特集　胸水貯留疾患のトピックス

胸膜中皮腫および良性石綿胸水

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要　旨

石綿曝露によって発生する胸膜病変である胸膜中皮腫と良性石綿胸水は慎重な鑑別診断が必要である。胸水はともに血性で，細胞成分ではリンパ球有意となる症例が多い。細胞診での鑑別診断率が低いので，胸部単純 X 線画像上腫瘍性胸膜肥厚がない症例には組織学的な確定が必要となる。その際には，胸腔鏡下胸膜生検が有用である。たとえ生検結果で悪性所見が得られなくても，胸膜中皮腫の可能性を否定せず，慎重な経過観察が必要である。

Key words：胸膜中皮腫，良性石綿胸水，ヒアルロン酸，可溶性メソテリン，胸腔鏡下胸膜生検/pleural mesothelioma, benign asbestos pleurisy, hyaluronic acid, soluble mesothelin-related peptides, thoracoscopic pleural biopsy

1 はじめに

胸膜中皮腫の発生が兵庫県尼崎のクボタ周辺の近隣曝露によって発生していることが社会問題化して，中皮腫に対しては労災補償されない症例に対しても救済されることになった。一方，中皮腫と同様に石綿曝露によって発症する良性石綿胸水は同地区からの報告は少ない。そこで，本稿では，胸膜中皮腫と良性石綿胸水の鑑別診断について解説する。

2 胸膜中皮腫

約 80%が職業性石綿曝露によって発生し，家庭内曝露や近隣曝露でも発生しうるが，低濃度の環境曝露では極めて発症リスクは低い。発生までの潜伏期間は初回曝露から平均 40 年以上を要することが報告されている。

1）臨床検査データによる鑑別診断

現在，日本には中皮腫診断あるいは臨床経過の指標となる血清マーカーはない。また，胸水中ヒアルロン酸は，100,000 ng/ml 以上を示す症例では診断価値はあるが，陽性率は約 40%である。

a . Soluble mesothelin-related peptides（SMRP）

Mesothelin は正常中皮細胞の膜表面に存在する 40 KD の糖蛋白であるが，その可溶性蛋白のモノクローナル抗体である SMRP[1]は中皮腫患者で 87%が陽性を示し，中皮腫以外

Pleural Mesothelioma and Benign Asbestos Pleurisy
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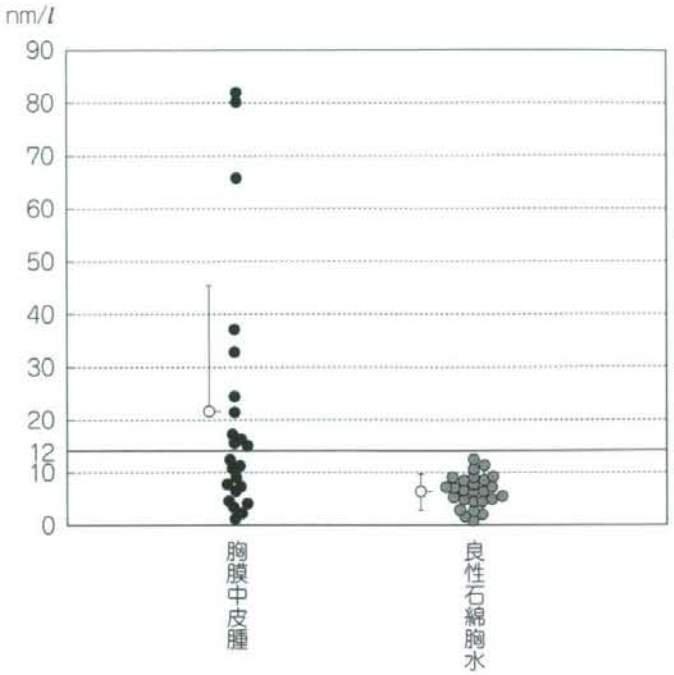


図 1　胸膜中皮腫症例（23 例）と良性石綿胸水症例（26 例）における胸水中 SMRP の比較

胸膜中皮腫症例では 20.96±23.99 nM/*l*，良性石綿胸水症例では 6.36±2.83 nM/*l* であった。良性石綿胸水では 12.0 nM/*l* を超える症例はなかった。

の胸膜疾患，胸膜以外の肺悪性腫瘍，胸膜以外の炎症性呼吸器疾患での陽性率は低く，中皮腫での特異性が高い。Scherpereel ら[2]は中皮腫，転移性胸膜炎，良性石綿胸水を対象として，血清 SMRP を測定し，中皮腫では平均 2.05 nM/*l* で，転移性胸膜炎や良性石綿胸水に比べて高値を示し，転移性胸膜炎では 1.85 nM/*l*，良性石綿胸水では 0.93 nM/*l* をカットオフ値にすると，感度および特異度がよいと報告している。しかし，組織型別では上皮型では高値を示すが，肉腫型やⅡ相型の一部では高値を示さず，診断的価値が低く，卵巣癌や膵臓癌でも高値となり，特異性に乏しいとする報告[3]もある。

一方，胸水中 SMRP については，カットオフ値を 10.4 nM/*l* にすると，転移性胸膜炎あるいは良性石綿胸水との鑑別の指標となると報告[4]されている。われわれも胸水中 SMRP を測定したが，カットオフ値を 12.0 nM/*l* にすると，良性石綿胸水 26 例ではすべて陰性であるが，中皮腫では 23 例中 11 例（47.8%）が陽性を示し鑑別に有用であると思われた（図 1）。

b．オステオポンチン

Pass ら[5]は血清オステオポンチンが胸膜中皮腫の場合には 133 ng/ml と石綿曝露者あるいは石綿曝露のない健常者より有意に高く，48.3 ng/ml をカットオフ値にすると感度が

77.6%，特異度が 85.5%であったと報告している。しかし，卵巣癌，大腸癌，乳癌などの多数の癌腫に陽性を示すことから転移性胸膜炎との鑑別にはならない[3]。Hiraki ら[6]は胸膜中皮腫 7 例の胸水中オステオポンチンが 22,692 ng/ml と高値を示し，11,436 ng/ml をカットオフ値とした場合に非悪性疾患の胸水に比較して，感度が 100.0%，特異度が 77.8%であり，鑑別診断に有用な検査方法であると報告している。しかし，反応中皮細胞にも発現しており，良性石綿胸水との鑑別にはならないとの報告もある[7]。

c．ヒアルロン酸

胸水中ヒアルロン酸の場合カットオフ値を 100,000 ng/ml とした場合，三浦[8]は感度 62%，特異度 98%と診断価値が高いと報告している。著者[9]の検査結果では，胸膜中皮腫であると診断した 50 例中 19 例（38.0%）が 100,000 ng/ml 以上であった。一方，癌性胸膜炎では 30 例中 1 例（3.3%）のみであった。その他良性石綿胸水 20 例，非悪性の胸膜炎 16 例ではすべて 100,000 ng/ml 以下であった。

d．CEA

胸水中あるいは血清 CEA は上昇しないのが悪性中皮腫の特徴であり，また，CEA のカットオフ値を 5.0 ng/ml とした場合は 3.1%の感度で陰性になると報告されており，CEA が 10 ng/ml 以上を呈する場合には腺癌を考慮すべきである。

2）画像診断

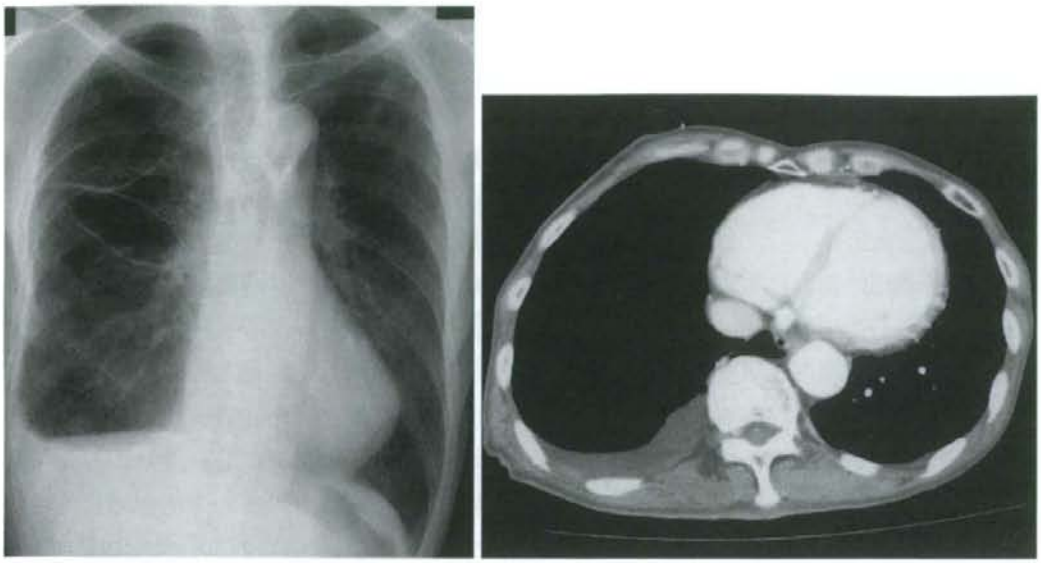
胸膜中皮腫の場合には，約 80%の症例では胸水貯留を伴う。典型例では胸部 CT 上，胸腔内に突出する腫瘤陰影，あるいは厚さ 1 cm 以上のびまん性胸膜肥厚像（pleural rind）を呈する。しかし，早期病変の場合には，腫瘍性胸膜肥厚所見を呈さないこともあるため，原因不明の胸膜炎あるいは結核性胸膜炎と診断され，経過観察あるいは抗結核薬の治療をされている症例もある。造影 CT は葉間胸膜の造影効果と腫瘍の横隔膜，縦隔の軟部組織，胸壁への浸潤の有無の検索に有用である。一方 MRI では，腫瘍は T1 強調像で低信号，T2 強調像で中等度信号を示す。早期病変の場合には葉間裂の限局性肥厚あるいは造影効果が認められる。また，造影 CT に比較して胸壁，横隔膜への浸潤の詳細な検出効果に優れている。最も鑑別を要する肺癌との鑑別には，肺野に原発となる腫瘍性病変があるかどうかについて詳細に検討しておく必要がある。

