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REVIEW ARTICLE

Practical approaches to diagnose and treat for T0 malignant pleural mesothelioma: a proposal for diagnostic total parietal pleurectomy

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Abstract Malignant pleural mesothelioma (MPM) remains suffering poor prognosis in spite of recent diagnostic and therapeutic progress. Although there is currently no established evidence, early diagnosis and early intervention may play a key role to improve prognosis of MPM, similarly to other malignancies. As pleural effusion is usually the first clinical sign of MPM, pleural effusion cytology is often the first diagnostic examination to be carried out. Since the sensitivity of pleural effusion cytology is approximately 60%, however, false-negative diagnosis is given to almost half of true MPM patients at this clinical step. One practical way to reduce the number of misdiagnosed MPM is to encourage performing thoracoscopic pleural biopsy unless definitive diagnosis other than MPM is established. There still remain a considerable number of patients with radiological/thoracoscopic T0 MPM who are misdiagnosed with nonspecific pleuritis after a complete investigation including thoracoscopic biopsies. Such patients will turn out to be malignant during follow-up period, although they have the best opportunity for long-term survival if only early therapeutic intervention is given. Currently, we are performing diagnostic total parietal pleurectomy in highly selected patients, who are characterized with strong clinical suspicion, positive pleural effusion cytology but uncertain pathological diagnosis, excellent cardiopulmonary reserve, and with written informed consent for highly invasive diagnostic surgery for pathologically unproven disease.

Keywords Malignant pleural mesothelioma · Pleurectomy · Extrapleural pneumonectomy · Pleural biopsy · Early stage · Thoracoscopy

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Introduction

Malignant pleural mesothelioma (MPM) is a quite aggressive thoracic malignancy and suffers pessimistic prognosis [1–7].

Early diagnosis and early intervention could offer better prognosis, since MPM does not metastasize until late in the disease, and these metastases are rarely the cause of death [8]. In fact, patients who underwent curative intent treatment in early stage of MPM are blessed with better survival [9–11]. Furthermore, there are sporadic case reports where patients with radiologically undetectable MPM survive long [12, 13].

Although there is currently no proof that early discovery of MPM will cure the patient or even improve their survival for many months [14], clinical evidence may be established as cases with early therapeutic intervention being piled up [15].

Definition of early MPM

Early MPM is an ambiguous term and sometimes makes arguments unfocused.

To make discussion clear, the following terms are used in this article.

Radiological T0 MPM No apparent tumor or pleural thickening other than pleural effusion is seen on chest X-ray and chest CT, and no abnormal uptake is found in 18F-fluorodeoxyglucose positron emission tomography (FDG-PET).

Thoracoscopic T0 MPM No apparent tumor is macroscopically visible at thoracoscopy or open biopsy.

Concepts of early MPM and mesothelioma in situ

In 1992, Whitaker and colleagues presented 7 cases of early MPM, and defined mesothelioma in situ (MIS) as the replacement of benign surface mesothelium by mesothelial cells that have cytoarchitectural feature of malignancy [16]. Acceptance of the above concept presented several challenges for both physicians and pathologists. The challenge for physicians was to accept stage 0 MPM defined as pathological evidence without gross tumor. Problem for pathologists was making diagnosis of MPM based on the cytological atypia. Later on, definition of MIS became less challenging particularly for pathologists in that they restricted diagnosis of MIS only when invasive tumor was present in other sites of the pleura [17–20]. Unfortunately, the term of MIS has not been widely used in clinical practice, because, once invasive tumor is found elsewhere, clinical decision is made regardless to the existence of MIS lesions [21].

Although most of Whitaker's cases were initially diagnosed as MPM by pleural effusion cytology [16], definitive diagnosis of MPM based on cytology alone is often difficult and is not recommended currently [14, 18].

Due to the above situation, difficult clinical decision is sometimes required in case with strong clinical suspicion for MPM, with positive pleural effusion cytology but without definitive pathological diagnosis on pleural biopsy specimen. Pathologists are quite anxious about overdiagnosis of MPM [18], because, once definitive diagnosis is established, highly invasive treatment will be started [22].

Difficulties in diagnosing early MPM

1. Because of low prevalence of MPM, any screening test will suffer from high false-positive/low true-positive rate [8, 23].
2. Currently, no biological markers are available as practical screening test for MPM [5, 14].
3. Unlike other solid tumors, MPM spreads out in whole pleural space since its earliest phase [24]. No apparent solid tumor is seen in this phase. Pleural effusion, only radiological feature in most of early MPM [14], has wide spectrum of differential diagnoses [25, 26]. This is why most of previous studies on asbestos-exposed population failed to prove any diagnostic benefit [15, 27–31]. For example, a CT screening study performed in Italy revealed only 10 (1%) malignancies but no MPM out of 1045 high-risk individuals [29].
4. As pleural effusion is usually the first clinical sign of MPM, pleural effusion cytology is often the first diagnostic examination to be carried out [14]. Series examining the diagnostic rate for malignancy of pleural cytology have reported a mean sensitivity of about 60% (range 40–87%) [25, 32]. Sensitivity of pleural effusion cytology is reduced to 20% in case of severe pleural adhesion [33]. Definitive diagnosis based on cytology alone is not recommended because of the high risk of diagnostic error [14, 18].
5. Surgical pleural biopsy, though it is invasive, remains to be the "gold standard" for definitive diagnosis of MPM [34–36]. One important issue is the significant possibility of sampling error at biopsy [37]. In most cases with other solid tumors, sampling error seldom occurs because biopsy target, the main lesion, is usually locatable. However, in radiological/thoracoscopic T0 MPM, it is uncertain whether a few pieces of the parietal pleura really represent the whole pleural lesions. Therefore, a diagnosis of MPM on thoracoscopic biopsy is useful but a benign histology can represent a sampling error or genuine benign pleuritis [37].
6. When early phase of solid tumors are strongly suspicious but results from biopsies are inconclusive, the best resolution is surgical resection of the whole lesion. Such surgery may be justified based on the risk/benefit ratio. In most of such cases, surgical resection is not very dangerous nor is very harmful to postoperative function. On the other hand, it always provides definitive pathological diagnosis regardless of benign or malignant one. Furthermore, such surgery is sometimes curative. On the contrary, in MPM, such surgery means total pleurectomy and is not usually performed.

Due to the above situation, therapeutic intervention in early MPM has been infrequently performed. Pathological stage I MPM consists of 8–10% of all surgical cases [10, 38].

Methodologies that might offer a strong assistance for early diagnosis of MPM in near future

Currently, we have not any non-invasive diagnostic tool that gives definitive diagnosis of radiological T0 MPM [25].

If some diagnostic tool with acceptable sensitivity and specificity will be developed, ethical barrier against invasive diagnostic surgery may be much reduced through strengthened clinical suspicion with support of such technique.

SMRP (soluble mesothelin-related peptide)

Robinson and colleagues first reported that SMRP in serum could be a useful marker for diagnosis of MPM [39]. It is notable that 3 of 7 non-malignant individuals with high SMRP developed MPM or lung cancer but none of 33 non-malignant individuals with normal SMRP developed malignancy thereafter. SMRP, in the first large-scale prospective study, failed to show clinical usefulness in screening MPM due to high false-positive rate [23, 40]. Creaney et al. [8] found that serum SMRP levels were absolutely elevated in up to 15% of subjects and were relatively elevated in 40% of individuals some months before diagnosis. Based on their longitudinal observation of serum SMRP level in asbestos-exposed individuals, they suggested early intervention in such patients could be studied. In a sporadic case report, elevated serum SMRP contributed to early surgical intervention after negative result of thoracoscopic pleural biopsy [41].

SMRP in pleural effusion is also a significant and promising marker in MPM [42–44]. Because it is believed that SMRP is released from MPM cells into the pleural effusion and probably absorbed subsequently into the systemic circulation, pleural fluid levels of SMRP may be a more sensitive test than those of serum SMRP in diagnosing MPM [45]. Surprisingly, Davies and others measured pleural fluid SMRP concentration taken from 167 undiagnosed patients and found that pleural fluid SMRP diagnosed MPM more reliably than cytological examination (71 vs. 35%) [46].

Immunohistochemical markers

Although several markers such as epithelial membrane antigen (EMA), desmin, glucose transporter-1 (GLUT-1), and p53, have been reported to be of use in distinguishing MPM and reactive mesothelial proliferation (RMP) in effusion cytology and histopathology, the results are various among reports [5, 47].

A recent study describes that CD146 is a sensitive and specific immunocytochemical marker enabling differential diagnosis of MPM from RMP [48].

Gene expression profiling [49–51]

It has been known that MPM often show CDKN2A [52–54] and NF2 [55–57] inactivation. More recent studies reported high frequencies of somatic or germline mutations in *BAP1* in MPM [58–60].

