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MAKING THE CASE FOR MOLECULAR STAGING OF MPM

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INTRODUCTION

Trimodality treatment consisting of surgical resection with chemotherapy and radiation leads to longer survival than non-surgical therapy in patients with malignant pleural mesothelioma (MPM). [1–13] This aggressive approach, however, benefits only a subset (~50%) of patients and entails substantial costs and adverse effects. Current methods for determining prognosis are insufficient to distinguish between individuals who will benefit from multimodality treatment and those who will not. There is a critical need for new predictive tools that can supplement or replace current methods of diagnosis, prognosis, clinical staging, and treatment design. Our laboratory has developed a simple predictive molecular test based on gene expression profiling that has prognostic value for identifying patients with MPM most likely to benefit from aggressive multimodality treatment.[14–16] This *4-gene ratio test* recently has been validated in a prospective clinical database at our institution.[17]

RATIONALE FOR PREDICTIVE TESTS AND EXISTING PROGNOSTIC MARKERS IN MPM

The median survival of patients undergoing trimodality therapy is 1–2 years, but some patients (20%) can live disease-free for 3–15 years. This represents a substantial subset of patients in whom an aggressive surgical approach is justified.[8,17] Given the wide range of survival among patients with seemingly identical tumors, however, there is a critical need for molecular markers that are capable of predicting survival. An exhaustive review of current knowledge about molecular markers expressed in MPM is beyond the scope of the article.[18] Suffice it to say that none of the biomarkers identified, to date, has been sufficiently developed for application or validation in clinical studies.

Clinical predictors

While conventional clinical findings and laboratory tests have some value in predicting survival in surgical MPM patients, these tools remain very limited. For example, prognosis in patients enrolled on clinical chemotherapy trials is predicted by the functional status, presence of intractable chest pain, advanced age, platelet count, and presence of distant metastases. These values are not useful in the surgical setting since, by definition, patients who register poorly in these categories, are excluded from aggressive surgical therapies.[19] Tumor pathology and

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stage-based predictive parameters for surgical candidates include histologic subtype, lymph node status, and to some degree, disease stage and tumor volume.

Histologic subtype

MPM is generally classified histologically as epithelial, biphasic (mixed), or sarcomatoid. Most tumors (~60%) are predominantly epithelial, and this cell type is associated with a somewhat better prognosis. Biphasic tumors have both epithelial and sarcomatoid elements. Fewer than 10% of MPM tumors are purely sarcomatoid, and patients with these tumors have substantially poorer outcome.[6,20,21]

Lymph nodes

Lymph node status correlates strongly with survival in surgical patients. The presence of tumor in mediastinal lymph nodes predicts nearly 0% 2-year survival. Consequently, preoperative staging by cervical mediastinoscopy or endoscopic ultrasound (EUS)-based lymph node biopsy [22] is routine at our institution and has been widely adopted at other centers. Patients with positive lymph nodes often are referred for neoadjuvant chemotherapy to down-stage their tumor burden before consideration of surgical therapy. Absence of positive mediastinal lymph nodes, however, is not a useful predictor of prognosis nor a sign that patients will necessarily benefit from trimodality therapy, because often such nodes are not surgically accessible for sampling before definitive resection.[6,20,23]

Staging

A major problem in the staging approach to MPM is that multiple non-inter-convertible staging systems are in use, most of which are indeterminable preoperatively because they are based on the pathological results of resection (see Dr. Richards' article in this issue). All such systems classify most patients as stage III, which prevents the subtle differentiation needed to predict treatment outcomes (i.e., which therapies are best for which patients).[8,23–31] Despite extensive study, conventional clinico-pathological classification strategies have proved inadequate for the task of predicting treatment outcomes and assigning therapies, underscoring the need for more sophisticated approaches.

MOLECULAR PREDICTIVE TESTS: GENE RATIO-BASED PREDICTIVE TEST FOR MPM

A number of studies have used either the whole-genome approach to profiling gene expression, comparative genomic hybridization (CGH), or methylation. Although these studies have led to proposed predictive markers for MPM, most have not demonstrated any ability to define clinically relevant subgroups of patients for treatment stratification.[14–16,32–37] We have recently developed a molecular diagnostic and predictive test for MPM that permits a more accurate diagnosis and a more rational basis for assigning therapies.[15,38] Below, we summarize the development and validation of the gene ratio-based predictive test for MPM.