3）病理診断

胸膜中皮腫は上皮型，肉腫型，II 相型の 3 型に大別される。線維形成型（desmoplastic type）は肉腫型の亜型と分類されるが，50%程度の組織で，著明な膠原線維の増生が存在する。そのため，良性石綿胸水（線維性胸膜炎）との鑑別が重要である。

組織型別頻度では，上皮型が最も多く，肉腫型が最も少ないが，腫瘍組織の顕微鏡下で観察する範囲が広くなるにつれて，II 相型の頻度が増加する。

上皮型胸膜中皮腫と鑑別が必要な腫瘍として，肺腺癌が問題となるため，免疫組織化学的な手法を用いた方法で染色性を検討し，総合的な評価の下で鑑別を行うことが推奨されている。陽性マーカーとしてはカルレチニン，サイトケラチン 5/6，WT-1，D2-40陰性マー



(a) 胸部単純 X 線写真正面像
右胸水とともに気胸像を認めるが，腫瘍性胸膜肥厚像は認められない。

(b) 胸部 CT 縦隔条件
右胸水と背側および心膜側の胸膜肥厚を認めるが，腫瘍性肥厚であるとは診断できない。

図 2　胸膜中皮腫早期病期症例
胸腔鏡下胸膜生検で，上皮型胸膜中皮腫と診断された。

カーとして，CEA，TTF-1 を検査しておく。また，肉腫型中皮腫では低分子ケラチンである AE1/AE3 あるいは CAM5.2 が有用であり，真の肉腫で陽性になる smooth muscle actin（SMA）やデスミン，s-100 などが陰性であることを確認することが必要である。II 相型悪性中皮腫では，少なくともいずれかの成分が 10%以上存在することを定義とする。鑑別すべき疾患としては，滑膜肉腫，肺癌肉腫，肺芽腫などII相性の増殖を示す腫瘍が問題となるが，免疫組織化学や遺伝子診断が鑑別に有用である。

4）鑑別診断

胸膜中皮腫の鑑別診断で最も重要な疾患は原発性肺癌である。肺癌のうちでも画像上，pseudomesothliomatous adenocarcinoma は鑑別できないので，病理組織学的な診断が必要である。一方，pleomorphic 型肺癌（肺癌肉腫）との鑑別は病理診断が困難なことも多いので，画像上の主病変部位が肺実質にあるのか胸膜にあるのかが重要である。しかし，石綿曝露歴のある症例で腫瘍性胸膜肥厚が存在しない症例では，良性石綿胸水との鑑別が最も重要である。すなわち，胸膜中皮腫早期病変（図 2）あるいは desmoplastic 型中皮腫が問題となる。病理組織学的に強い炎症所見を伴う胸膜炎の場合には，反応性中皮細胞の増殖が強く，腫瘍のようにみえることがある。また，desmoplastic 型中皮腫は病理組織上，炎症性変化に類似することがある。この際には，免疫組織学的に EMA，デスミン，p-53 の所見が有用である。EMA，p-53 が陽性でデスミンが陰性の場合には中皮腫である可能性

が高い[10]。胸水貯留があり炎症が強い壁側胸膜側に細胞成分が多く，肺側に近いほど浸潤細胞数が少なくなるという zonation を認める際には炎症である可能性が高く，鑑別点になる。

3 良性石綿胸水

良性石綿胸水は石綿胸膜炎ともいい，通常は片側で少量の胸水を認める疾患であり，1964 年に Eisenstadt[11]が初めて報告した。診断基準としては Epler ら[12]の 4 項目が有名である。すなわち，① 石綿曝露歴があること，② 胸部単純 X 線写真あるいは胸水穿刺で胸水の存在が確認されること，③ 石綿曝露以外に胸水の原因がないこと，④ 胸水確認後 3 年以内に悪性腫瘍を認めないことの 4 項目を満たす場合であり，良性とは悪性でないという意味で，臨床経過は必ずしも良性であるということではない。Hillerdal ら[13]は胸部 CT 等の画像診断で詳細な臨床経過を観察した場合には発症後 1 年の経過観察でよいと述べているが，現在までに新たな診断基準は提唱されていない。

発生機序は不明であるが，石綿の機械的刺激や胸膜の線維化による壁側胸膜のリンパ排出孔の閉塞説と石綿繊維のアジュバント効果による自己免疫機序などが推測されている。

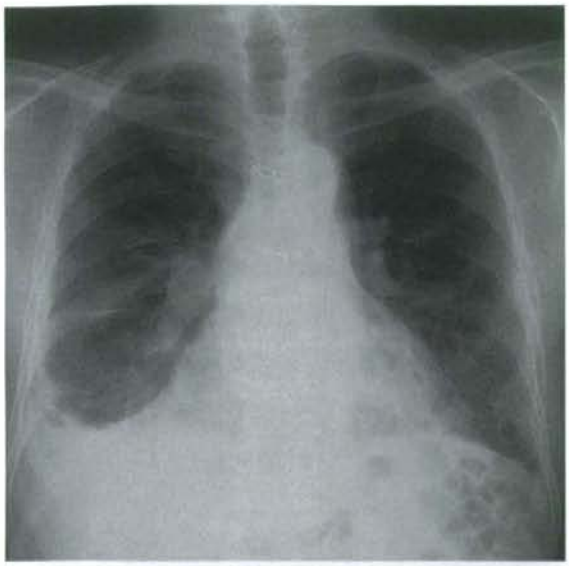
1）臨床所見

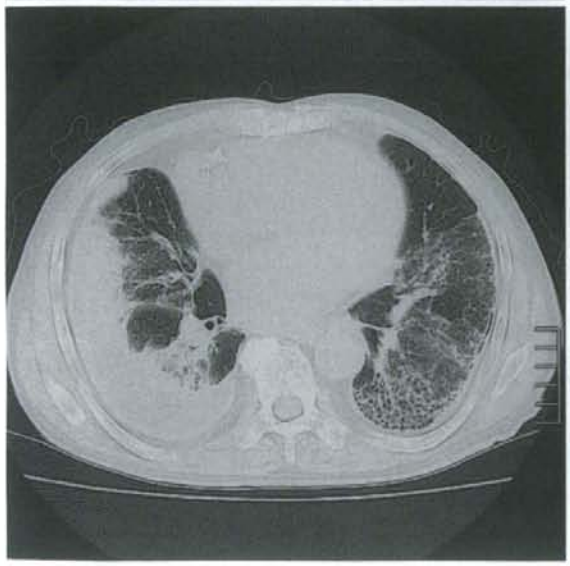
性別では圧倒的に男性が多い。発生年齢では石綿高濃度曝露が示唆される石綿工場での症例は平均 51.5 歳と比較的若い症例が多く[14]，造船業者や建設業者のように石綿中等度曝露を受けた症例では平均 66 歳と比較的高齢者が多い[15]。この原因としては石綿曝露濃度によって発生年齢が異なり，高濃度曝露では発生までの潜伏期間が短いことが示唆されている。臨床症状としては，半数は自覚症状がなく，検診等で偶然発見される。自覚症状としては胸痛，発熱，咳嗽，呼吸困難等が挙げられるが，これらは胸膜中皮腫の初発症状と同様である。自覚症状の頻度では労作時呼吸困難が最も高く，胸痛，発熱の順である[15]。また，臨床検査所見として，胸水の量は通常わずかであるが，500 ml 以上の大量胸水を約 10％に認める。胸水の性状は滲出液で，半数以上が血性あるいは淡血性である。細胞成分ではリンパ球優位あるいは好酸球増多であり，リウマチ性胸膜炎あるいは結核性胸膜炎等との鑑別が必要である。胸水中のヒアルロン酸値は 100,000 ng/ml 以下の場合が大半である。また，adenine deaminase（ADA），CEA も高値を示すことはない。