Genetic expression profiling is promising because it may, in near future, lead to definitive diagnosis of MPM that other diagnostic methodologies fail to give.

Circulating tumor cells (CTCs) and circulating endothelial cells (CECs)

CTCs, shed from primary site and present in the peripheral blood, can be a clinically useful surrogate of distant metastasis in various solid tumors including lung cancer [61, 62].

As MPM is characterized by aggressive angiogenesis, CECs can be a useful clinical marker.

Application of CTCs [63] and CECs [64] in detecting early MPM is currently under investigation to overcome unsatisfactorily low sensitivity.

A practical scenario of diagnosing radiological T0 MPM

We sometimes encounter possible radiological T0 MPM patients in whom minimal radiological changes such as small amount of pleural effusion are seen without visible tumors. Practical diagnostic steps using currently available methodologies in such cases are as follows.

Step 1. Non-invasive screening tests are performed in patients with clinical suspicion of MPM. Aspiration cytology is performed whenever possible.

Step 2. Since the sensitivity of pleural effusion cytology is approximately 60%, false-negative diagnosis is given to almost half of true MPM patients at step 1. One practical way to reduce the number of misdiagnosed MPM is to encourage performing pleural biopsy with medical thoracoscopy or video-assisted thoracoscopic surgery (VATS) unless definitive diagnosis other than MPM is established.

Group 2-1: In case where pleural nodules are found at thoracoscopy (thoracoscopic > T1a MPM), targeted biopsy can be performed. Pathological diagnosis is usually reliable regardless to benign or malignant one.

Group 2-2 (Figs. 1, 2, and 3 Diagnostic procedures of a representative case with radiological/thoracoscopic T0 MPM. No significant radiological features including chest X-ray, chest CT, and FDG-PET were notable in this case): If nonspecific inflammatory changes are the only findings at thoracoscopy (possible thoracoscopic T0 MPM), a 2 × 1 cm whole layer of the parietal pleura is taken from 3 different sites as a specimen [65]. Maximum care should be taken to prevent damaging specimen, since pathological differential diagnosis between RMP and MPM is highly delicate [16–18, 20, 51]. Pleural specimen is immediately stretched and fixed to a rubber plate.

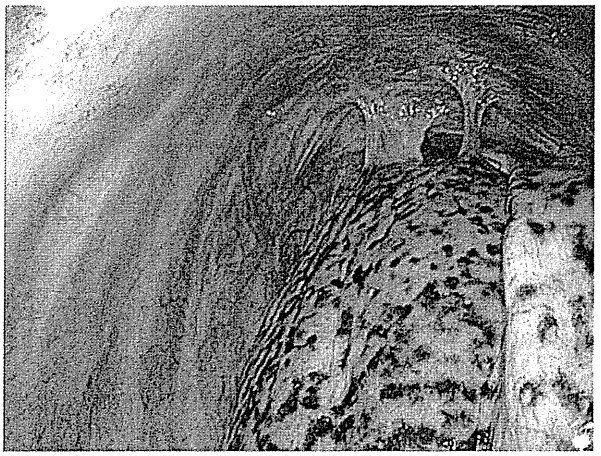

Fig. 1 Only nonspecific inflammatory changes of the parietal pleura were seen at VATS biopsy. Three pieces of whole layer parietal pleura with each size of 2 × 1 cm were sampled from lateral chest wall, the diaphragm, and the costophrenic fold

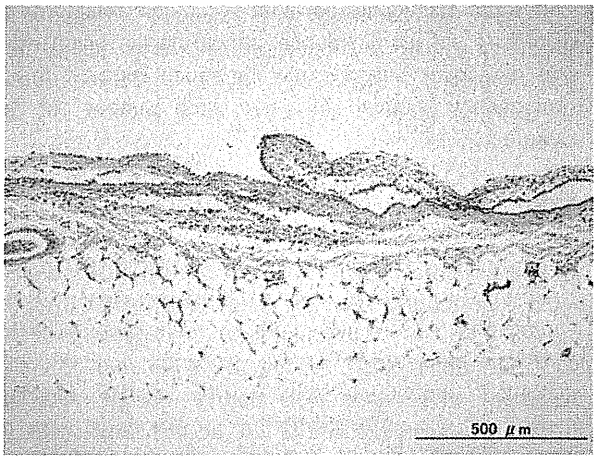

Fig. 2 Pleural biopsy. Cytologically atypical mesothelial cells expand as a single layer on the serosal surface of parietal pleural with limited invasion within submesothelial fibrous tissues. In addition, organizing fibrous tissues partially covered with atypical mesothelial cells are present on the serosal surface of parietal pleura. No invasion into subpleural adipose tissues is observed in all three biopsy specimens

Step 3. According to the results from pathological examination, there may be three possible further scenarios in Group 2-2 patients.

Group 3-1: No atypical mesothelial proliferation (AMP) is seen in pathological examination. Repeated pleural biopsy or CT follow up is indicated in this case.

Group 3-2: Microscopic foci of invasive mesothelioma are confirmed in biopsy specimen. This is a pathologically proven radiological/thoracoscopic T0 MPM, and will be the best candidate for curative intent treatment.

Group 3-3 (Fig. 2): Although AMP is seen in biopsy samples, there is no evidence of fat invasion that is currently considered to be a key for definitive diagnosis of MPM [18, 20]. This is the most controversial situation. Follow up with interval CT or repeated biopsy is recommended by guidelines [14, 66]. However, repeated biopsies often result in the same uncertain diagnosis. Hooper and others reported that 8.3% of the patients who were diagnosed with nonspecific pleuritis after a complete investigation including thoracoscopic biopsies turned out to be malignant over a 2-year follow-up period [25]. As a lengthy follow-up may miss a golden opportunity of early intervention, it creates significant anxiety for patients [37].

Currently, we perform diagnostic total parietal pleurectomy (DTPP) in highly selected patients from Group 3-3, who are characterized with strong clinical suspicion, positive pleural effusion cytology, excellent cardiopulmonary reserve, and with written informed consent for highly invasive diagnostic surgery for pathologically unproven disease (Fig. 3).

Comments

There may be a lot of arguments with regards to performing pleurectomy without definitive pathological diagnosis of MPM. The most important issue in diagnosing radiological/thoracoscopic T0 MPM is that a few biopsy specimens may not be the most representative part of the disease. Reported false-negative rate with medical thoracoscopic pleural biopsy in thoracoscopic T0 MPM ranges 4–12% [25, 37, 67–69]. It is notable that, in Davies' series [37], there was no false-negative outcome in cases with a low pre-thoracoscopic clinical suspicion of malignancy and all five false-negative outcomes occurred in patients with high level of suspicion prior to thoracoscopy. This episode clearly indicates that physicians should consider further diagnostic procedures in patients with high level suspicion but without definitive diagnosis of MPM [66].

To increase the number of biopsy site might provide a better diagnostic rate in radiological/thoracoscopic T0 MPM. Boutin [65] took a total of 10–20 biopsies from the parietal, diaphragmatic, and costovertebral gutter. However, in 188 Boutin's cases, EPP and pleurectomy/decortication (P/D) were performed in only 2 and 11 patients, respectively. Since thoracoscopic T0 MPM is the best candidate for curative intent surgery, we restrict biopsy sites up to 3 to prevent reducing curability.

Mortality of P/D is approximately 3% [10, 70], which is apparently too high if it is performed as a diagnostic procedure. In DTPP, however, mortality/morbidity may be

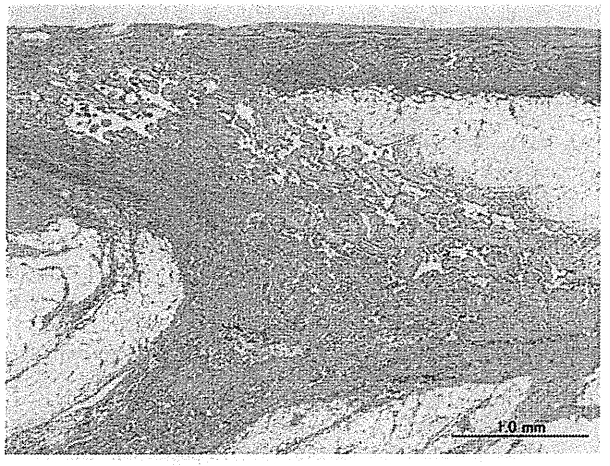

Fig. 3 DTPP surgery. Several small nodules with maximum size of 1–2 mm were found during macroscopic examination of the whole parietal pleura from the DTPP surgery. In a small nodule, atypical mesothelial cells proliferate and invade into subpleural adipose tissues with papillary and trabecular pattern. Accordingly, this case is pathologically diagnosed as epithelioid type MPM

considerably reduced. This is because DTPP skips visceral pleurectomy that, in comparison with parietal pleurectomy, is more responsible to postoperative adverse events such as massive bleeding, prolonged air leakage, and empyema.

