Table 2. Components of tumor cells observed in pathological and cytological specimens

Case	Methods	Component of tumor cells in pathological specimens				Component of tumor cells in cytological specimens			
		spindle cells	giant cells	AD	LA	spindle cells	giant cells	AD	LA
1	TI	present	present	present	present	X	present	present	present
2	TI	present	present	present	present	X	present	present	present
4	Cr	present		present		X		present	
7	TI	present	present	present		present	present	X	
8	TI	present		present		present		present	
9	Br	present	present			present	present		
10	Br	present	present	present		present	present	X	
12	Br		present				present		

AD = Adenocarcinoma; Br = brushing; Cr = curettage; LA = large-cell carcinoma; TI = touch imprint; X = absent.

Table 3. Summary of cytological features of sarcomatoid component of pleomorphic carcinoma and giant cell carcinoma

Background	necrosis	present	2/8 (25%)
	type of cells	lymphocytes, neutrophils	7/8 (88%)
Amount of tumor cells		large	5/8 (63%)
Clusters	size	small	4/8 (50%)
	nuclear overlapping	not obvious	8/8 (100%)
	arrangement	2-dimensional	6/8 (75%)
Cells	shape	spindle, pleomorphic, variable	8/8 (100%)
	size	large	7/8 (88%)
	variability in size	5 times or more	4/8 (50%)
	pleomorphism	marked	7/8 (88%)
	margin	demarcated	5/8 (63%)
	cell adhesion	poor	7/8 (88%)
Cytoplasm	color	green/blue	8/8 (100%)
	nature	translucent or vacuole, thick	8/8 (100%)
Nucleocytoplasmic ratio		increased	7/8 (88%)
Nucleus	location	centrifugal	5/8 (63%)
	shape	irregular, oval	8/8 (100%)
	size	5 times of lymphocyte or more	4/8 (50%)
	variability in size	5 times or more	4/8 (50%)
	nuclear membrane	thin, slightly thick	8/8 (100%)
	hyperchromatism	present	8/8 (100%)
	chromatin texture	coarsely granular	7/8 (88%)
	distribution of chromatin	uneven	5/8 (63%)
Nucleolus	shape	round	7/8 (88%)
	size	medium	4/8 (50%)
	number	single	7/8 (88%)

ily from the glass slide during the staining process. On the other hand, the adenocarcinoma component was not observed in cytological specimens from cases 7 and 10. Pathological specimens from case 7 revealed that the adenocarcinoma component was a solid adenocarcinoma with mucin that had bizarre nuclei. Giant cells and spindle cells were marked in this case, and mucin in the cytoplasm was difficult to discern in cytological specimens. Pathological specimens in case 10 revealed that the adenocarcioma component comprised a small percentage of the tumor. This may be the reason why the adenocarcinoma component did not appear in cytological specimens from case 10.

There have been only a few cytological studies of GC [12, 13]. GC cytology specimens have exhibited numerous mono- or multinucleate giant cells with significant pleomorphism in size and shape. The cytoplasm of the giant cells is abundant, eosinophilic, microvesicular, and well demarcated. Most of the tumor cells have round, oval or irregularly shaped macronuclei with coarse, granular chromatin and large, prominent nucleoli. Their cytoplasm is occasionally infiltrated with neutrophils. The tumor cells usually occurred singly, and the background contains tumor diathesis with numerous polymorphonuclear leukocytes [12, 13].

Giant cells are one component of PC or GC [1]. However, there is no clear definition of how large these giant cells are. Fishback et al. reported that the single large pleomorphic nucleus of GC measured greater than the diameter of four small resting lymphocytes [14]. Guillan and Zelman reported that the giant cells varied in size from 50 to 120 μm in diameter [15], and Hellstrom and Fisher reported that the giant cells measured from 80 to 100 μm [16]. This vague definition of giant cells causes confusion among pathologists. In our study, the mononucleated giant tumor cells had large nuclei, the size of which was greater than the diameter of 5 resting lymphocytes in half of the cases. There was variability in the size of the nuclei, and the size of the largest nucleus was 5 times greater than that of the smallest nucleus of the tumor cells in half of the cases.

It has been reported that the prognosis for PC patients is worse than that for patients with other NSCLC in surgically resected cases [2–4]. In contrast, Nakajima et al. reported similar clinical behaviors and prognosis between PC and other NSCLC [7]. Pelosi et al. reported that stage I PC behaves more aggressively than ordinary NSCLC; however, the differences were not statistically significant for both overall and disease-free survival curves [6]. Yamamoto et al. reported that the overall 5-year survival rate of surgically resected PC was 80.0% and the disease-free survival rate was 63.3%, which were both far better than rates reported elsewhere [5].