2）診断

除外診断であるので，胸水を来すあらゆる疾患を除外する必要がある。すなわち，リウマチ，SLE 等の膠原病，結核性あるいは癌性胸膜炎等を否定しなければならない。胸水の量はごくわずかな貯留から 500 ml を超えて穿刺を繰り返さなければならないほど大量貯留を認める症例もあるので，胸水の貯留量からの鑑別はできない[16]。胸部画像所見では，胸水貯留のみで軽度胸膜肥厚を伴う症例もあるため，胸膜中皮腫早期病変との鑑別が必要であるため，胸腔鏡検査が必須となる（図 3）。

胸腔鏡による肉眼所見で壁側胸膜は，表面が平滑な胸膜肥厚の頻度が高く，次いでややびまん性に発赤色調を呈する症例が多く，胸





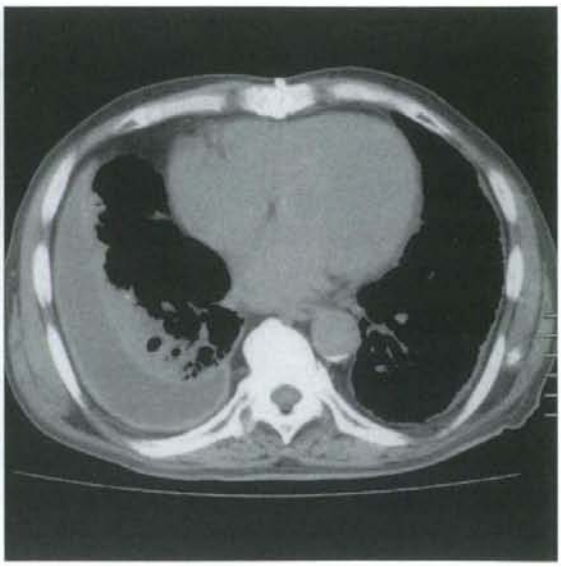


図 3　石綿肺に良性石綿胸水を合併した症例
(a) 胸部単純 X 線写真正面像
PR2 型の石綿肺と右胸水を認める。
(b) 胸部 CT 肺野条件
左肺野には小さな蜂巣肺を伴う肺の線維化所見を認める。
(c) 胸部 CT 縦隔条件
右胸水貯留と両側胸膜肥厚像が認められる。右胸膜肥厚は平滑で肥厚の程度も軽く，腫瘍性変化であるとは診断できない。

a
b | c

膜中皮腫の症例のような隆起性病変は認められない。難治性胸水で石綿曝露が明らかな症例では積極的に胸腔鏡を行い，上述の病理学的な検査によっても，胸膜中皮腫ではないと診断した場合には良性石綿胸水として，慎重な経過観察をする。そして，中皮腫の発生のみならずびまん性胸膜肥厚への移行についても経過観察することが求められる。

3）治療と臨床経過

無治療で約半数が自然軽快する[12]。胸水の持続期間は 1〜10 カ月である。最近のわれわれ[17]の調査結果によれば，良性石綿胸水 45 症例のうち 33 例（73%）では，胸水ドレナージのみで胸水がコントロールされていた。ドレナージの回数は 1 回のみから多い症例では 5 回のドレナージが行われ，胸水のドレ

ナージ量は 200～3,500 ml であったが，うち 6 例では 1,000 ml 以上の大量の胸水をドレナージしていた。一方，副腎ステロイドで奏効する症例もある。上述の 45 例中 8 例ではプレドニソロン 20 mg が投与されており，5 例では奏効していた[17]。

しかし，治療効果がない場合には胸水が残存する。その際には胸膜中皮腫との鑑別が重要である。特に画像上胸膜肥厚を認める症例にはその程度が軽微であっても，早期に胸腔鏡による壁側胸膜の観察とともに生検が必要である。良性石綿胸水 22 例を 23 年にわたって経過観察したが，悪性中皮腫を 1 例も認めなかったという報告がある[16]。一方，良性石綿胸水として診断されて経過観察中，3 年以上を経過して悪性胸膜中皮腫を発生したとの報告[18]もある。また，良性石綿胸水 77 例を 6 年以上経過観察中にその 10 例（13%）に悪性胸膜中皮腫が発生したとの報告[19]もあり，良性であることに固執せず，中皮腫が発生する可能性を想定して経過観察を行うべきである。

一方，良性石綿胸水は再発率が高く，25～40%と報告されている[20]。短期間に 3 度胸水を繰り返したとの報告[21]もある。また，再貯留率が 5 年間で 58%であったとする報告もある。上述の 45 例中でも 12 例（26.6%）では胸水の再貯留を来しており，再貯留までの期間の中央値は 37.2 カ月であった[17]。胸水が長い間残存する症例あるいは胸水が軽快した後，約半数にびまん性胸膜肥厚を残す[22]。その際には組織学的に慢性胸膜線維症を起こし，壁側胸膜との癒着が起こり，横隔膜の運動範囲が制限されるため，肺活量の低下を来す症例がある。びまん性胸膜肥厚を合併した症例では通常肺拡散能は正常であるが，肺活量および全肺気量の低下を主体とする拘束性呼吸機能障害を来し，慢性呼吸不全等により死亡する症例も少なくない[23]。

4）石綿曝露（曝露期間と潜伏期間）と労災補償

良性石綿胸水は石綿高濃度曝露の場合には発生率が約 9%であるが，中等度曝露で 3.7%，低濃度曝露で 0.2%であり，石綿高濃度曝露の場合に発生頻度が高い[24]。一方，Epler ら[12]は間接曝露でも 1,000 例中 3.7 例は発生すると報告している。石綿曝露期間は 20 年以上の場合が多いため，石綿肺を合併することもあるが，石綿低濃度曝露を示唆する胸膜プラークを伴う症例の方が多い[13]。発症までの潜伏期間は石綿関連疾患中一番短く，曝露後 10 年以内に発生する疾患は良性石綿胸水のみであるともいわれている。しかし，実際には平均潜伏期間は 28.7～34.5 年[14][15]と報告されており，上述の調査[17]では，平均 50.5 年であった。

良性石綿胸水は労災補償の対象疾患であるため，職業性石綿曝露とともに臨床経過と治療の必要性を詳細に記載して，各労働基準監督署に申請を行う。現在，労災認定は厚生労働省に協議したうえで決定されている。

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The aberrant promoter methylation of *BMP3b* and *BMP6* in malignant pleural mesotheliomas

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Abstract. Bone morphogenetic proteins (BMPs) belong to the transforming growth factor-ß superfamily. Recent studies have showed that aberrant methylation of *BMP* genes is present in several types of human cancer. We examined the expression and methylation status of *BMP3b* and *BMP6* in malignant pleural mesotheliomas (MPMs). The expression status of *BMP3b*, and *BMP6* mRNAs were examined in seven MPM cell lines by RT-PCR assay. The expression of *BMP3b* was completely suppressed in 2 and partially suppressed in 2 of 7 cell lines and expression of *BMP6* was partially suppressed in 2 cell lines. Methylation status of *BMP3b* in cell lines was determined by methylation-specific assay to find aberrant methylation in 6 cell lines which include 4 cell lines with suppressed *BMP3b* expression. Partial methylation of *BMP6* was found in 2 cell lines whose expression was partially suppressed. Treatment with 5-Aza-dC restored *BMP3b* expression in methylated cell lines. Next, we examined the methylation status in 57 surgically resected MPM cases and found aberrant methylation of *BMP3b* in 9 (53%) out of 17 cases from Japan and 3 (8%) of 40 cases from USA and that of *BMP6* in 4 (24%) cases from Japan and 12 (30%) cases from USA, showing significant difference in frequency of *BMP3b* methylation between MPMs of the two countries (P=0.0004). Our study indicated that *BMP3b* and *BMP6* genes were suppressed by DNA methylation and methylation of *BMP3b* is significantly frequent in Japanese MPMs, suggesting its pathogenic role and the ethnic difference in MPMs.

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Key words: DNA methylation, bone morphogenetic proteins, mesothelioma

Introduction

Bone morphogenetic proteins (BMPs) are multifunctional cytokines involved in skeletal development and bone formation (1,2). They are members of the transforming growth factor-ß (TGF-ß) superfamily and critical mediators of early embryonic patterning. BMPs have been shown to inhibit cellular proliferation and be involved in organogenesis, particularly of the lung, heart and kidney. Inactivation of *BMP* genes has been implicated as important in several cancer types (3-8). Recent studies have shown that *BMP* genes including *BMP3*, *BMP3b* and *BMP6* are epigenetically inactivated in various kinds of cancers, suggesting that inactivation of BMPs may play an important role in carcino-genesis. *BMP3b* is methylated in colorectal neoplasms and non-small cell lung cancers (NSCLCs), while *BMP6* is methylated in breast cancer and NSCLCs.

Malignant pleural mesothelioma (MPM) is an aggressive tumor that develops from the pleural surface. The strong association with exposure to asbestos is known and implication of SV40 infection has been revealed recently (9,10). Regarding molecular alteration of MPM, homozygous deletion of *p16* gene and mutation of neurofibromatosis type 2 gene are well known as genetic alteration of MPM. DNA methylations of several tumor suppressor genes are also known as epigenetic alteration of MPMs (11-13).