By definition, macroscopic complete resection is always achieved when DTPP is performed in radiological/thoracoscopic T0 MPM [71, 72]. It means that, in such cases, aggressive cytoreductive surgery is performed for the earliest MPM ever diagnosed. Thus, DTPP is not only an ultimate diagnostic procedure but also a therapeutic one.

Surgical specimen of DPTT may contain the earliest phase of MPM. Fortunately, such MPM lesions are not biochemically modulated by chemotherapy or radiation. Analysis of such specimen will lead to deeper understanding of carcinogenesis and growth pattern of MPM.

Since DPTT has considerable ethical barrier, it should be performed as a clinical trial [73].

Conclusion

Consideration of DPTT in patients with strong clinical suspicion but without definitive pathological diagnosis after a complete investigation including thoracoscopic biopsies may lead to improved survival of MPM.

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Conflict of interest No author has any conflict of interest.

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REVIEW ARTICLE

Pathological and molecular biological approaches to early mesothelioma

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Abstract Malignant mesothelioma is an asbestos-related malignancy that arises primarily from mesothelial cells on the serosal surfaces of the pleural, peritoneal, and pericardial cavities. Malignant pleural mesothelioma (MPM) is most common, and its incidence is dramatically increasing worldwide as a result of widespread use of asbestos. Morphological discrimination between MPM and reactive mesothelial hyperplasia is difficult, and the most reliable pathological criterion for malignancy is mesothelial proliferation invading deeply into subpleural adipose tissues. To establish radical cure of MPM, it is crucial to find early-stage MPM of epithelial type, in which mesothelial proliferation is localized on the serosal surface of parietal pleura or limited within the submesothelial fibrous tissues of parietal pleura. The initial clinical presentation for patients with MPM is frequently dyspnea and/or chest pain due to large pleural effusion, and cytological analysis of pleural effusions is valuable to find patients with early-stage MPM of epithelial type. Recently, cytological features of MPM in pleural effusion, molecular markers for MPM, and genetic alternations of MPM have been reported. In this review, we discuss major issues on pathological and molecular biological approaches for diagnosis of early-stage MPM of epithelial type.

Keywords Mesothelioma · Mesothelioma in situ · Atypical mesothelial proliferation · Reactive mesothelial hyperplasia · $p16^{INK4a}/p14^{ARF}$ (*CDKN2A*)

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Introduction

Malignant mesothelioma (MM), considered to be closely associated with asbestos exposure, is an aggressive tumor arising from mesothelial cells on the serosal surfaces of the pleural, peritoneal, and pericardial cavities and tunica vaginalis testis [1]. MM arises most frequently in the pleura, and the incidence of malignant pleural mesothelioma (MPM) is dramatically increasing worldwide as a result of widespread use of asbestos in the last century [2]. In most MPM cases, a clinically overt tumor is diagnosed in the 30–40 years after exposure to asbestos, indicating a long latency for tumor development [3]. MPM is characterized by high resistance to conventional therapies and poor prognosis with a median survival duration of 9–17 months after diagnosis [4]. The poor prognosis is due to the fact that early-stage MPM is notoriously difficult to diagnose clinically and pathologically [5].

MPM grows in the pleural cavity diffusely more often than as a localized mass. According to WHO histological classification of tumors of the pleura, diffuse MM is classified into four types; epithelioid, sarcomatoid, desmoplastic, and biphasic mesothelioma [6]. Epithelioid mesothelioma (MPM of epithelial type) is most common, and better prognosis has been reported for patients with MPM of epithelial type than those with MPM of other types [6]. An extrapleural pneumonectomy (EPP), considered to

be a curative intent surgery for MPM, is characterized by stripping of the affected parietal pleura from the endothoracic fascia of the chest wall and en bloc removal of the affected parietal pleura and lung [7]. To establish radical cure of MPM, it is therefore indispensable to find early-stage MPM of epithelial type, in which mesothelial proliferation is localized on the serosal surface of parietal pleura or limited within the submesothelial fibrous tissues of parietal pleura. Morphological discrimination between MPM of epithelial type and reactive mesothelial hyperplasia (RMH) is difficult, and the most reliable pathological criterion for malignancy is mesothelial proliferation invading deeply into subpleural adipose tissues [8, 9].

The initial clinical presentation for patients with MPM of epithelial type is frequently dyspnea and/or chest pain due to large pleural effusion, and cytological analysis of pleural effusion may be useful to identify early-stage MPM of epithelial type with the potential for radical cure. However, it has been considered that MPM should not be diagnosed by pleural effusion cytology, since the reliable cytological criteria for the diagnosis of MPM has not been established so far and invasion cannot be assessed with specimens used in effusion fluid cytology [6, 10–12]. Recently, characteristic cells of MPM of epithelial type in pleural effusion have been collected and analyzed [13, 14]. In addition, several molecular markers, which may assist diagnosis of early-stage MPM of epithelial type in pleural effusion cytology and histology, have been developed [15–19]. In this review, we focus on pathological and molecular biological approaches for diagnosis of early-stage MPM of epithelial type.

Pathological approach to diagnose early-stage MPM of epithelial type

Issues on the pathological diagnosis

It is important to distinguish malignant and benign mesothelial proliferation to determine treatment strategy. The submesothelial basal lamina, unlike the subepithelial counterpart, is immature and allows non-tumor mesothelial cells to infiltrate into the submesothelial fibrous tissues. Non-tumor mesothelial cells can be also entrapped in a solitary, trabecular, and/or grandular pattern during organizing serosal inflammatory processes [6, 8, 9]. Therefore, it is very difficult to distinguish between genuine invasion of MPM and benign inflammation-induced infiltration and entrapment of mesothelial cells. To eliminate overtreatment of patients resulting from inappropriate diagnosis of MPM, MPM is generally diagnosed pathologically when invasion of atypical mesothelial cells into subpleural adipose tissues and/or deeper chest wall structures is established [6, 8, 9, 20, 21]. The difficulty of pathological diagnosis until MPM invades into subpleural adipose tissues and/or deeper chest wall structures certainly results in poor prognosis of MPM. However, given the general principle of the morphologic stages leading to malignancy in other organs, the stage in which the invasion of atypical mesothelial cells has not reached subpleural adipose tissues should exist in MPM.

Atypical mesothelial proliferation

Mesothelial proliferation on the serosal surface proceeds in various ways forming a single layer of cells with various degrees of cytologic atypia or heaped up aggregates of cells without papillary cores. Cells expanded as a single layer on the serosal surface are cuboidal and often show elongated forms with nuclei placed apically toward the pleural cavity. Atypical mesothelial cells usually carry distinctly enlarged nuclei and prominent nucleoli (Fig. 1a). However, benign and malignant mesothelial proliferation on the serosal surface are hardly distinguished morphologically [9].

Even if mesothelial proliferation with cytologic atypia on the serosal surface of parietal pleura appears malignant or represents part of MPM, Churg et al. [8, 9] recommend that this noninvasive mesothelial proliferation with cytologic atypia should not be diagnosed as malignant by itself and should be designated simply as atypical mesothelial proliferation (AMP) or atypical mesothelial hyperplasia, that requires further investigation and close follow-up, if the process is clinically suspicious (Fig. 1b). The term of AMP for mesothelial proliferation with cytologic atypia on the serosal surface of parietal pleura, where the discrimination between benign and malignant mesothelial proliferation is problematic, is usually accepted, but the use of atypical mesothelial hyperplasia would be discouraged for such mesothelial proliferation as hyperplasia denotes a benign process by definition [22].