Development and description[15]

Our original goal was to develop a predictive MPM gene expression ratio test that could be used to translate comprehensive (RNA) expression profiling data into a simple, clinically useful predictive test based on the expression levels of a relatively small number of genes. This algorithm identifies genes that are differentially expressed between two clinically distinct conditions and calculates ratios of gene expression for gene pairs that predict the condition, either alone or in combination. This approach has major advantages in comparison with traditional approaches. First, both genes in such a given gene-pair ratio are informative. Second, by using a ratio, which has no units of measure, this technique is microarray platform-

independent and easy to use with individual specimens. The latter properties are extremely important elements of our translational research for developing predictive molecular tests, because they ensure the widespread and easy clinical introduction of the method.[14] The principal utility of this test, in contrast with many proposed predictive tests in cancer, is that it does not depend on highly specific instrumentation and the test can be performed in any laboratory and still deliver a consistent, reproducible result. We and others have demonstrated the utility of this bioinformatic technique in the diagnosis of many malignancies including lung cancer, MPM, bladder cancer, and prostate cancer as well as in the prognosis of medulloblastoma, breast cancer, lung cancer, and MPM.[16,39–44]

Principle of the MPM predictive test

The purpose of the predictive gene ratio test is to compare the differences in gene expression in tissue samples of MPM stratified by patient outcome (survival) after surgical therapy. The test ratio is calculated from the relative expression levels of each of four genes, analyzed by quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) or microarrays. The geometric mean then is calculated for the following three gene-pair expression ratios: *TM4SF1/PKM2*, *TM4SF1/ARHGDIA*, and *COBLL1/ARHGDIA*. The resulting prognosis is assigned as *good* (geometric mean >1) or *poor* (geometric mean <1). As demonstrated in multiple reports, the test differentiated reliably between patients with good versus poor prognosis, on the basis of post-surgical survival and cancer-specific survival in two independent retrospective cohorts of MPM patients.[14–16]

Validation in a prospective trial [17]

Before a predictive test can be approved for clinical use, it must be validated in a prospective clinical trial. Such prospective validation, however, has rarely been reported for the various molecular predictive tests for cancer that have been proposed or approved for clinical use. [17,45] To further validate the gene ratio predictive test for MPM, we tested its ability to predict overall survival and cancer-specific survival in a prospective clinical trial of patients undergoing EPP for MPM at our institution. In this same prospectively consented cohort, we also measured the technical assay performance characteristics of the test.[17]

Patient sample

Between 2001 and 2006, a total of 120 consecutive patients who were scheduled for and ultimately underwent EPP surgery for MPM at Brigham and Women's Hospital provided written informed consent, under an IRB-approved protocol, to submit their surgical specimens for analysis of gene expression and to correlate those data with clinical outcomes. All of the patients were preoperatively staged by standard clinical and radiologic criteria and considered to be candidates for surgery.[9,13] None of the patients had received pre-operative chemotherapy or radiation therapy. The tumors from this independent, prospectively enrolled cohort had not previously been subjected to this type of molecular analysis and were distinct from those tumors used to develop the original test.[15,16] The clinical data were collected by an independent clinical research staff that was blinded to the test results. This information included diagnosis, age, sex, survival, and other demographic and outcome data. Clinical and gene expression datasets, including gene ratio test predictions, were merged at the final stage of data analysis by a statistician, who was not involved in the clinical or gene-expression data collection.

Results

At the time of analysis, the median follow-up among the 38 patients who were still alive was 15 months and the minimum follow-up was 4 months after surgery. The analysis included 32 patients still alive without evidence of disease, four patients alive with recurrent disease, 65

patients dead of disease, 16 patients dead of other causes, one patient who died of unknown cause, and 2 patients lost to follow-up. The median overall survival of the entire cohort (120 patients) was 12.9 months (95% CI = 11.1 to 16.8 months).[17] The gene ratio test was performed and applied exactly as previously described,[15] and used to assign the patients to two groups. Seventy (58%) of the 120 patients were assigned to the *good* outcome group and 50 (42%) were assigned to the *poor* outcome group. Overall survival of the *good* outcome group was significantly ($P < .001$) greater than that of the *poor* outcome group (median values: *good* outcome group 16.8 months, 95% CI = 12.4–25.8 months; vs. *poor* outcome group 9.5 months, 95% CI = 7.2–13.6 months) (Fig. 1). The gene ratio test also detected a significant ($P = .007$) improvement in cancer-specific survival in the *good* outcome group (median cancer-specific survival 21.9 months, 95% CI = 16.7–40.7 months) vs. that of the *poor* outcome group (15.9 months, 95% CI = 8.6–21.0 months). Two well-established prognostic factors for MPM--lymph node status and histological subtype--were also strongly related to outcome in the univariate analysis[17]

Multivariate analysis

To assess further the robustness of the gene ratio test, we used a multivariable model to adjust for the effects of histological subtype, tumor stage, and lymph node status as covariates in the patient survival data. It is noteworthy that the prognostic contributions of both lymph node status (hazard ratio [HR] = 1.97, 95% CI = 1.15–3.38, $P = .013$) and histological subtype (HR = 1.88, 95% CI = 1.14–3.10, $P = .013$) remained statistically significant in the multivariable model, as this is evidence that the gene ratio test (HR for death = 2.09, 95% CI = 1.27 to 3.45, $P = .004$) provides predictive information not obtainable by current pathological staging methods. The hazard ratio for the gene ratio test also was in the same range (HR = 2.0 – 4.6) as that found in our initial studies,[15,16] which used several different platforms for gene expression analysis. These results support the robustness of the gene-ratio test.