In this study, we examined the methylation and expression status of *BMP3b* and *BMP6* genes in primary MPMs and MPM cell lines to investigate the implication of *BMP* genes in MPMs.

Materials and methods

Tumor samples and cell lines. Seven MPM cell lines (NCI-H2452, NCI-H2373, NCI-H2058, NCI-H2052, NCI-H290,

NCI-H28, HP1) were kindly gifted from Ad.F. Gazdar (Department of Pathology, University of Texas Southwestern Medical Center, Dallas, TX) or Harvery I. Pass (Department of Cardiothoracic Surgery, NYU School of Medicine). All these cell line samples were maintained in RPMI-1640 medium (Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal bovine serum. Surgically resected specimens of 57 MPMs were obtained after informed consent from each patient. Tumor tissues were obtained from 40 patients with MPMs resected at Karmanos Cancer Center, MI, 6 patients resected at Okayama Rousai Hospital (Okayama, Japan), 5 patients at NHO Sanyo National Hospital, Yamaguchi Japan, and 6 patients at Okayama University Hosipital, Okayama Japan. The study was approved by the Institutional Review Board of each institution and informed consent was obtained from the patients.

Reverse transcription polymerase chain reaction (RT-PCR). The expression status of *BMP3b* and *BMP6* mRNAs were examined in seven MPM cell lines by RT-PCR assay. Total-RNA was isolated using RNeasy mini kit™ (Qiagen, Valencia, CA). cDNA was synthesized from the RNA using the SuperScript II kit (Invitogen, Carlsbad, CA). RT-RCR amplification of *BMP3b* and *BMP6* cDNA was performed using primers designed with Primer 3. *BMP3b* primers used were: 5'-GGTGGACTTCGCAGACATCG-3' (sense) and 5'-GATGGTGGCATGGTTGGATG-3' (antisense). *BMP6* primers were: 5'-ACAGCATAACATGGGGCTTC-3' (sense) and 5'-CTCGGGGTTCATAAGGTGAA-3' (antisense). *GAPDH* was used as an internal control to confirm the success of the RT reaction (sense primer, 5'-ACAGTCCATGCCA TCACTGCC-3' and antisense primer, 5'-GCCTGCTTCA CCACCTTCTTG-3') (14). The PCR mixture contained 10X PCR buffer, dNTPs (200 μM of each), primers (1 μM of each) and 0.25 μl TaqGold. cDNA was amplified using an annealing temperature at 60˚C and two different cycles of 17 and 35 cycles. PCR product was separated by electrophoresis and visualized on an ethidium bromide-stained agarose gel.

DNA extraction and bisulfite treatment. Genomic DNA was isolated from cell lines and frozen tissues of primary tumors by homogenization by digestion with 100 μg/ml, SDS/proteinase K followed by standard phenol-chloroform (1:1) extraction, and ethanol precipitation. One microgram of extracted DNA was modified with bisulfate treatment using the EZ DNA Methylation kit™ (Zymo Research, Orange, CA) according to the manufacturer's instructions.

Methylation-specific PCR assay. Aberrant methylation of *BMP3b* and *BMP6* genes was determined by MSP assay using DNAs treated with sodium bisulfite as described previously. The primer sets for both methylated and unmethylated forms of each gene were prepared. The primers to *BMP3b* promoter region were: 5'-CGGCGTCGATATATAGGAGTC-3' (sense) and 5'-AAATCGTCCCTAACCCGACT-3' (antisense) for methylated form and 5'-TGGTGTTGATATA TAGGAGTT-3' (sense) and 5'-AAATCATCCCTAACCCA ACT-3' (antisense) for unmethylated form. The primers for *BMP6* were: 5'-GGTTTGTTGGGTAGTCGGG-3' (sense) and 5'-GCCCCTCCCCAAATCG-3' (antisense) for methylated form and 5'-TTGGGTAGTTGGGTGATTGTT-3' (sense) and 5'-ACACCCCTCCCCAAATCA-3' (antisense) for unmethylated form. Bisulfite-modefied DNAs were mixed with 10X PCR Buffer, 150 μM of deoxynucleotide triphosphates, 0.4 μM of primers and 1 unit of HotStar Taq (Qiagen). The PCR condition for methylated alleles of *BMP3b* consisted of 12 min at 95˚C, 40 cycles of 30 sec of 95˚C, 60 sec of 61˚C and 60 sec of 72˚C followed 7 min elongation. The PCR condition for unmethylated alleles of *BMP3b* was similar to that for methylated allele: 12 min at 95˚C, 40 cycles of 30 sec of 95˚C, 60 sec of 56˚C and 60 sec of 72˚C followed 7 min elongation. The PCR condition for methylated alleles of *BMP6* consisted of 12 min at 95˚C, 40 cycles of 30 sec of 95˚C, 60 sec of 62˚C and 60 sec of 72˚C, followed 7 min elongation. The PCR condition for unmethylated alleles of *BMP6* was 12 min at 95˚C, 40 cycles of 30 sec of 95˚C, 60 sec of 60˚C and 60 sec of 72˚C, followed 7 min elongation. PCR products were separated by electrophoresis and visualized on an ethidium bromide-stained 2.5% agarose gel. DNA from non-malignant lung tissue was treated with SssI methyltransferase (New England BioLabs, Beverly, MA) and then subjected to bisulfite treatment was used as a positive control for methylated alleles.

5-Aza-2'-deoxycytidine treatment. Cell lines were treated with 5-aza-2'-deoxycytidine (5-Aza-CdR) (Sigma-Aldrich, St. Louis, MO) at a concentration of 1-2 μg/ml for 6 days with medium changes on days 1, 3 and 5. Treated or untreated cell from individual triplicate flasks were harvested to detect expression level using RT-PCR as described earlier. Two MPM cell lines (NCI-H290, NCI-H28) with weak and negative *BMP3b* expression were used for this assay.

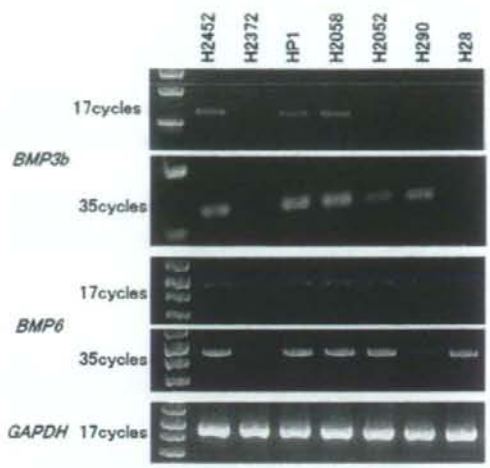

Figure 1. mRNA expression of *BMP3b* and *BMP6* in MPM cell lines. RT-PCR for each gene was carried out using two cycles, 17 and 35. The expression of *BMP3b* was completely suppressed in NCI-H2372 and NCI-H28. But in two weak expressing cell lines; NCI-H2052 and H290, *BMP3b* amplicon was not detected using 17 cycles but observed using 35 cycles. *BMP6* amplicon in NCI-H2372 and NCI-H290 was not detected using 17-cycle amplification. *GAPDH* was used as internal control.

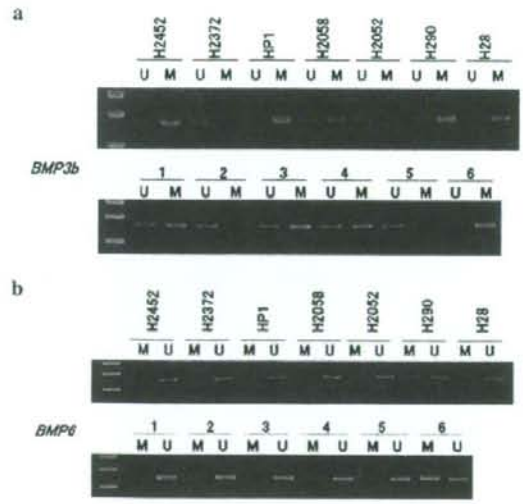

Figure 2. Methylation specific PCR for *BMP3b* (a) and *BMP6* (b) in MPM cell lines and representative examples of primary tumors. Result of testing for the methylated (M) and unmethylated (U) forms for each sample are illustrated. Samples from 1 to 7 are primary MPMs.

Data analysis. A comparison of the proportion of gene methylation was done using Fisher's exact test. A P-value <0.05 was defined as being statistically significant. All data were analyzed with the use of Survival Tools for StatView (Adept Scientific Inc., Acton, MA).