Mesothelioma in situ

Considering the definition of carcinoma in situ, an entity of mesothelioma in situ is histologically defined as malignant mesothelial cells arrayed along the serosal surface of parietal pleura with no evidence of invasive tumor. Previously, Whitaker et al. [23] have proposed criteria for morphologic diagnosis of mesothelioma in situ; a single row of highly atypical mesothelial cells that are quite pleomorphic, sometimes with a picket fence appearance, and appear cytologically malignant. Later, the same group [24] has proposed that mesothelioma in situ should be

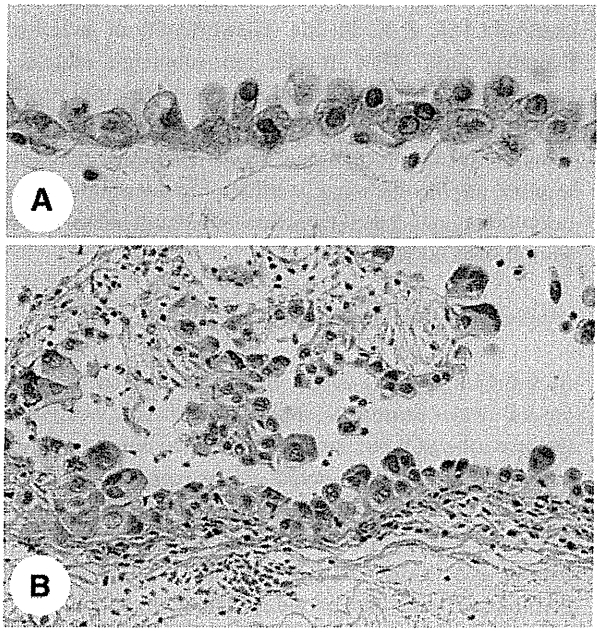

Fig. 1 Proliferation of atypical mesothelial cells at the serosal surface of parietal pleura. **a** Cytologically atypical mesothelial cells expand as a single layer on the serosal surface of parietal pleural without invasion into submesothelial fibrous tissues. These cells are cuboidal and often elongate with nuclei placed apically toward the pleural cavity. Binucleate cells are also observed. This lesion is considered to be an AMP [8, 9]. **b** Atypical mesothelial cells proliferate with heaped up and aggregated features at the serosal surface of parietal pleura without invasion into submesothelial fibrous tissues. Such a lesion has been proposed to be called AMP by Churg et al. [8, 9]. However, invasion into subpleural adipose tissues was observed later in this case, which is regarded as mesothelioma in situ according to Whitaker et al. [24]. In addition, this case shows AMP with an exophytic papillary architecture at the serosal surface of pleural biopsy, supporting the idea that complex exphytic mesothelial proliferation does not usually occur at the serosal surface of parietal pleura as part of benign inflammation-induced mesothelial proliferation

diagnosed only when unequivocal invasion is identified in a different area of the pleura or at a different time and the patient has experienced exposure to asbestos (Fig. 1b). On the other hand, since complex exophytic mesothelial proliferation does not usually occur at the serosal surface of parietal pleura as part of benign inflammation-induced mesothelial proliferation, the presence of such proliferation raises a suspicion of mesothelioma in situ where an invasive component has not been sampled by biopsy [22]. Hammar et al. [25] consider that AMP with an exophytic papillary architecture at the serosal surface of parietal pleura should not be dismissed as benign and require close clinical follow-up and/or further cytologic or biopsy investigation (Fig. 1b). In addition, an obvious bulk tumor spreading along the serosal surface would be malignant, but ancillary techniques with molecular markers for MPM

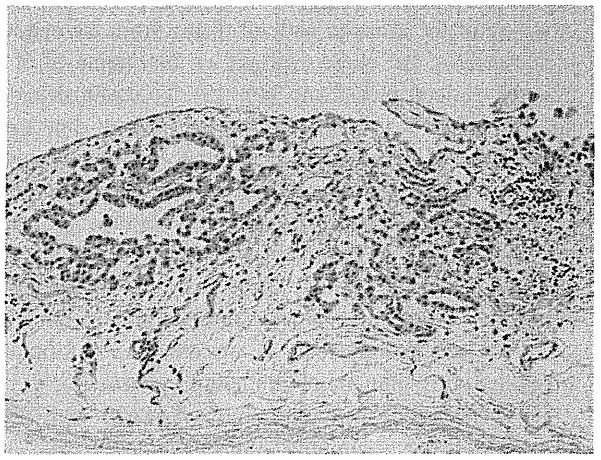

Fig. 2 Early-stage MPM of epithelial type with invasion within submesothelial fibrous tissues. There was no obvious invasion into subpleural adipose tissues, but mesothelial proliferation extends from the serosal surface to the junction with adipose tissues. In addition, an outward branching gland structure is observed in this lesion. These histological features are indicative of malignancy

are required to precisely diagnose malignant mesothelial proliferation on the serosal surface of parietal pleura.

Invasion within the submesothelial fibrous tissues

Limited invasion within the submesothelial fibrous tissues, as well as malignant mesothelial proliferation on the serosal surface, would represent early-stage MPM of epithelial type, whereas invasion of mesothelial cells into the subpleural adipose tissues or deeper chest wall structures is usually a decisive indicator of malignancy. There are certain features which are hardly considered as benign mesothelial entrapment in the fibro-inflammatory process, even if there is no evidence of invasion into the subpleural adipose tissues; first, mesothelial proliferation that extends from the serosal surface to the junction with subpleural adipose tissues, second, the presence of proliferating mesothelial cells only in the deep portion of the thickened parietal pleura and not near the surface of the thickened parietal pleura, third, the outward branching gland structure, a typical feature of MPM of epithelial type, and finally, nodular stromal expansion, a feature of mesothelioma and not of reactive mesothelial processes (Fig. 2). These histological features are indicative of malignancy and useful to find early-stage MPM of epithelial type with invasion into the submesothelial fibrous tissues [22, 25]. Particularly, the outward branching gland structure and nodular stromal expansion are helpful to identify early-stage MPM of epithelial type with only superficial invasion into the submesothelial fibrous tissues, but it should be confirmed that the superficial invasion is genuine by ancillary techniques with diagnostic biomarkers.

Pleural effusion cytology

Approximately 85% of patients with MPM develop pleural effusion, and its examination is usually the first step for diagnosis of MPM [6, 12]. The pleural effusion obtained from patients for the first time may contain a high ratio of MPM cells to other types of cells, such as inflammatory cells. Iatrogenic modifications resulting from repeated puncture and/or drainage are not seen in the first time pleural effusion, and the cellular morphology of MPM cells is well conserved in it. Accordingly, the first time pleural effusion is useful to diagnose early-stage MPM of epithelial type. MPM cells are more likely to be observed in the pleural effusion obtained at the beginning of pathological changes (T0 or T1 stage of International Mesothelioma Interest Group classification) [21, 26], in contrast to lung cancer cells which appear in the pleural effusion following penetration of the visceral pleural of lung and dissemination into the pleural cavity at an advanced stage [27]. Therefore, pleural effusion cytology is valuable to explore patients with early-stage MPM of epithelial type when a tumor mass is clinically undetectable.

MPM cells are usually much larger than the average mesothelial cells and characterized by round or oval nucleus with one or two prominent nucleoli. Although not all MPM cells contain prominent nucleoli, some RMH cells are found to carry prominent nucleoli [6, 12]. Cytopathologically, MPM are characterized by mesothelial cells with multinucleation, window-formation, hump-like cellular processes, cell-in-cell engulfment, thick basophilic cytoplasm, and blurring of cell contour [6, 12, 14, 28]. In addition, MPM cells often form mirror ball-like or papillary cell clusters [6, 12, 21, 28]. These cytological features are useful to diagnose MPM.

Molecular biological approach to diagnose early-stage MPM of epithelial type

Immunocytohistochemistry

The pathological diagnosis of MPM is based on the demonstration of mesothelial and malignant nature of cells. Calretinin, podoplanin (D2-40), Wilms' tumor 1 protein, cytokeratin 5/6, and mesothelin are useful as positive markers for the mesothelial nature, and carcinoma markers, epithelial-related antigen (MOC-31) and carcinoembryonic antigen, are useful as negative markers [1, 6, 21, 29–31]. In addition, thyroid transcription factor-1, Napsin A, and surfactant, which are usually expressed in lung epithelium, are useful to distinguish between MPM and lung carcinoma [29, 32, 33].

Distinction between MPM (malignant proliferation) and RMH (benign proliferation) is challenging. Immunocytohistochemistry of epithelial membrane antigen (EMA), p53, and desmin are somewhat useful to discriminate between MPM and RMH [34–41]. Attanoos et al. [36] studied 60 MM and 40 RMH cases and found that 10% MM were positive for desmin, 80% for EMA, and 45% for p53, while 85% RMH were positive for desmin, 20% for EMA, and 0% for p53. Although strong, thick, linear membrane staining of EMA in atypical mesothelial cells suggests malignancy, the diagnostic sensitivity and specificity for EMA was less than 80%. Telomerase transcriptase expression was once suggested to be applicable for discriminating between hyperplastic and neoplastic mesothelium [42], but this was found to be of limited use in later. GLUT-1 immunoreactivity was found in 40 of 40 MM cases, whereas all 40 cases of RMH were negative. Other studies also showed that GLUT-1 is a useful marker for MM [15]. Recently, CD146, a cell adhesion molecule, was reported to be useful for the discrimination between MPM and RMH with more than 90% sensitivity for detection of MPM and 100% specificity for discrimination between MPM and RMH [16]. In addition, insulin-like growth factor II messenger ribonucleic acid-binding protein 3 (IMP3), an oncofetal protein, has been shown to exhibit strong cytoplasmic staining in 33 of 45 (73%) MM cases and undetectable staining in all 64 cases of benign RMH [18].