PCs have been reported to be highly metastatic. In our study, some patients had a recurrence even though the tumor was stage I or II; the patient with a stage IA tumor had a recurrence in the lung 31 months after surgery (case 7), and 1 patient with a stage IIB tumor had a brain metastasis 21 months after the surgery (case 8). In contrast, some patients had a favorable prognosis. One patient with a stage IIB tumor is alive 5 years after surgery without any adjuvant therapy (case 9). One patient with a stage IIIA tumor underwent thoracic radiotherapy and chemotherapy (CDDP + GEM) and is alive without recurrence 40 months after the surgery (case 10). One patient (case 12) had an enlarged right adrenal gland the size of which was 15 mm, and its size had become 53 mm six months later. It was surgically removed and confirmed to be metastasis from a pulmonary PC. The patient is alive 23 months after the surgery of the lung tumor.

The contradictory prognoses of PC in different studies may be due to the different criteria of PC used among pathologists. Because ours is a multidisciplinary study, we selected cases that underwent pathological review by pathologists specialized for lung cancers. We did not include patients treated with chemotherapy or radiotherapy before the surgery, because these therapies may modify the tumor cells and enlarge them even further. The present study, by analyzing carefully selected PC or GC cases, suggests that some patients with PC or GC can expect long survival after resection of the tumor with adjuvant therapy. We could not address the pathological or molecular differences between long-survivors and short-survivors suffering from PC or GC. Further studies are needed to clarify the mechanisms of different biological behaviors among this type of lung carcinoma.

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診断病理の精度管理

分子病理診断の標準化と精度管理

畑中　豊　　久保田佳奈子　　松野吉宏

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特集　診断病理の精度管理

分子病理診断の標準化と精度管理

畑中　豊*　久保田佳奈子*
松野吉宏*

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．

*北海道大学病院病理部/北海道大学大学院医学研究科分子病理診断学

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

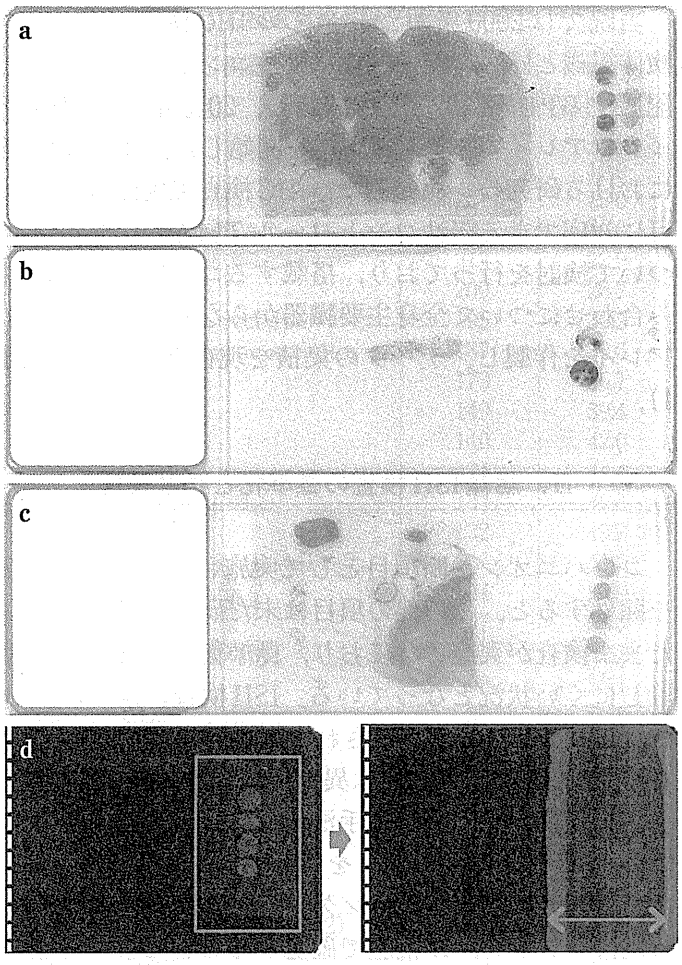

図1　内部精度管理用組織マイクロアレイ（TMA）
a, b：現在試験的に作製・使用している補助診断項目用 TMA．上位項目用は扁桃，虫垂，肝臓，膵臓を含めたΦ2 mmの8コア TMAで検討を進めている（a）．また使用頻度の高い悪性リンパ腫マーカー用には扁桃と虫垂からなるΦ3 mmの2コア TMAを使用している（b）．これらの TMAは1週間分に相当する枚数をまとめて薄切し，スライドグラスに TMAを事前に載せた状態で保管するようにし負担軽減を図っている．
c, d：コンパニオン診断用は HER2，ER，PgR，EGFRの4項目をカバーした4コア TMA（乳癌3コアと大腸癌1コア）を作製し使用している（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