Results

The expression of *BMP3b* and *BMP6* was examined in 7 MPM cell lines by RT-PCR (Fig. 1). RT-PCR is not a quantitative assay for expression, but 2 different amplification cycles, 17 and 35 cycles; of PCR distinguished complete suppression or partial suppression of these genes. The expression of *BMP3b* was completely suppressed in 2 cell lines; NCI-H2372 and NCI-H28; and partially suppressed in 2 cell line; NCI-H2052 and NCI-H290, while expression of *BMP6* was partially suppressed only in 2 cell lines. Methylation status of *BMP3b* in cell lines was determined by MSP assay (Fig. 2). DNAs which were amplified with primers for methylated alleles but not with primers with unmethylated alleles were determined to be heavily methylated. DNAs which were amplified with both methylated and unmethylated primer sets were considered to be partially methylated. In 7 MPM cell lines, 2 cell lines (NCI-H290 and NCI-H28) were strongly methylated and 4 cell lines (NCI-H2452, HP1, NCI-H2058 and NCI-H2052) were partially methylated in the *BMP3b* gene. NCI-H28 in which *BMP3b* expression was not present was strongly methylated, but NCI-H2372 with negative expression was not methylated. Two cell lines with weak expression of *BMP3b* were heavily (H290) and partially methylated (H2052). By contrast, expression of *BMP3b* was not suppressed in 3 cell lines with partial methylation (NCI-H2452, HP1 and NCI-H2058). In the *BMP6* gene, only two cell lines (NCI-H2373 and NCI-H290) were partially methylated and no cell lines were heavily methylated. The expression of these two cell lines was partially suppressed.

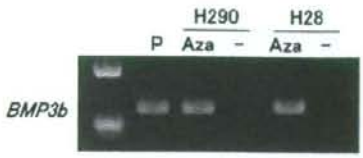

Figure 3. The restoration of *BMP3b* expression by 5-Aza-CdR in MPM cell lines. *BMP3b* amplicon was detected using 17 cycles in both H290 and H28 treated with 5-Aza-CdR. Aza, 5-Aza-CdR treated; -, untreated; P, positive control.

Next, methylation status in 57 surgically resected MPM cases was tested by MSP assay (Fig. 2). Because non-malignant cells were contained in surgically resected samples, all primary samples were amplified with primers for unmethylated alleles. Aberrant methylation of *BMP3b* were found in 9 (53%) out of 17 cases from Japan and 3 (8%) of 40 cases from USA and that of *BMP6* were in 4 (24%) cases from Japan and 12 (30%) cases from USA (Table I). While the frequency of *BMP6* methylation was similar in MPMs in Japan and USA, but *BMP3b* methylation was significantly frequent in Japanese patients (P=0.0004).

To confirm the responsibility of DNA methylation for *BMP3b* silencing, we treated two methylated cell lines (NCI-H290, NCI-H28) with 5-Aza-CdR. *BMP3b* expression was significantly up-regulated by 5-Aza-CdR treatment in methylated cell lines (Fig. 3).

Discussion

Bone Morphogenetic Proteins (BMPs) are multifunctional cytokines involved in skeletal development and bone

Table I. The frequency of *BM3b* and *BMP6* methylation in MPM cell lines and tumors.

	BMP3b methylation (%)	P-value	*BMP6* methylation (%)	P-value
Cell line (n=7)	6 (86)		2 (29)	
Primary tumor				
USA (n=40)	3 (8)	0.0004	12 (30)	0.75
Japan (n=17)	9 (53)		4 (24)	

formation. They are members of the transforming growth factor-ß (TGF-ß) superfamily and critical mediators of early embryonic patterning. BMPs have shown to inhibit cellular proliferation and involved in organogenesis, particularly of the lung, heart and kidney. Recent work has shown that *BMP3b* and *BMP6* are epigenetically inactivated in several malignancies. *BMP3b* has been demonstrated to be methylated in NSCLCs, and colorectal cancer, and *BMP6* gene in NSCLCs, prostate cancer, malignant lymphomas and breast cancer (9-13).

We have screened for aberrant methylation of *BMP* genes in MPMs. In spite of the limitation of the number of cell lines and primary samples, we showed the suppression of *BMP3b* and *BMP6* expression in MPM cell lines and DNA methylation was one of the mechanisms of gene suppression. Aberrant methylation of *BMP3b* and *BMP6* was also detected in primary MPMs. The frequency of methylation of *BMP6* was quite similar in cell lines, primary tumors in USA and those in Japan, but methylation of *BMP3b* gene was significantly more frequent in MPMs of Japan than those of USA. These results indicate that the methylation of *BMP3b* may be influenced by ethnic or pathogenic differences. Of note, we have reported that the methylation of insulin-like growth factor binding protein-3 was significantly more frequent in Japanese MPMs than in those of USA (15).

As shown in Fig. 1, methylated and unmethylated bands were observed in 4 cell lines, indicating partial methylation. In addition, even partial methylated cell lines, expression status was not identical. These finding suggest heterogeneity of methylation among individual cells even in the same cell lines. The importance of partial methylation of *BMP3b* is not clear and further study is necessary. Of note, the seven cell lines examined were established in USA, but the frequency of methylation in primary tumors in USA is not frequent. Thus, partial methylation in cell lines might be artificial phenomenon in the process of establishing cell lines. Furthermore, there must be other mechanisms for gene suppression of *BMP3b* because it was completely suppressed in NCI-H2372, in which DNA methylation was absent.

Recent work of others and our study strongly support that *BMP3b* and *BMP6* have a role as tumor suppressor genes in several malignancies. Other members of BMPs are shown to be involved in malignancies but their roles in carcinogenesis are still controversial. Methylation and loss of expression of *BMP2* was observed in gastric carcinomas, but it was also shown to promote tumor growth in A549, NSCLC cell lines (16). The roles of BMP proteins in carcinogenesis may be different according to the organ and further studies are necessary.

In conclusion, we showed that DNA methylation cause the suppression of *BMP3b* and *BMP6* expression in MPMs. In addition, there was an ethnic difference of frequency of the methylation in *BMP3b*, suggesting the etiology of MPM may be different between Japanese and USA.

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A Useful Antibody Panel for Differential Diagnosis Between Peritoneal Mesothelioma and Ovarian Serous Carcinoma in Japanese Cases

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Abstract

Malignant mesothelioma is increasing in incidence worldwide, including in Japan. However, the accurate pathologic diagnosis of pleural or peritoneal mesothelioma (PM) is sometimes difficult if adequate histologic and immunohistochemical analyses are not undertaken. The aim of this study was to identify a useful antibody panel for distinguishing PM from ovarian serous papillary adenocarcinoma (SC). We obtained 29 PMs (23 epithelioid mesotheliomas and 6 biphasic mesotheliomas) and 20 SCs from our surgical pathology files. Immunohistochemical analysis was undertaken using 13 commercially available antibodies. No significant sex differences in antigen expression among the 29 PMs were observed. The results identified calretinin and thrombomodulin as positive markers and Ber-EP4, MOC-31, CA19-9, and estrogen receptor as negative markers with relatively high sensitivity and specificity for the differential diagnosis of PM and SC. The combination of these positive and negative markers may contribute to accurate diagnosis and adequate therapy for PM and ovarian SC.

Malignant mesothelioma is a relatively rare tumor in Japan; however, its incidence has been increasing owing to the past importation and use of asbestos in Japan and other countries.[1-3]

The majority of mesotheliomas occur in the pleura, followed by the peritoneum, pericardium, and tunica vaginalis testis. The percentage of peritoneal mesotheliomas (PMs) is rather small, accounting for only 10% of all mesotheliomas.[4,5] The diagnosis of malignant mesothelioma is difficult owing to its rare frequency, histologic variety, and heterogeneity. Four histologic subtypes, including epithelioid, sarcomatoid, desmoplastic, and biphasic, are described in the 2004 World Health Organization classification.[6] Differential diagnosis depends on the tumor location and its histologic type. For example, pleural mesothelioma must be differentiated from pulmonary adenocarcinoma, pulmonary pleomorphic carcinoma, reactive mesothelial hyperplasia, fibrous pleuritis, and "true" sarcoma invading the pleura and chest wall.

On the other hand, PM is often disseminated in the peritoneal cavity with formation of multiple nodules and ascites, occasionally forming a localized abdominal mass, including in the ovaries.[7] Therefore, serous papillary adenocarcinoma (SC) of ovarian or peritoneal origin[8] is the most important and difficult malignant tumor from which it must be differentiated owing to these clinical and histologic similarities.[9] The differential diagnosis of these tumors from PM is important because chemotherapy and/or radiotherapy can significantly improve patient survival and decrease recurrence, especially for primary and secondary SC.[10-12]

There are a relatively large number of immunohistochemical studies of PM focused on differential diagnosis from pulmonary adenocarcinoma[13-17]; however, immunohistochemical

studies on the differential diagnosis of PM and ovarian carcinoma are limited[18-22] because of the rare frequency of PM.

In this study, therefore, immunohistochemical analyses were conducted using commercially available antibodies to elucidate the usefulness of immunohistochemical analysis for the differential diagnosis of PM and ovarian SC in Japanese cases.

Materials and Methods

We selected 29 cases of PM, including 23 of the epithelioid type and 6 of the biphasic type (from 23 men and 6 women), and 20 cases of ovarian SC, collected from 1987 to 2007, from the surgical archives of the Department of Pathology, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan. The average patient age was 63 years (range, 22-84 years) for men with mesothelioma, 50 years (range, 22-78 years) for women with mesothelioma, and 59 years (range, 23-84 years) for women with ovarian SC. The diagnosis in each case was based on the recommended criteria listed in the 2004 World Health Organization classification.[6] All of the patients were Japanese.