$p16^{INK4a}/p14^{ARF}$ (cyclin-dependent kinase inhibitor 2A, *CDKN2A*)

The $p16^{INK4a}/p14^{ARF}$ (*CDKN2A*) gene at chromosome 9p21 has been reported to be frequently deleted in MM [43–45]. Fluorescence in situ hybridization (FISH) analysis using locus-specific $p16^{INK4a}/p14^{ARF}$ probe shows homozygous deletion of this locus in 50–70% primary MM samples or MM cells cultured for less than 5 days. Recent FISH analysis has also demonstrated homozygous deletion of the $p16^{INK4a}/p14^{ARF}$ locus in 35 of 40 cases with MM (88%), but in no cases of RMH [46]. Importantly, other studies confirmed no homozygous deletion of the $p16^{INK4a}/p14^{ARF}$ locus in benign cases [47, 48]. Since the $p16^{INK4a}/p14^{ARF}$ gene is conserved in all non-tumor mesothelial cells and is essential for normal cell cycle control, FISH analysis of the $p16^{INK4a}/p14^{ARF}$ locus can be a definitive diagnosis method for early-stage MPM of epithelial type.

The $p16^{INK4a}/p14^{ARF}$ (*CDKN2A*) gene encodes two cell cycle regulatory proteins which function as tumor suppressor, the p16 protein and, in an alternative reading frame, the p14ARF protein. The p16 protein controls the cell cycle via the cyclin-dependent kinase 4 (CDK4)/cyclin D-RB pathway, while the p14ARF protein regulates p53

through inactivation of human homolog of mouse double minute 2 (HDM2), which is an upstream regulator of p53. Accordingly, homozygous deletion of $p16^{INK4a}/p14^{ARF}$ locus results in the inactivation of two major tumor suppressing pathways of RB and p53. Recent studies showed that mice deficient for either $p16^{Ink4a}$ or $p19^{Arf}$, a mouse homolog of human $p14^{ARF}$, were susceptible to asbestos-induced MM. In addition, mice doubly deficient for $p16^{Ink4a}$ and $p19^{Arf}$ showed accelerated asbestos-induced MM formation relative to mice deficient for $p16^{Ink4a}$ or $p19^{Arf}$ alone, indicating that inactivation of both $p16^{Ink4a}$ and $p19^{Arf}$ accelerates asbestos-induced tumorigenesis [49].

Neurofibromatosis type 2

The neurofibromatosis type 2 (*NF2*) tumor suppressor gene at 22q12 was originally identified as a gene involved in NF2 familial cancer syndrome [50, 51]. The characteristic tumors of this syndrome are vestibular schwannoma and meningioma, and accordingly the protein product of the *NF2* gene was called Merlin or Schwannomin [52]. The *NF2* gene is inactivated by homozygous deletion, nonsense mutation, or missense mutation in 40–50% of MM [50, 51]. Recent studies have revealed multiple downstream signaling cascades, including mTOR and Hippo signaling cascades, regulated by underphosphorylated (activated) Merlin [53]. The Hippo signaling cascade regulates the yes-associated protein (YAP), a transcriptional coactivator involved in transcription of multiple genes regulating proliferation and anti-apoptosis. More recently, the large tumor suppressor homolog 2 (*LATS2*) gene, which encodes a component of the Hippo cascade, has also been shown to be mutated in MM [54]. It is therefore likely that dysregulation of the Merlin-Hippo signaling plays a key role in MM development and/or progression via constitutive activation of YAP. In an animal model [55], Nf2 (+/−) knockout mice were shown to develop MM earlier after asbestos exposure than asbestos-treated wild-type littermates. Loss of the wild-type *Nf2* allele, leading to biallelic inactivation, was observed in all asbestos-induced MM from Nf2 (+/−) mice. Taken together, FISH analysis of the *NF2* locus is not as valuable for diagnosis of MPM as the $p16^{INK4a}/p14^{ARF}$ locus, but analysis of the NF2 signaling pathway in MPM has a potential to develop new diagnostic and therapeutic tools for MPM including its early stage.

BAP-1

BRCA1-associated protein 1 (BAP1) has been initially identified as a protein that binds to the RING finger domain of BRCA1 and exhibits tumor suppressor activity. BAP1 is one of cysteine proteases, called deubiquitinating enzymes, which catalyze the removal of ubiquitin chains from ubiquitinated proteins [56, 57]. BAP1 seems to regulate deubiquitination in the DNA damage response and the cell cycle, thus influencing S-phase progression, cell necrosis, and apoptosis. Mutations that abolish the deubiquitination activity of BAP1 and/or its nuclear localization result in the loss of BAP1 tumor suppressor activity. It has been reported that *BAP1* gene is frequently mutated in metastasizing uveal melanoma, but not in low metastatic uveal melanoma [58].

It has recently been reported that BAP1 somatic mutations are found in 25% of sporadic MM [59] and that individuals with heterozygous BAP1 germline mutations are affected by a novel cancer syndrome characterized by very high risk of developing MM, uveal melanoma, and other cancers [60]. In addition, more recent studies using array-based comparative genomic hybridization (CGH) have shown that 11 of 14 cases (79%) of MM of epithelial type exhibits homozygous and heterozygous deletions of 3p21.1, in which nine genes including *BAP1* gene are located [61]. Therefore, there is a possibility that BAP1 becomes a useful marker to distinguish MM of epithelial type from RMH.

Other molecular abnormalities

In addition to homozygous deletion of 9p21, 22q12, and 3p21.1 regions, it has been reported that genomic aberrations with gains were present in 1q, 5p, 7p, 8q24, and 20p regions and those with losses in 1p36.33, 1p36.1, 1p21.3, 3p21.3, 4q22, 4q34-qter, 6q25, 10p, 13q33.2, 14q32.13, and 18q regions [62]. The effect of these genomic abnormalities on pathogenesis of MPM and their usefulness as molecular diagnostic markers for MPM remain to be investigated.

Constitutive activation of receptor tyrosine kinase (RTK), which often results from gene amplification or gain-of-function mutations that do not require their ligand binding, participates in proliferation of tumor cells, but no gain-of-function mutations of RTK genes have been identified in MM [63]. On the other hand, it has been reported that the downstream signaling cascades of RTK, including the mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3-kinase (PI3K)-AKT cascades, are activated in most cases of MM [64]. Activation of mammalian target of rapamycin (mTOR), a downstream molecule of the AKT pathway, has also been shown in MM with active status of AKT [64]. Homozygous deletion of phosphatase and tensin homolog (PTEN), which is responsible for AKT activation, has been reported in a small subset of MM cell lines [64, 65]. However, whether this deletion is present in primary MM is unclear, since one previous study found no *PTEN* mutation in 18 primary MM [66].

Mutations of the *p53*, *Ras*, and *RB* genes found commonly in other malignancies have not been reported in MM [67, 68], except for one study showing mutation of *N-ras* gene in three of 38 MM cases [69].

Conclusions

Histopathological features of early-stage MPM of epithelial type are beginning to be understood, but it is still difficult to morphologically distinguish early-stage MPM of epithelial type from RMH. It is generally accepted that homozygous deletion of the $p16^{INK4a}/p14^{ARF}$ locus is of value as a molecular marker for diagnosis of MPM. In order to precisely diagnose early-stage MPM of epithelial type, a more in-depth understanding of molecular pathogenesis of MPM, including sequential accumulation of genetic/epigenetic alterations during MPM development, is urgently needed.

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Conflict of interest No author has any conflict of interest.

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Prognostic Impact of Circulating Tumor Cells in Patients with Small Cell Lung Cancer

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Background: Enumeration of circulating tumor cells (CTCs) may be valuable for prognostic assessment in lung cancer patients. In this study, we report the clinical significance of CTCs in small cell lung cancer (SCLC).

Methods: In total, 51 consecutive patients newly diagnosed as having SCLC and starting chemotherapy or chemoradiotherapy were prospectively enrolled. Blood samples were drawn at the baseline, after chemotherapy, and at relapse. CTCs were isolated using the CellSearch System (Veridex LLC). Thresholds of 1 to 100 cells at the baseline were systematically correlated with the overall survival. The optimal cutoff was determined by comparing the Cox proportional hazard ratios (HRs).

Results: Two or more CTCs were detected at baseline in 35 patients (68.6%; 95% confidence interval, 55.0–79.7). The HR signifying the difference between the unfavorable (more than or equal to threshold) and favorable (less than threshold) groups was maximal at the threshold of 8 CTCs (HR, 3.50; 95% confidence interval, 1.45–8.60). Patients with ≥8 CTCs had worse survival than those with <8 CTCs at baseline ($p = 0.0014$). Patients with ≥8 CTCs posttreatment or at relapse also showed worse survival than those with <8 CTCs ($p = 0.0096$ and <0.0001). Patients whose baseline and posttreatment CTC levels remained <8 tended to show better survival than those whose CTC level converted from ≥8 to <8 cells ($p = 0.0288$) or whose posttreatment CTC level was ≥8 cells ($p = 0.0047$).