Analytical performance criteria and properties

Another key step in validating predictive tests for routine clinical use is to characterize assay performance according to standard performance criteria. The reported performance testing for the various proposed and currently approved molecular predictive tests for cancer, however, has been uneven.[41,46–48] Accordingly, we determined the technical assay performance of the gene ratio test in a set of 253 tissue specimens from 51 consecutive patients, a subset of the 120 enrolled patients, analyzed by quantitative RT-PCR (5 specimens from each of 49 patients, and 4 from each of 2 patients). Tumors from all patients were informative and contributed to the analysis. Below we summarize these results.

Repeatability—Repeatability (run-to-run, within-specimen) was 88.5% (95% CI = 84.0%–92.2%). We found that we could improve this value by defining gene ratio combined scores that were close to 1 as technically unacceptable (uninterpretable due to margin of error). Excluding values of log(geometric mean score) between -0.1 and $+0.1$ improved repeatability to 91.9% (95% CI = 87.4%–95.1%). When we applied this criterion, 32 (12.6%) of the 253 specimens were excluded. However, this still left multiple specimens for each of the 51 patients in the series and produced a valid test result for each patient. We therefore adopted the criteria of 5 samples per case and refined test threshold log values of < -0.1 and $> +0.1$ as standard practice.[17]

Tumor cell requirements—Repeatability was unaffected by low levels of tumor content in tissue specimens. Tumor content in 252 specimens ranged from 0%–90%, with a median of 43% (unknown in 1 specimen). Among these, only a total of 5 specimens had no microscopically detectable tumor (based on 2 H&E-stained slides each), and these were included in the analysis; 22 (9% of specimens) contained $< 10\%$ tumor cells; in 20 (8% of

specimens), tumor cell content was 10%; and in 144 (57% of specimens), it was $\geq 40\%$. [17] We suspect that one reason for the accuracy of the test with low tumor content is the high signal-to-noise ratio generated by the method. Another explanation may be that the microenvironment of the tumor also is detected by the predictive test, as suggested by reports of similar findings in breast cancer. [49–51]

To determine within-patient reproducibility, we computed the concordance of test results from among the 4–5 specimens obtained from each of the 51 patients. Test results of the 2 independent runs per specimen were consistent for at least 4 of the within-patient specimens in 79% of the 51 patients. Among the 51 patients, 51% exhibited full concordance of test results among all of their 4–5 within-patient specimens tested. The gene ratio-based predictions for individual patients in 2 independent determinations were reproducible in 96.1% (95% CI = 86.5%–99.5%) of patients. After exclusion of the 32 specimens with values in the range of log (geometric mean score) between -0.1 and $+0.1$, reproducibility was 100% among the 44 patients definitively assigned to a prognosis group by both determinations. Exclusion of these 32 specimens resulted in equivocal test results (prognosis) for 7 (14%) patients. [17]

Summary of MPM predictive gene ratio test

Our initial retrospective studies [15,16] showed that the 4-gene ratio binary test predicted post-surgical outcome with significant accuracy for patients with MPM, and our prospective clinical trial [17] validated its use for that purpose. We found the test repeatable and reproducible for a given specimen set in independent tests, performed by different personnel, using different instruments, in different laboratories. These properties have not been formally tested for other molecular predictive tests, but the technology and laboratory independence of the gene-ratio algorithm is a major advantage for its prospective clinical use. We optimized repeatability between independent determinations and reproducibility within patients by introducing test result exclusion limits (log score criteria of < -0.1 and $> +0.1$). We demonstrated that pleural biopsy specimens from visible tumors were generally adequate for analysis even when tumor cell content was relatively low. This is relevant because it indicates that tumor specimens selected by clinicians on the basis of appearance will suffice to ensure consistent test results. For predictive analysis, we found that sampling 5 minimally invasive pleural biopsy specimens per patient from visible tumors provides adequate numbers of specimens having the criterion tumor cell content $\geq 10\%$.