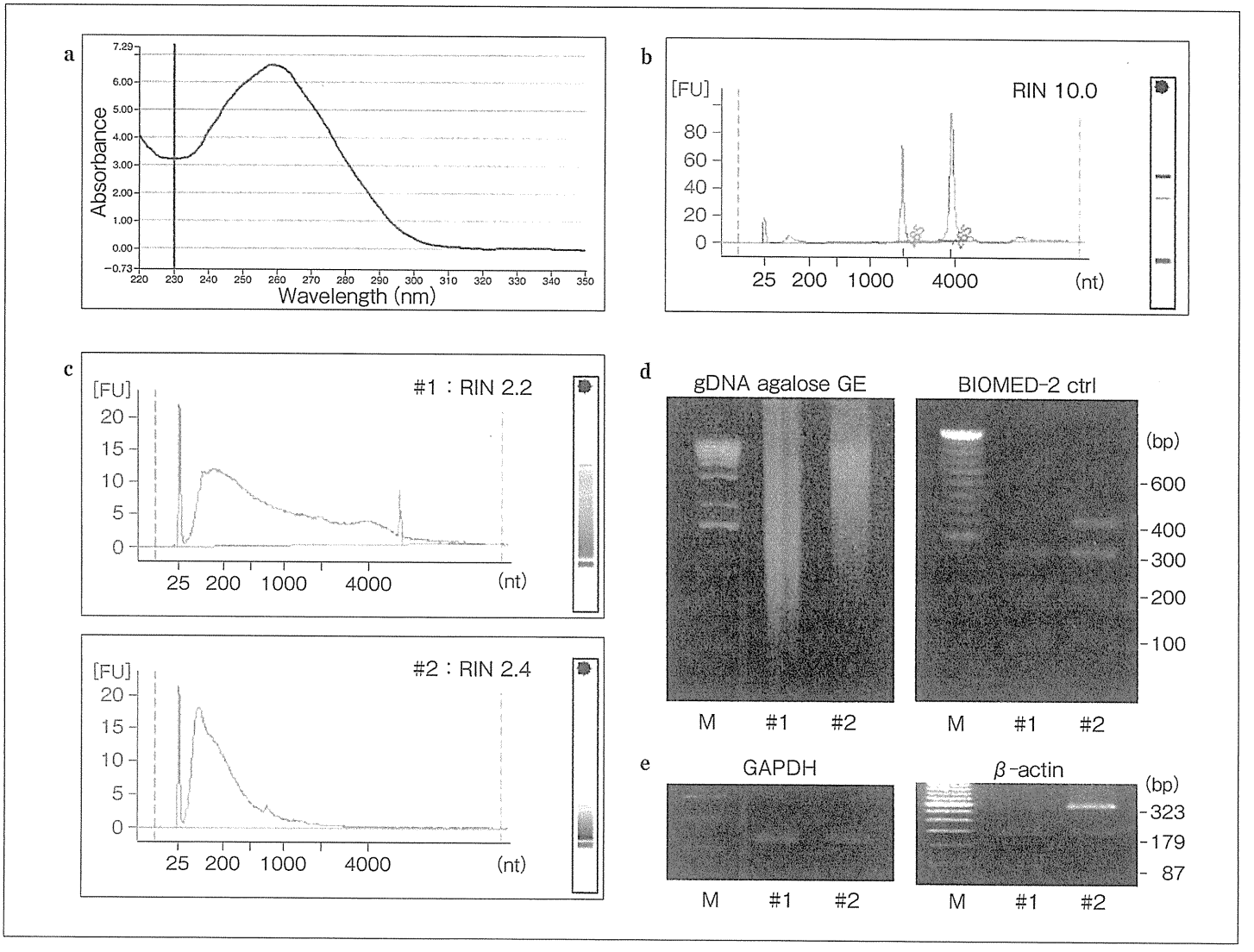

図2　核酸の品質評価　a：吸光度A260/A280比による核酸品質確認．カラム法を用いたFFPE検体からのDNA抽出液では比はおよそ1.9～2.1となる（JCCLS・遺伝子関連検査標準化専門委員会調査）．抽出に問題があった場合はA230の値が高値を示すためA260/A230比が低くなる場合があるため，この比の確認も必要である．b：マイクロチップ型電気泳動装置を用いたRIN値によるRNA品質確認．培養細胞などから良好にRNA抽出ができた場合などは高いRIN値を示す．c：RIN値を用いたFFPE検体等の品質確認．通常のFFPE検体ではしばしばRIN値は低値を示す（上段，#1）．核酸安定性が高いとさ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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Original Article