Conclusions: CTCs were highly detectable in SCLC, and higher CTC levels were strongly associated with worse survival. Consistently favorable CTC levels were associated with favorable outcomes.

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Small cell lung cancer (SCLC) accounts for 15% of all lung cancer diagnoses and is characterized by aggressive tumor growth, often presenting with metastases in the regional lymph nodes and distant organs. Because SCLC is highly sensitive to chemotherapy and radiotherapy, early diagnosis followed by appropriate treatment can be expected to yield favorable outcomes.[1,2] Circulating tumor cells (CTCs) are known to circulate in the peripheral blood in patients with several types of malignancies,[3–6] while rarely being detected (0.3–1.0%) in healthy control subjects or patients with non-malignant diseases.[3,7,8] The CellSearch system (Veridex LLC, Raritan, NJ) is a well-validated system for quantitative evaluation of CTCs, in which CTCs are immunomagnetically captured using an antibody against epithelial cell adhesion molecules (EpCAMs).[9,10] A growing body of evidence suggests the existence of a correlation between CTC level as measured by the CellSearch system and the progression-free survival (PFS) and overall survival (OS) in patients with metastatic breast, colorectal, castration-resistant prostate, and non-small cell lung cancers (NSCLC).[7,11–15] In SCLC, the detection rate of CTCs by the Cell Search system has been reported to be relatively high, with 67 to 86% of the patients being reported to have ≥2 CTCs per 7.5 ml of blood.[8,16,17] However, the prognostic impact of CTCs and their relationship to the presence of metastases in patients with SCLC remain unknown. We conducted this study to evaluate the relationship of CTC levels to the disease extent and prognosis and to determine the optimal CTC level cutoff for predicting the outcomes in SCLC patients.

METHODS

Study Design

This prospective study was conducted at two institutions (Shizuoka Cancer Center and Hyogo College of Medicine) to evaluate the usefulness of measurement of the CTC

levels for predicting the OS. Patients with chemotherapy-naive, pathologically confirmed SCLC scheduled to commence first-line standard chemotherapy with or without thoracic radiotherapy were eligible. All patients were enrolled at the Shizuoka Cancer Center and had an Eastern Cooperative Oncology Group performance status (ECOG-PS) of 0 to 2. The institutional review boards at each center approved the study protocol, and all patients provided written informed consent. Before the start of the new treatment, the patients underwent an evaluation of metastatic sites by means of standard imaging studies, including contrast-enhanced computed tomography of the chest to lower abdomen, contrast-enhanced magnetic resonance imaging of the brain, and bone scan or positron emission tomography, along with the collection of blood sampled for counting of the baseline CTCs. The post-treatment blood samples were collected 3 weeks after completion of the last chemotherapy cycle or completion of sequential thoracic radiotherapy. The samples were collected 2 weeks after relapse had been diagnosed by imaging and before administration of the second-line chemotherapy. The sampling date could be adjusted depending on the type of treatment and the visit schedule, with allowance for ±2 weeks. Reevaluations of the disease status were conducted using the same techniques as those applied at the baseline, every 8 to 12 weeks, depending on the type of treatment the patient had received and the treatment schedule. Disease status was assessed according to the RECIST[18] by examiners with no knowledge of the CTC levels. Serum lactate dehydrogenese (LDH) levels and the levels of other biomarkers, including neuron-specific enolase (NSE) and progastrin-releasing peptide (ProGRP), were measured at the same time point as the baseline CTC measurement. The blood samples for the serum biomarker measurements were obtained by venous puncture, and the sera were stored at −40°C until use. The ProGRP concentration was measured using an ELISA kit (FUJIREBIO Inc., Tokyo, Japan), and the NSE concentration was measured using the radioimmunoassay solid-phase method (SRL Inc., Tokyo, Japan).

Counting of CTCs

Blood samples were drawn into 10-ml vacuum tubes (CellSave, Immunicon, Huntingdon Valley, PA). Samples were maintained at room temperature, mailed overnight, and processed within 96 hours of collection. The results were reported quantitatively as the number of CTCs per 7.5 ml of blood. All CTC evaluations were performed without knowledge of the patient clinical status in one of two laboratories (Hyogo College of Medicine, Japan, or the laboratory of SRL Inc.). The CellSearch system was used for the CTC counting, the technical details of which, including accuracy, precision, linearity, and reproducibility, have been previously described.[3] CTCs were defined as EpCAM-isolated intact cells showing positive staining for cytokeratin and negative staining for CD45. At each time point, the favorable and unfavorable groups were defined as those with CTC levels less than or more than or equal to the selected threshold, respectively.

Statistical Analysis

The primary analysis was a comparison of the OS between the unfavorable and favorable groups stratified according to the selected threshold of CTC level. The study was designed to enroll 50 patients for a statistical power of 80% with a two-sided log-rank test at a level of 0.05 to detect an absolute difference of 40% points between the two groups in the 1-year estimates of OS (20% in the unfavorable group versus 60% in the favorable group). To select the threshold CTC level that most clearly distinguished patients with an unfavorable prognosis from those with a favorable prognosis, thresholds of 1 to 100 cells at baseline were systematically correlated with the OS. The Cox proportional hazard ratio (HR), goodness-of-fit, and discriminatory power of each threshold were compared. The Bonferroni correction was applied for multiple testing for 14 thresholds, and a p value of <0.0036 was set to obtain a statistical significance of $p < 0.05$. The goodness-of-fit of the model was assessed by the coefficient of determination (R^2) defined as 1 − {(log likelihood of the estimated model)/(log likelihood of the model with only the intercept)}. The discriminatory power was assessed by the accuracy rate ([AR] defined as the rate of correct diagnosis among all predictions of 1-year survivors) and the area under the receiver operator characteristics curve (AUROC). The treatment-free interval (TFI) was defined as the time between the completion of first-line chemotherapy and the diagnosis of relapse. Patients with a TFI of 90 days or more were considered to have treatment-sensitive disease, and those with a TFI of less than 90 days were considered to have treatment-refractory disease. For all survival analyses, the time to death was defined as the time between the date when the blood sample was obtained and the date of death or date of the last follow-up visit. Separate Kaplan-Meier survival plots were generated based on the CTC levels at baseline and the results in the follow-up blood collections. Survival curves were compared using the log-rank test. Cox proportional hazards regression was used to determine the HRs for the OS adjusted for age, gender, pretreatment stage (extensive disease [ED] versus limited disease [LD]), and ECOG-PS at the time of blood collection. The discriminatory power of the baseline CTC, LDH, NSE, and ProGRP for predicting 1-year survivors was compared by AUROC. The χ^2 test or Fisher exact test was used to compare categorical variables. For comparison of the means, the nonparametric Wilcoxon's test or analysis of variance was used. We tested the correlations between variables by calculating the Spearman's rank correlation coefficients. Calculations were carried out using the statistical program, JMP version 9.0 for Windows (SAS Institute Inc., Cary, NC).

RESULTS

Patient Characteristics

In total, 51 consecutive patients met the inclusion criteria and were prospectively enrolled between July 2009 and September 2010. The cutoff date for analysis was August 31, 2011. The median age of the patients was 67 years, and 44 of the patients (86.3%) were men (Table 1). Nineteen of the

TABLE 1. Baseline Characteristics

Characteristics	All (n = 51)	Extensive Disease (n = 24)	Limited Disease (n = 27)
Age, median (range)	67 (34–92)	66.5 (57–80)	68 (34–92)
Gender (female:male)	7:44	3:21	4:23
ECOG-PS, n (%)			
0	21 (41.2)	6 (25.0)	15 (55.6)
1	21 (41.2)	10 (41.7)	11 (40.7)
2	9 (17.6)	8 (33.3)	1 (3.7)
No. of organs with metastasis, median (range)	0.5 (0–3)	1 (0–2)	None
Brain metastasis, n (%)	7 (13.7)	7 (29.2)	None
Liver metastasis	8 (15.7)	8 (33.3)	None
Bone metastasis	3 (5.9)	3 (12.5)	None
Malignant effusion	12 (23.5)	11 (45.8)	1 (3.7)
Serum biomarkers (mean ± SE)			
NSE (ng/ml)	75.7 ± 24.3	131.2 ± 49.5	26.4 ± 5.5
ProGRP (pg/ml)	657.2 ± 205.7	1071.3 ± 419.1	289.0 ± 66.7
LDH (IU/L)	360.5 ± 79.9	529.7 ± 164.4	210.1 ± 8.8
Treatments, n (%)			
Chemotherapy alone	32 (62.7)	24 (100.0)	8 (29.6)
Chemoradiotherapy	19 (37.3)	None	19 (70.4)
Regimens, median cycle (range)	4 (1–6)	4 (1–6)	4 (1–5)
Cisplatin + etoposide, n (%)	23 (45.1)	16 (66.7)	7 (25.9)
Carboplatin + etoposide	21 (41.2)	2 (8.3)	19 (76.4)
Cisplatin + irinotecan	7 (13.7)	6 (25.0)	1 (3.7)

ECOG-PS, Eastern Cooperative Oncology Group performance status; SE, standard error; NSE, neuron-specific enolase; ProGRP, progastrin-releasing peptide; LDH, lactate dehydrogenese.