Advantages of the MPM gene ratio test over widely used gene expression–based predictive algorithms

The MPM gene ratio test has important advantages over earlier and alternative approaches and systems for cancer diagnosis, prognosis, and predictive tests based on molecular signatures. [51,52] For example, data from previous approaches have not been readily reproduced, probably for several reasons. First, specimen quality, which could dramatically influence results, has rarely been assessed. Second, the algorithms used have typically been quite complex, requiring analysis of the expression of many genes (often hundreds) and the use of training sets of specimens to develop predictive algorithms, followed by validation in a comparable independent set of specimens. [52] Third, most specimen sets tested previously in such studies have been assembled retrospectively from tumor banks. [53] Very few tests have been validated using prospectively obtained specimens, [46,54] and none have been tested for reproducibility using alternative technology platforms to measure gene expression. In contrast, the MPM gene ratio test has proved to be highly reproducible, probably owing in large part to its simplicity and its technology platform--and instrumentation--independence. The simplicity of the ratio-based approach obviates the use of complex algorithms involving many genes and the need to use training sets of specimens, while also providing a high level of internal control and independence of overall absolute gene expression levels and tissue RNA yields. The quality

of specimens was closely documented in our multiple studies by using highly annotated specimen sets from our own tumor bank,[55] and unlike the majority of other tests proposed or already approved for clinical use, the clinical performance of the MPM gene ratio test has been validated by prospective clinical trials, distinguishing it from previous tests.

Development of a predictive model by combining gene ratio test with independent pathology-based predictors

We have previously demonstrated by multivariate analysis that the pathological measures, histological subtype and lymph node status, are independent predictors of survival and add predictive value to the gene ratio test. We therefore constructed a predictive model that included all three parameters to see if we could enhance patient stratification. For each of the three predictive parameters, we assigned a value of 0 or 1. A value of 1 was assigned for each of the following: gene ratio test result of *poor*; mixed or sarcomatoid histology; and cancer presence in a lymph node. A value of 0 was assigned for each of the following: gene ratio test result of *good*; epithelial histology; and absence of cancer-positive lymph nodes. This approach segregated the patients into four distinct survival groups: low risk (median overall survival 31.9 months; 95% CI = 21.9–41.7 months), low intermediate risk (13.6 months; 95% CI = 11.5–20.2 months), high-intermediate risk (11.1 months; 95% CI = 8.3–16.2 months), and high-risk (6.9 months; 95% CI = 2.6–8.9 months). The intermediate low-risk and intermediate high-risk groups were combined into a single intermediate-risk group because of similar overall survival. Within this three-subgroup model, median overall survival was 31.9 months (95% CI = 21.9–41.7 months) in the low-risk group, 12.9 months (95% CI = 9.9–16.4 months) in the intermediate-risk group, and 6.9 months (95% CI = 2.6–8.9 months) and in the high-risk group. These values corresponded with overall survival 3-year survival rates of 42%, 12%, and 0% (Fig. 2). All the survival differences were statistically significant.[17]

CONCLUSION

On the basis of these promising results, we propose adding this multi-factorial predictive model to standard practice as a staging method for mesothelioma. Our successful application of this test indicates that it can be performed on specimens obtained in a minimally invasive biopsy performed prior to major surgical intervention, can accurately predict post-surgical outcome, and can reliably inform the clinical decision of whether to perform major surgical or trimodal therapies. For example, although the specimens tested in our studies were obtained during EPP surgery, for clinical application of this procedure, tissue specimens for the gene ratio test can be obtained at the time of pleuroscopy and mediastinoscopy, which are routinely performed preoperatively to confirm diagnosis and surgical staging. Sufficient biopsy specimens are usually taken during routine patient workup and provide adequate tissue for the gene ratio analysis. Patients assigned to the predicted poor outcome group would be counseled to forego major surgery, which would not benefit them, and to seek best supportive care, or alternatively to participate in available clinical trials of relevant non-surgical modalities.

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Figure 1.

Overall survival of subgroups of patients classified by the gene ratio test. Hash marks indicate censored patients; N is the number of patients at risk; S is the Kaplan-Meier survival point estimate as percent; CI 95% is the confidence interval for the survival estimate [Reproduced with permission from Gordon et al. Four-gene expression ratio test for survival in patients undergoing surgery for mesothelioma. *J Natl Cancer Inst* 2009; 101:1-9. (Figure 1B)]

Figure 2.

Overall survival of patients with malignant pleural mesothelioma after surgery from (A) four subgroup model of risk (Low, Low Intermediate, High Intermediate, and High) and (B) three subgroup model of risk (Low, Intermediate, High). Hashmarks indicate censored patients; N is the number of patients at risk; S is the Kaplan-Meier survival point estimate as percent; CI 95% is the confidence interval for the survival estimate [Reproduced with permission from Gordon et al. Four-gene expression ratio test for survival in patients undergoing surgery for mesothelioma. *J Natl Cancer Inst* 2009; 101-1-9. (Figure 2)]