_020796
Deleted in esophageal cancer 1	*DEC1*	BC030567
Phosphatidylinositol 3,4,5-trisphosphate–dependent Ras-related C3 botulinum toxin substrate 1 exchanger 1	*PREX1*	NM_020820
Breast cancer antiestrogen resistance 3	*BCAR3*	NM_003567
Myeloid cell nuclear differentiation antigen	*MNDA*	NM_002432
Integrin, β7	*ITGB7*	NM_000889
Hypothetical protein LOC199725	*LOC199725*	AK023628
Up-regulated in MT-2Rsts compared with MT-2Org		
Hypothetical LOC728701	*LOC728701*	BC011779
Mediator complex subunit 19	*MED19*	NM_153450
Norrie disease (pseudoglioma)	*NDP*	NM_000266
Protein phosphatase 6, regulatory subunit 1	*SAPS1*	NM_014931
Phosphoprotein enriched in astrocytes 15	*PEA15*	NM_003768
cDNA clone: 6386006	*BU587941*	BU587941
Hypothetical protein FLJ11348	*AK002210*	AK002210
FLJ00217 protein	*AK074144*	AK074144
cDNA clone: 5451514	*BM045853*	BM045853
F-box protein 2	*FBXO2*	NM_012168
cDNA clone: 1917130	*SSR2*	AI344752
AF4/FMR2 family, member 3	*AFF3*	NM_002285
Breakpoint cluster region	*BCR*	NM_021574
cDNA clone CS0DM002YA18	*CR608907*	CR608907

(*Continued*)