27 patients with LD had received chemoradiotherapy, while the remaining 8 patients could not receive radiotherapy for the following reasons and were treated by chemotherapy alone. The first patient was a 73-year-old man with a treatment history of thoracic chemoradiotherapy for esophageal cancer 6 years before the current treatment. Reirradiation was avoided because of the potential late adverse effects of radiotherapy. The second patient was a 79-year-old man with poor pulmonary functions who was scheduled for sequential radiotherapy after chemotherapy. However, his tumor progressed, with the development of contralateral pulmonary metastases after the first course of chemotherapy, and radiotherapy could not be administered. The remaining six patients had interstitial lung disease before the start of the treatment. Thoracic radiotherapy was withheld because of the potential risk of severe radiation pneumonitis. Twenty-four patients (47.1%) were still alive at the time of analysis. The median follow-up period for determining the survival was 13.0 months after the baseline blood sample collection. All 51 patients were evaluable for the baseline CTC level. Blood samples were not obtained during follow-up from two patients who died of interstitial lung disease and cancer progression. The remaining 49 patients were evaluable for the posttreatment CTC levels. The median time between the baseline and posttreatment blood collections was 3.4 months. Thirty-eight patients (74.5%) exhibited tumor progression; 37 were evaluable for the CTC level at the time of relapse, and 1 woman refused to provide blood samples.

Circulating Tumor Cells

Two or more CTCs were detected in 68.6% of the patients (95% confidence interval [CI], 55.0–79.7) at baseline, in 26.5% of the patients (95% CI, 16.2–40.3) posttreatment, and in 67.6% of the patients (95% CI, 51.5–80.4) at the time of relapse (Table 2). The CTC counts at baseline were higher in patients with ED, who showed a median of 9.5 cells (range, 0–5648), than in those with LD, who showed a median of 1 cell (range, 0–58; $p = 0.0001$, Figure 1*A*). Fourteen of the 16 patients (87.5%) who had a baseline CTC level of ≤1 had LD. The median CTC levels at baseline in patients with 0, 1, and ≥2 organs showing metastases were 2.0 (range, 0–58), 7.5 (1–799), and 21.0 (0–5648), respectively, showing a statistically significant correlation of the CTC count with the number of organs showing metastases (Spearman's rho, 0.72, $p < 0.0001$, Figure 1*B*). Patients with liver metastasis had higher CTC levels than those without liver metastasis (64 [range, 5–5648] versus 3 [range, 0–799]; $p = 0.0007$). There was no association between brain or bone metastasis and the CTC levels (data not shown).

Stratification According to Levels of Circulating Tumor Cells

The baseline CTC level was predictive of the OS when it was stratified by the threshold of 8 cells ($p = 0.0029$; Table 3). The Cox proportional HR signifying the difference between the unfavorable (more than or equal to threshold) and favorable (less than threshold) groups showed a waxing and waning pattern with the peak at the level of 8 CTCs. The HR associated with a CTC level of 8 cells was 3.50 (95% CI, 1.45–8.60) after adjustment for stage (ED or LD), age, gender, and ECOG-PS at the baseline. The Cox proportional hazard model at this level also showed a favorable goodness-of-fit and discriminatory power with the highest R^2, AR, and AUROC among all the thresholds examined. Thus, a cutoff level of 8 CTCs was chosen for the subsequent analyses. Analyses based on the stage (ED or LD) and therapy type

TABLE 2. CTC Levels at the Baseline, Posttreatment, and at the Time of Relapse

	Baseline	Posttreatment	At Relapse
Total[a]	51	49	38
Evaluable[b]	51	49	37
CTC, median (range)	4 (0–5648)	0 (0–253)	1 (0–510)
CTC, mean ± SE	203.2 ± 118.5	10.2 ± 5.9	44.6 ± 16.8
CTC ≥ 2, % (95% CI)	68.6 (55.0–79.7)	26.5 (16.2–40.3)	67.6 (51.5–80.4)

[a] Number of patients alive and evaluable.
[b] Number of patients with nonmissing data for CTCs at the time-point indicated.
CTC, circulating tumor cell; SE, standard error; CI, confidence interval.

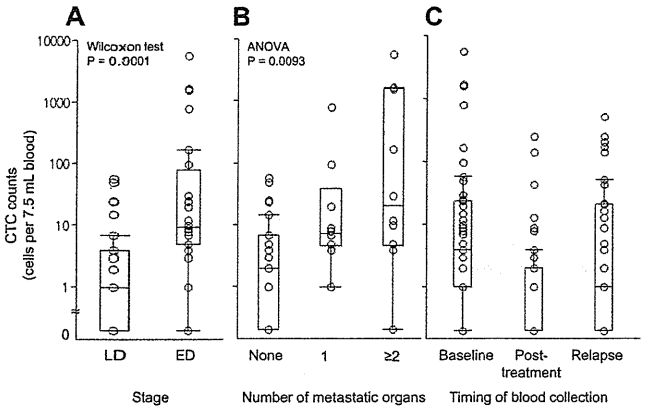

FIGURE 1. Box plots were drawn using the minimum and maximum values and the 25th, 50th, and 75th percentiles. *A*, Circulating tumor cell (CTC) levels at the baseline and the disease stage. ED, extensive disease; LD, limited disease. *B*, CTC levels at the baseline and number of metastatic organs. *C*, CTC levels at the baseline and the timing of blood sampling. *p* values calculated by Wilcoxon's test and analysis of variance (ANOVA) are presented.

TABLE 3. Baseline CTC and Prognosis

CTC Level[a]	Adjusted HR (95% CI)[b]	p^c	R^2	AR	AUROC (95% CI)
1	0.74 (0.26–2.40)	0.0604	0.06	0.49	0.55 (0.43–0.65)
2	0.67 (0.25–1.87)	0.0532	0.06	0.51	0.55 (0.42–0.67)
3	0.76 (0.27–2.11)	0.0606	0.06	0.55	0.58 (0.45–0.71)
4	0.85 (0.25–2.79)	0.0656	0.05	0.61	0.63 (0.48–0.75)
5	1.59 (0.61–4.29)	0.0481	0.06	0.67	0.68 (0.53–0.80)
6	2.97 (1.24–7.31)	0.0063	0.08	0.73	0.73 (0.58–0.84)
7	2.97 (1.24–7.31)	0.0063	0.08	0.73	0.73 (0.58–0.84)
8	3.50 (1.45–8.60)	0.0029	0.09	0.76	0.74 (0.59–0.85)
9	2.90 (1.20–7.04)	0.0072	0.08	0.73	0.71 (0.57–0.83)
10	2.41 (0.99–5.81)	0.0151	0.07	0.71	0.69 (0.54–0.80)
15	3.00 (1.19–7.40)	0.0079	0.08	0.71	0.68 (0.54–0.79)
25	2.02 (0.74–5.04)	0.0318	0.06	0.67	0.62 (0.50–0.73)
50	3.49 (1.23–9.79)	0.0107	0.08	0.67	0.62 (0.50–0.72)
100	3.97 (0.90–15.59)	0.0181	0.07	0.65	0.58 (0.48–0.67)

[a] CTC levels are expressed as the number of cells per 7.5 ml of blood.
[b] The Cox proportional hazard ratios were adjusted for stage, age, gender, and ECOG-PS at the baseline.
[c] The level of significance calculated by the Bonferroni method was $p < 0.0036$.
HR, hazard ratio; CTC, circulating tumor cell; CI, confidence interval; AR, accuracy rate for predicting 1-year survivors; AUROC, area under the receiver operator characteristics curve for predicting 1-year survivors.

(chemotherapy alone or chemoradiotherapy) showed that the prognostic significance of the CTC level was significant only in the ED subset and in the patients treated by chemotherapy alone (Supplemental Table 1, Supplemental Digital Content 1, http://links.lww.com/JTO/A204).