Immunohistochemical staining of sections from formalin-fixed, paraffin-embedded tissue samples was performed using the streptavidin-biotin-peroxidase method (SAB) with the Histofine SAB-PO kit (Nichirei, Tokyo, Japan) with or without antigen retrieval. The list of primary antibodies, including the 13 antibodies, clone, source, dilution, and antigen retrieval, is shown in **Table 1**.

The scoring of immunohistochemical staining was semiquantitative as follows: 0, no or trace staining; 1+, fewer than 25% of tumor cells positive; 2+, 26% to 50% of tumor cells positive; and 3+, 51% or more of tumor cells positive. The definition of a positive case in this study is a case having a score of 1+, 2+, or 3+. The scoring was performed on the epithelioid component of the epithelioid and biphasic mesotheliomas. The immunohistochemical evaluation for the sarcomatoid component of the biphasic mesotheliomas was excluded in this study. The abbreviation "PM" indicated in the present study includes epithelioid mesothelioma and the epithelioid component of biphasic mesothelioma.

Statistical analyses were performed by using the Fisher exact test and Mann-Whitney U test. Sensitivity and specificity were calculated for each marker by using a simple 2×2 table.

Results

Positivity of Antibodies for PM and Ovarian SC

The positive results of each antigen for PM and ovarian SC are given in **Table 2**. Representative immunohistochemical staining panels for PM and SC are shown in **Image 1** and **Image 2**. The staining pattern for each antibody for the 2 tumor types is described in brief in the following paragraphs.

Calretinin

All PM cases were positive, with most in the 3+ staining category. The staining pattern in the PM cases was fairly strong staining in the nucleus and weak in the cytoplasm. The SC cases showed the same staining pattern as the PM cases; however, most of the positive cases had a lower staining score (ie, 1+ or 2+).

D2-40

PM cases and SC cases showed an intense membranous staining pattern. All positive cases of PM had a high

Table 1
Antibodies Used in the Study

Antibody	Source	Clone	Pretreatment	Dilution
Calretinin	Zymed, San Francisco, CA	Polyclonal	Autoclave	1:50
D2-40	Nichirei BioScience, Tokyo, Japan	D2-40	Autoclave	Prediluted
WT1	DAKO, Glostrup, Denmark	6F-H2	Autoclave	1:400
Thrombomodulin	DAKO	1009	None	1:500
CK5/6	DAKO	D5/16B4	Autoclave	1:50
Mesothelin	Novocastra, Newcastle upon Tyne, England	5B2	Autoclave	1:20
Ber-EP4	DAKO	Ber-EP4	None	1:100
MOC-31	DAKO	MOC-31	None	1:50
CD15	DAKO	C3D-1	Autoclave	1:30
CA19-9	TFB, Tokyo, Japan	NS19-9	Autoclave	Prediluted
CEA	Nichirei BioScience	COL-1	Autoclave	Prediluted
h-Caldesmon	DAKO	H-CD	Microwave	1:50
ER	DAKO	1D5	Autoclave	1:75

CEA, carcinoembryonic antigen; CK, cytokeratin; ER, estrogen receptor.

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Table 2
Immunohistochemical Findings for Mesothelioma and Ovarian Serous Carcinomas for Various Antibodies

	Peritoneal Mesothelioma					Serous Papillary Adenocarcinoma						
		Staining Grade*					Staining Grade*					
Marker	No./Total (%)	0	1+	2+	3+	No./Total (%)	0	1+	2+	3+	$P^{\dagger}$	$P^{\ddagger}$
Calretinin	29/29 (100)	0	2	1	26	8/20 (40)	12	5	3	0	<.0001	<.0001
D2-40	22/23 (96)	1	0	8	14	9/20 (45)	11	7	2	0	.002	<.0001
WT1	22/24 (92)	2	8	3	11	18/20 (90)	2	1	10	7	.623	.741
Thrombomodulin	21/22 (95)	1	11	7	3	1/20 (5)	19	1	0	0	<.0001	<.0001
CK5/6	19/21 (90)	2	6	6	7	16/20 (80)	4	9	4	3	.307	.040
Mesothelin	20/20 (100)	0	2	3	15	19/20 (95)	1	1	3	15	.5	.972
Ber-EP4	0/21 (0)	21	0	0	0	20/20 (100)	0	2	5	13	<.0001	<.0001
MOC-31	1/19 (5)	18	1	0	0	18/20 (90)	2	3	7	8	<.0001	<.0001
CD15	0/12 (0)	12	0	0	0	12/20 (60)	8	10	2	0	.012	<.001
CA19-9	3/23 (13)	20	3	0	0	16/20 (80)	4	9	4	3	<.0001	<.0001
CEA	0/29 (0)	29	0	0	0	7/20 (35)	13	5	0	2	<.001	<.001
h-Caldesmon	0/17 (0)	17	0	0	0	0/20 (0)	20	0	0	0	NA	NA
ER	0/15 (0)	15	0	0	0	16/20 (80)	4	1	5	10	<.0001	<.0001

CEA, carcinoembryonic antigen; CK, cytokeratin; ER, estrogen receptor; NA, not available.
* The scoring of immunohistochemical staining was semiquantitative, as follows: 0, no or trace staining; 1+, fewer than 25% of tumor cells positive; 2+, 26%-50% of tumor cells positive, and 3+, 51% or more of tumor cells positive.
† The difference in the positive rate between peritoneal mesothelioma and serous papillary adenocarcinoma for each antibody calculated by the Fisher exact test.
‡ The difference in the distribution of reactivity scores between peritoneal mesothelioma and serous papillary adenocarcinoma for each antibody calculated by the Mann-Whitney *U* test.

staining score (ie, 2+ or 3+); however, most positive cases of SC had a low score.

WT1

For WT1 staining, 90% or more of the PM and SC cases were positive. The staining location in each tumor was the nucleus. Little or trace cytoplasmic staining was observed in some positive cases.

Thrombomodulin

More than 90% of the PM cases showed a primarily membranous staining pattern. However, half of the positive cases had a 1+ staining score. Only 1 SC case showed focal membranous staining.

Cytokeratin 5/6

For cytokeratin (CK)5/6, 80% or more of the PM and SC cases were positive. The staining location was the cytoplasm.

Mesothelin

All PM cases and 19 (95%) of 20 SC cases were positive. The distribution of scores for PM and SC was similar. Most of the positive cases showed a membranous staining pattern.

Ber-EP4 and MOC-31

No PM case was positive for Ber-EP4, whereas all SC cases were positive. Most of the positive SC cases were graded 3+. Only 1 PM case was weakly positive for MOC-31, whereas 18 (90%) of 20 SC cases were positive for this antibody. The location of expression of both antigens was the cell membrane for both tumor types.

CA19-9, CD15, and Carcinoembryonic Antigen

Only 3 (13%) of 23 PM cases were focally or weakly positive (1+) for CA19-9, whereas 16 (80%) of 20 SC cases showed a cytoplasmic staining pattern for this antibody. No PM cases were positive for CD15 or carcinoembryonic antigen (CEA). For SC cases, the positive rates for these antibodies were 60% (12/20) and 35% (7/20), respectively. The staining location was the cytoplasm in both tumor types. Most of the CD15+ SC cases had a 1+ score.

Estrogen Receptor and h-Caldesmon

No PM cases were positive for estrogen receptor (ER), whereas 16 (80%) of 20 SC cases showed a strong nuclear staining pattern for ER. No cases were positive for h-caldesmon for either tumor type.

Statistical Results

The *P* values for the difference in positive rates (cases with a score of 1+, 2+, or 3+) by the Fisher exact test between PM and ovarian SC are shown in Table 2. Calretinin, D2-40, and thrombomodulin showed a significantly higher expression rate in PM; however, Ber-EP4, MOC-31, CA19-9, CD15, CEA, and ER showed significantly lower expression than in SC.