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

Shuko Nojiri[1], Kenichi Gemba[2], Keisuke Aoe[3], Katsuya Kato[4], Takuhiro Yamaguchi[5], Tsugumichi Sato[6], Kiyoshi Kubota[1,*] and Takumi Kishimoto[2]

[1]Department of Pharmacoepidemiology, Faculty of Medicine, University of Tokyo, Tokyo, [2]Department of Respiratory Medicine, Japan Labor Health and Welfare Organization Okayama Rosai Hospital, Okayama, [3]Department of Respiratory Medicine, National Hospital Organization Yamaguchi—Ube Medical Center, Yamaguchi, [4]Department of Radiology, Okayama University Hospital, Okayama, [5]Department of Clinical Trial Data Management, Graduate School of Medicine, University of Tokyo and [6]NPO (Nonprofit Organization) Drug Safety Research Unit Japan, Tokyo, Japan

*For reprints and all correspondence: Kiyoshi Kubota, Department of Pharmacoepidemiology, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan. E-mail: kubotape-tky@umin.net

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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

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.

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 developed pleural effusion during the observation period after diagnosis. Treatments given to 314 patients with MPM

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)

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 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

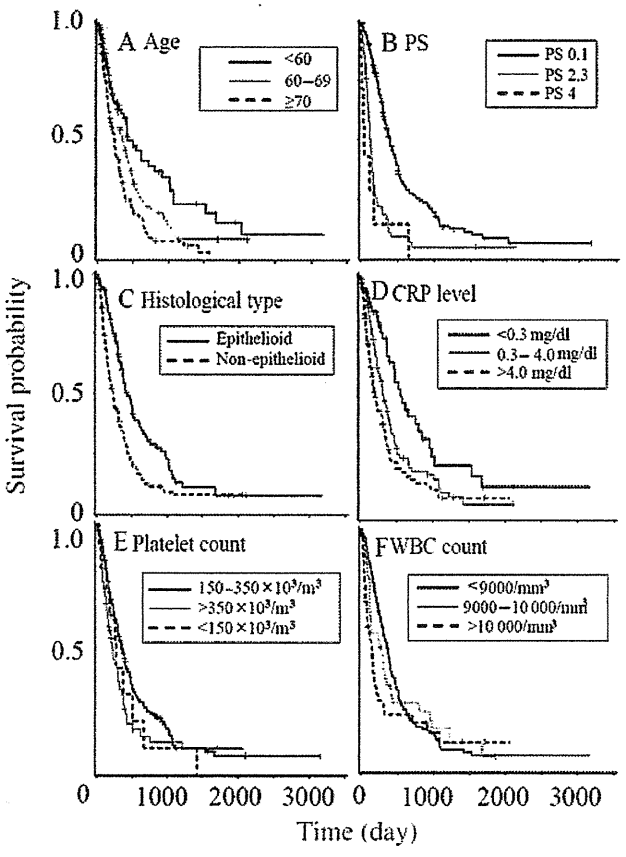

Figure 1. Kaplan–Meier survival curves for prognostic factors (log-rank test). (A) Three age groups ($P < 0.001$), (B) three groups for performance status ($P < 0.001$), (C) two groups for histological type ($P = 0.001$), (D) three groups for C-reactive protein (CRP) level ($P < 0.001$), (E) three groups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

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 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

Table 3. Association between clinicopathologic variables and survival

	Crude analysis						Multivariate analysis[a]	
Prognostic factors	*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 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 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

Megumi Maeda[1], Yasumitsu Nishimura[1], Hiroaki Hayashi[1], Naoko Kumagai[1], Ying Chen[1], Shuko Murakami[1], Yoshie Miura[3], Jun-ichi Hiratsuka[2], Takumi Kishimoto[4], and Takemi Otsuki[1]

[1]Department of Hygiene, and [2]Department of Radiation Oncology, Kawasaki Medical School, Okayama, Japan; [3]Division of Molecular and Clinical Genetics, Department of Molecular Genetics, Medical Institute of Bioregulation, Kyushu University, Fukuoka, Japan; and [4]Department at Okayama Rosai Hospital, Okayama, Japan

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

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).

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.

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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Correspondence and requests for reprints should be addressed to Megumi Maeda, Ph.D., Department of Hygiene, Kawasaki Medical School, 577 Matsushima, Kurashiki, Okayama 7010192, Japan. E-mail: mmaeda@med.kawasaki-m.ac.jp

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