Baseline CTC and Prognosis

Figure 2*A* shows the Kaplan-Meier curves for the OS according to the baseline CTC levels. Patients in the unfavorable group had significantly shorter survival than those in the favorable group ($p = 0.0014$). The 1-year survival rates and the median OS in the unfavorable and favorable groups were 31.6% versus 78.0% and 8.5 versus 17.2 months, respectively. The sensitivity, specificity, AR, and AUROC for predicting 1-year survivors using the cutoff level of 8 CTCs were 0.81, 0.65, 0.75, and 0.73 (95% CI, 0.58–0.84), respectively. The 1-year survival rates in the unfavorable and favorable groups were 21.4 and 70.0% ($p = 0.0282$), respectively, in the ED subset, and 60.0 and 81.6% ($p = 0.4387$), respectively, in the LD subset (Figures 2*B*, *C*).

Posttreatment CTC and Prognosis

During the posttreatment period, the CTC levels were measured in the 49 patients who were available for the evaluation. Of these 49 patients, 7 (14.3%) with ≥8 CTCs had a significantly shorter posttreatment survival than the remaining 42 (85.7%) with <8 CTCs ($p = 0.0096$, Figure 2*D*). The HR of the threshold CTC count adjusted for stage, age, and posttreatment PS was 2.76 (95% CI, 0.97–6.92, $p = 0.0562$). The median posttreatment survivals in the unfavorable and favorable groups were 4.1 and 13.9 months, respectively. At the time of relapse, CTC levels were measured in 37 patients. Of these 37 patients, the 13 (35.1%) with ≥8 CTCs had a significantly shorter postrelapse survival than the remaining 24 (64.9%) with <8 CTCs ($p < 0.0001$, Figure 2*E*). The HR of the threshold CTC adjusted for stage, age, TFI (<90 versus ≥90 days), and PS at the time of relapse was 6.20 (95% CI, 2.39–17.52, $p = 0.0002$). The median postrelapse survivals in the unfavorable and favorable groups were 4.0 and 11.8 months, respectively.

Posttreatment CTC Status and Prognosis

Among the 42 patients with posttreatment CTC levels of <8, 29 had a baseline CTC level also of <8 (group A), and in the remaining 13, the baseline CTC level was ≥8 (group B). Among the seven patients with posttreatment CTC levels of ≥8 (group C), four had a baseline CTC level also of ≥8, and the remaining three had a baseline CTC level of <8. As shown in Figure 3, the survival impact of conversion from an unfavorable to favorable CTC level was assessed by using the Kaplan-Meier curve for posttreatment survival according to the posttreatment CTC status. The median posttreatment survival was >18.8 months in group A, 7.2 months in group B, and 4.1 months in group C ($p = 0.0066$). The difference in the survival between group A and group C was significant ($p = 0.0047$ by log-rank test; level of significance calculated by the Bonferroni method, $p = 0.0166$). Conversely, there was no significant difference between group A and group B ($p = 0.0288$), or group B and group C ($p = 0.2489$). The HR adjusted for the pretreatment stage, posttreatment ECOG-PS, and TFI was 3.08 (95% CI, 1.03–8.90; $p = 0.0450$) in group B and 3.29 (95% CI, 1.01–10.07; $p = 0.0479$) in group C, both calculated using group A as the reference (Table 4).

Discriminatory Power of CTCs and Serum Biomarkers for Predicting the Prognosis

Figure 4 shows the receiver operator characteristics curves for CTCs, and the serum levels of LDH, NSE, and ProGRP measured at the baseline. Data on survival at 1 year

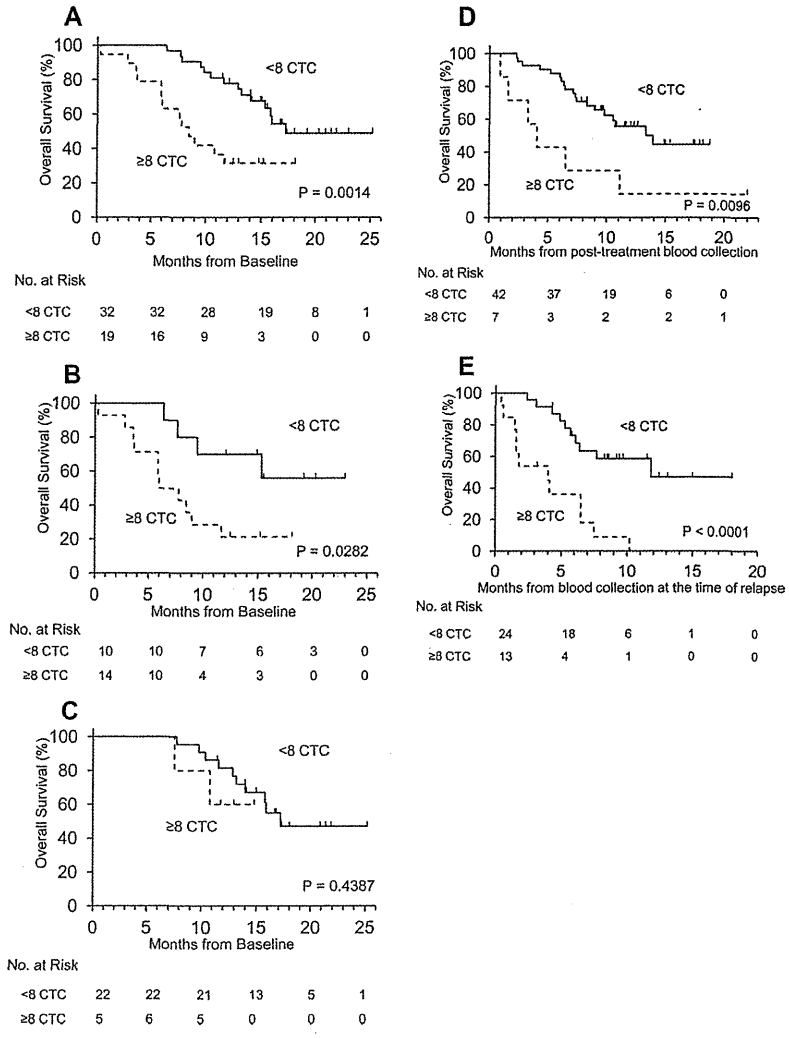

FIGURE 2. Kaplan-Meier curves for overall survival in patients with <8 and ≥8 circulating tumor cells (CTCs) at the baseline in the full set of data (*A*), extensive disease subset (*B*), and limited disease subset (*C*). Kaplan-Meier curves for posttreatment survival and postrelapse survival in patients with <8 and ≥8 CTCs posttreatment and at relapse (*D* and *E*). *p* values calculated by the log-rank test are presented.

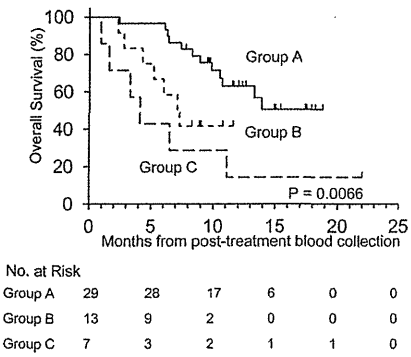

FIGURE 3. Kaplan-Meier curves for posttreatment survival in three groups, including patients in whom the baseline and posttreatment circulating tumor cell (CTC) levels remained at <8 (group A), patients in whom the CTC level converted from ≥8 to <8 cells (group B), and patients in whom the posttreatment CTC level was ≥8 cells (group C). *p* values calculated by the log-rank test are presented.

TABLE 4. Hazard Ratios of the Posttreatment Status of CTC Level

Posttreatment CTC Status	CTC Level[a]	*n*	MST (mo)	Adjusted HR (95% CI)[b]	*p*
Group A	<8–<8	29	NR	Reference	
Group B	≥8–<8	13	7.2	3.08 (1.03–8.90)	0.0450
Group C	≥8–≥8 or <8–≥8	7	4.1	3.29 (1.01–10.07)	0.0479

Group A: patients whose baseline and posttreatment CTC levels remained <8 cells; group B: patients whose CTC level converted from ≥8 to <8 cells; and group C: patients whose posttreatment CTC level was ≥8 cells.

[a] CTC levels are expressed as the number of cells per 7.5 ml of blood.

[b] The Cox proportional hazard ratios were adjusted for the pretreatment stage, posttreatment ECOG-PS, and treatment-free interval.

CTC, circulating tumor cell; HR, hazard ratio; MST, median survival time; CI, confidence interval; NR, not reached.

were available for all 51 patients. The baseline CTC level showed a favorable discriminatory profile, showing an AUROC of 0.70 (95% CI, 0.52–0.83), as compared with that of 0.67 (0.49–0.82) for LDH, 0.68 (0.52–0.82) for NSE, and 0.46