The *P* values according to the Mann-Whitney *U* test for differences in the distribution of staining grades between PM and SC for each antibody are also shown in Table 2. Nearly

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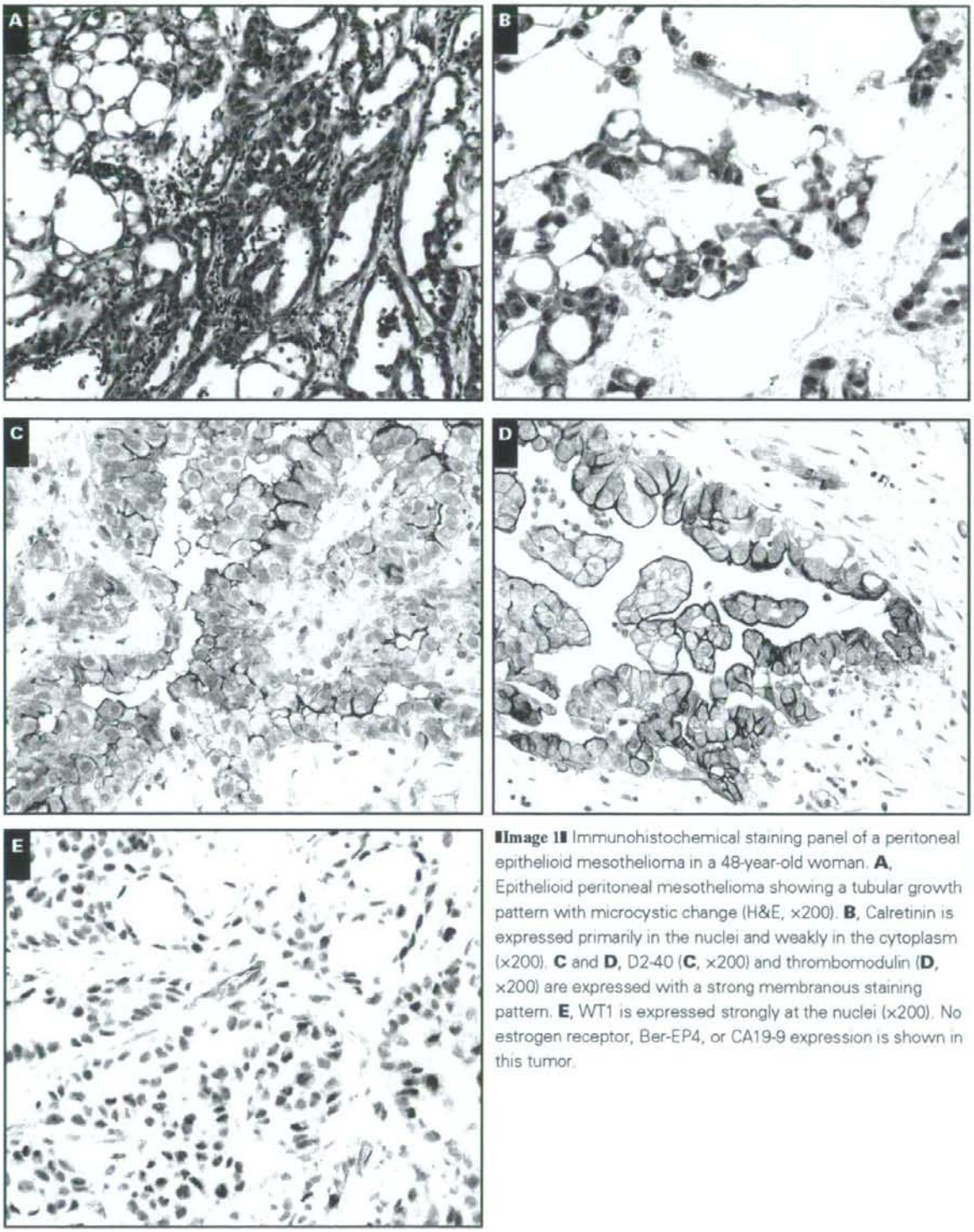

Image 1 Immunohistochemical staining panel of a peritoneal epithelioid mesothelioma in a 48-year-old woman. **A**, Epithelioid peritoneal mesothelioma showing a tubular growth pattern with microcystic change (H&E, ×200). **B**, Calretinin is expressed primarily in the nuclei and weakly in the cytoplasm (×200). **C** and **D**, D2-40 (**C**, ×200) and thrombomodulin (**D**, ×200) are expressed with a strong membranous staining pattern. **E**, WT1 is expressed strongly at the nuclei (×200). No estrogen receptor, Ber-EP4, or CA19-9 expression is shown in this tumor.

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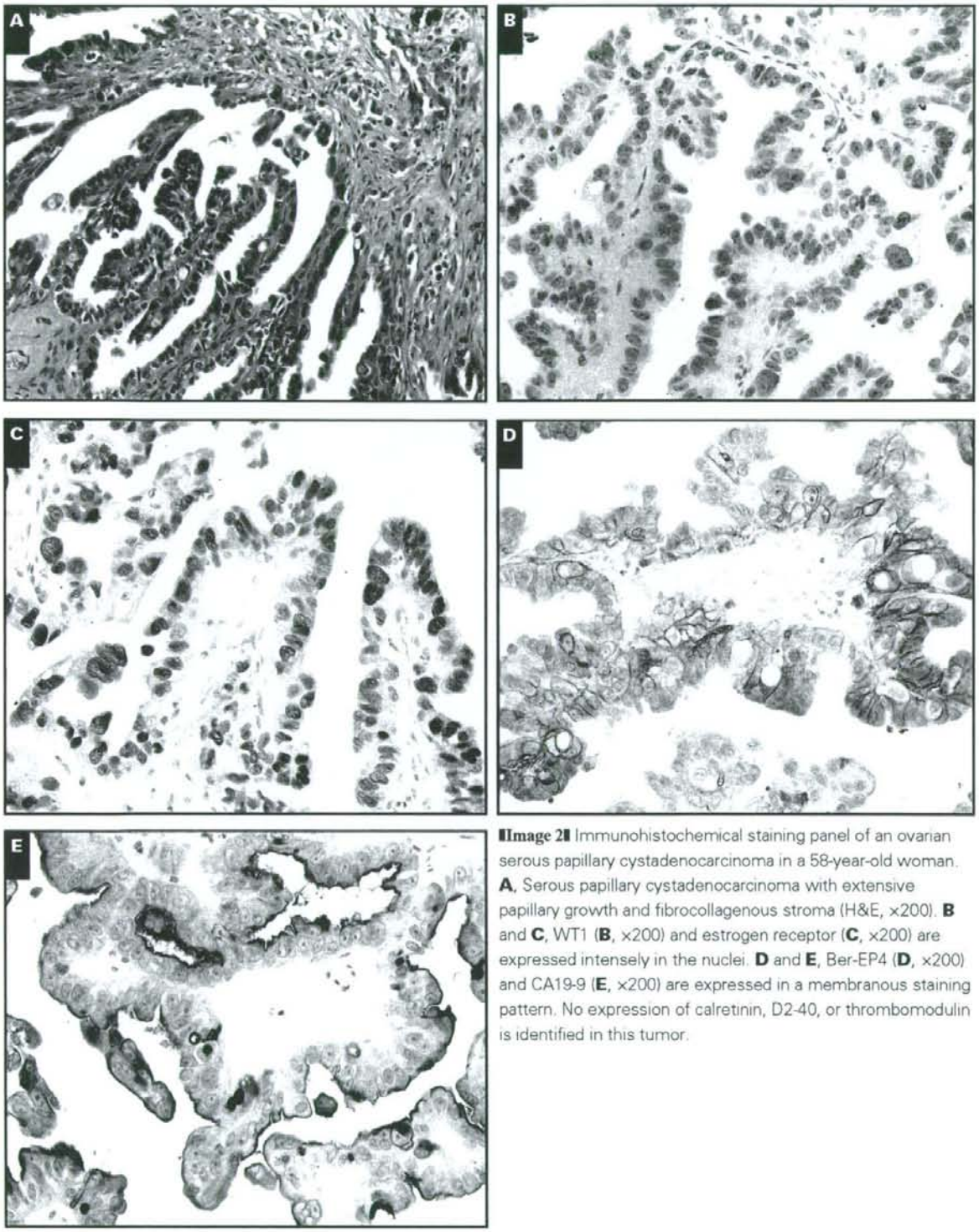

Image 2 Immunohistochemical staining panel of an ovarian serous papillary cystadenocarcinoma in a 58-year-old woman. **A**, Serous papillary cystadenocarcinoma with extensive papillary growth and fibrocollagenous stroma (H&E, ×200). **B** and **C**, WT1 (**B**, ×200) and estrogen receptor (**C**, ×200) are expressed intensely in the nuclei. **D** and **E**, Ber-EP4 (**D**, ×200) and CA19-9 (**E**, ×200) are expressed in a membranous staining pattern. No expression of calretinin, D2-40, or thrombomodulin is identified in this tumor.

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the same pattern of significant differences was observed among the antibodies as for the simple positive rate described in the preceding paragraph.

Sex Differences in Expression of Various Markers for PM

In this study, there was a relatively higher number of cases among men than among women (23 men and 6 women). Therefore, to exclude the possibility of sex bias in the comparisons between PM and SC, differences according to sex were analyzed. As indicated in ▮Table 3▮, no significant sex differences were observed for any antibody, although the number of cases was limited.

Sensitivity and Specificity of Each Antibody for Differential Diagnosis Between PM and Ovarian SC

The sensitivity and specificity of each antibody in terms of differential diagnosis between PM and ovarian SC cases are indicated in ▮Table 4▮. For SC, calretinin and thrombomodulin as positive markers and Ber-EP4, MOC-31, CA19-9, and ER as negative markers showed relatively high sensitivity and specificity (>60%). Although the negativity of CD15 showed the same sensitivity and specificity as calretinin, this antigen was excluded as a negative marker because many of the CD15+ SC cases had a low staining score.

▮Table 3▮
Sex Differences in Expression of Various Antibodies in Peritoneal Mesothelioma Cases*

	Male	Female	$P^{\dagger}$
Calretinin	23/23 (100)	6/6 (100)	NA
D2-40	17/18 (94)	5/5 (100)	.783
WT1	18/19 (95)	4/5 (90)	.38
Thrombomodulin	16/17 (94)	5/5 (100)	.772
Cytokeratin 5/6	14/16 (88)	5/5 (100)	.571
Mesothelin	15/15 (100)	5/5 (100)	NA
Ber-EP4	0/16 (0)	0/5 (0)	NA
MOC-31	1/14 (7)	0/5 (0)	.737
CD15	0/8 (0)	0/4 (0)	NA
CA19-9	2/18 (11)	1/5 (20)	.893
Carcinoembryonic antigen	0/23 (0)	0/6 (0)	NA
h-Caldesmon	0/13 (0)	0/4 (0)	NA
Estrogen receptor	0/11 (0)	0/4 (0)	NA

NA, not available.
* Data are given as number/total (percentage).
† For sex differences in expression calculated by the Fisher exact test.

▮Table 4▮
Sensitivity and Specificity of Each Antibody for Differential Diagnosis Between Mesothelioma and Ovarian Serous Carcinoma

Marker	Sensitivity (%)	Specificity (%)
Calretinin+	100	60
D2-40+	95.7	55
WT1+	91.7	10
Thrombomodulin+	94.4	95
Cytokeratin 5/6+	90.5	20
Mesothelin+	100	5
Ber-EP4–	100	100
MOC-31–	94.7	90
CD15–	100	60
CA19-9–	87	80
Carcinoembryonic antigen–	100	35
h-Caldesmon–	100	0
Estrogen receptor–	100	80

Discussion

The accurate diagnosis of PM and exclusion of other peritoneal malignant tumors is sometimes difficult when pathologists are limited to observing histologic features such as H&E and classical mucin staining, even when associated with clinical information. This distinction is especially difficult because disseminated SC of the ovary and SC originating from the female peritoneum are among the most important malignant neoplasms to be differentiated.[9]

Baker et al[9] described the morphologic differences between PM and SC based on their experience with 75 PMs in females. Specifically, PM might show more papillary growth with prominent, often hyalinized stromal cores and have more conspicuous eosinophilic cytoplasm than SC. On the other hand, SC might have more frequent slit-like spaces and psammoma bodies, more hierarchical branching, cellular stratification and detached cell clusters, a greater degree of nuclear atypia, and more mitotic figures than PM. However, the differential diagnosis between PM and SC is difficult through purely histologic observations, especially when higher grades of these tumors are encountered.

Recently, many so-called mesothelial markers, including calretinin, D2-40, WT1, thrombomodulin, mesothelin, and others, have been certified.[23,24] However, to date, no mesothelioma-specific markers that are expressed only in mesothelioma cells have been identified. This fact indicates that a combination of positive and negative mesothelial markers is important to accurately diagnose mesothelioma. Several comparative immunohistochemical studies for differential diagnosis between PM and SC have been reported.[18-22,24,25] Attanoos et al[19] reported that calretinin and Ber-EP4 are useful discriminant markers for distinguishing PM in women from serous papillary ovarian and peritoneal carcinoma. They also indicated that thrombomodulin, cytokeratin 5/6, and CD44H as mesothelial markers and CEA and CD15 as carcinoma markers have too low a sensitivity for practical use.[19] Ordonez[24] reported that a combination of the best positive markers (D2-40 and calretinin) and negative markers (Ber-EP4 and MOC-31) is useful for discriminating between 2 tumors. Ordonez[20] and Barnetson et al[25] also reported that ER immunohistochemical analysis is very useful for the differential diagnosis owing to its high sensitivity and specificity,

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ie, high expression in serous carcinoma and no expression in epithelioid mesothelioma. In the present study, the combination of positive and negative markers, such as calretinin and thrombomodulin as positive markers and Ber-EP4, MOC-31, CA19-9, and ER as negative markers, was helpful.

Calretinin is a 29-kDa calcium-binding protein that is a member of the large family of EF-hand proteins and is expressed especially strongly in epithelioid mesotheliomas of the pleura or peritoneum.[26] Calretinin positivity in PM has been reported as 100%,[26] 88%,[19] 100%,[24] 100%,[22] and 100% (present study), and positivity in SC has been reported as 6%,[26] 0%,[19] 31%,[24] 12.5%,[22] and 40% (present study). The calretinin positivity of SC in the present study is the highest among this and the previous studies,[19,22,24,26] considering its sensitivity, specificity, and positive intensity. These discrepancies of positivity, especially for SC, may be due to differences in antigen-retrieval and immunoscoring methods. In comparing the degree of positivity between the 2 tumor types in the present study, the PM cases with positive scores were mainly 3+; however, the SC cases had a relatively lower score (ie, 1+ or 2+). By considering the positive rate and positive staining grade, calretinin could be a "positive marker" for differential diagnosis between PM and SC, even though the specificity was relatively low (ie, 60%).

Commercially available D2-40 has recently been used as an antibody that reacts with the 40-kDa antigen of the M2A oncofetal membrane antigen originally detected in germ cell neoplasia.[27] This antibody is also expressed in the lymphatic endothelium, so is applied to the study of lymphatic invasion in many tumors.[28,29] Chu et al[30] first described the usefulness of D2-40 in the diagnosis of mesothelioma, showing 100% positivity in epithelioid mesothelioma, 96% in reactive pleura, 7% in pulmonary adenocarcinoma, and 65% in ovarian serous carcinoma; they concluded that it is a useful positive marker for differential diagnosis between epithelioid mesothelioma and pulmonary adenocarcinoma. Its relatively high expression in ovarian serous adenocarcinoma may be related to the close histogenetic relationship between the mesothelium and serous lining cells of müllerian origin.[30] Ordonez[23,24] reported that 93% of PMs and 13% of SCs were positive for the D2-40 antibody and that the positive intensity in PM was higher than that in SC and concluded that D2-40 is a good discriminator between the 2 tumors. Comin et al[22] also reported that 100% of PMs and 40% of SCs were positive for D2-40 and concluded that D2-40 is a good marker, although with a slightly lower sensitivity and specificity than shown by h-caldesmon and calretinin.

The present study on D2-40 in PM and SC had almost the same results as these previous studies.[23,24] However, most PMs showed higher expression compared with SCs in terms of the positive staining pattern, and D2-40 was expressed with a focal or weak pattern in SC in the 1+ scoring category. The difference in expression pattern and the degree of expression between PM and SC may be useful for differential diagnosis. However, it was not possible to determine the usefulness of this antibody for diagnosis of PM and SC because of its relatively low specificity. Further studies are necessary to draw a definite conclusion.

WT1 has been reported to be expressed in rat and human mesotheliomas[31,32] and to be a very useful marker for differentiating between epithelioid mesothelioma and pulmonary adenocarcinoma.[15,17] Ordonez[15] summarized the positivity of epithelioid mesothelioma as 71% to 95% and pulmonary adenocarcinoma as 0% to 22% in a review article. He also summarized data for serous adenocarcinoma as having a positivity of 83% to 100%. In the present study, the positivity for WT1 was 22 (92%) of 24 cases for PM and 18 (90%) of 20 cases for SC. WT1 also showed an intense nuclear staining pattern in PM and SC. Therefore, this antibody is not useful for distinguishing between PM and SC. Acs et al[33] reported that WT1 expression was seen in 24 of 28 SCs, 4 of 18 clear cell adenocarcinomas, and in none of 11 endometrioid adenocarcinomas or 11 mucinous carcinomas. These results suggest that WT1 is differentially expressed depending on cellular differentiation among the tumors of the female genital tract, including the peritoneum.[33] Accordingly, WT1 could not be used for differential diagnosis between PM and SC, in contrast with use for differential diagnosis between pulmonary adenocarcinoma and pleural mesothelioma.[17]

Thrombomodulin, also known as CD141, is a relatively old positive marker for mesothelioma.[34] The positivity of thrombomodulin was reported as 33% to 73% in PM and 2% to 35% in SC in a review article by Ordonez.[24] Ordonez reported that 73% of PMs and 4% of SCs are positive for thrombomodulin and concluded that it is a less sensitive and more specific marker than calretinin or D2-40. In the present study, the positivity of thrombomodulin was 21 (96%) of 22 cases for PM and 1 (5%) of 20 cases for SC. These results also indicated that thrombomodulin is a relatively highly specific and sensitive marker for distinguishing between PM and SC compared with findings of other studies.[19,24] The disadvantage of this antibody is that most of the positive cases had a lower staining grade (ie, 1+, 11 cases; 2+, 7 cases). Although thrombomodulin is a classical positive marker with a relatively weak positive staining pattern, its usefulness as a positive marker is suggested by its high specificity.

CK5/6, a high-molecular-weight keratin, has been reported as a positive mesothelioma marker in differentiating from pulmonary adenocarcinoma,[13,15] although the data are not consistent in our experience.[17] The CK5/6+ percentage reported for PM is 53% to 100% and for SC is 24% to 31%.[18,19,21] Also, the present results showed no significant difference in staining pattern between PM and SC.

Ber-EP4 and MOC-31 have been recognized as epithelial cell adhesion molecules[35] and reported to be useful negative

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