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Blinded Comparator Study of Immunohistochemical Analysis versus a 92-Gene Cancer Classifier in the Diagnosis of the Primary Site in Metastatic Tumors

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Accurate tumor classification is fundamental to inform predictive biomarker testing and optimize therapy. Gene expression—based tests are proposed as diagnostic aids in cases with uncertain diagnoses. This study directly compared the diagnostic accuracy of IHC analysis versus molecular classification using a 92-gene RT-PCR assay for determination of the primary tumor site. This prospectively defined blinded study of diagnostically challenging cases included 131 high-grade, primarily metastatic tumors. Cases were reviewed and reference diagnoses established through clinical correlation. Blinded FFPE sections were evaluated by either IHC/morphology analysis or the 92-gene assay. The final analysis included 122 cases. The 92-gene assay demonstrated overall accuracy of 79% (95% CI, 71% to 85%) for tumor classification versus 69% (95% CI, 60% to 76%) for IHC/morphology analysis ($P = 0.019$). Mean IHC use was 7.9 stains per case (median, 8; range, 2 to 15). IHC/morphology analysis accuracy was 79%, 80%, and 46% when 1 to 6 ($n = 42$), 7 to 9 ($n = 41$), and >9 ($n = 39$) IHC stains were used, respectively, versus 81%, 85%, and 69%, respectively, with the 92-gene assay. Results from this blinded series of high-grade metastatic cases demonstrate superior accuracy with the 92-gene assay versus standard-of-care IHC analysis and strongly support the diagnostic utility of molecular classification in difficult-to-diagnose metastatic cancer. *(J Mol Diagn 2013, 15: 263—269; http://dx.doi.org/10.1016/j.jmoldx.2012.10.001)*

In the United States, >270,000 patients present with metastatic cancer each year [SEER Cancer Statistics Review, 1975-2009 (Vintage 2009 Populations), *http://seer.cancer.gov/csr/1975_2009_pops09*. Accessed August 15, 2012.]. Accurate identification of the primary site of tumor origin is fundamental for optimal patient care. Outcomes have improved with the use of site-specific chemotherapy, predictive biomarker testing, and appropriate molecular-targeted therapies[1–9]; however, each of these relies primarily on a definitive diagnosis for site of tumor origin. Likewise, clinical practice guidelines recommend treating patients based on site of origin [NCCN Clinical Practice Guidelines in Oncology (NCCN Guidelines), *http://www.nccn.org/professionals/physician_gls/f_guidelines.asp*, last accessed August 15, 2012]. Metastatic cancers often pose diagnostic challenges: despite advances in imaging and pathologic techniques, the primary site of tumor origin remains unknown or uncertain in a quarter to a third of new metastatic cases.[10,11]

Immunohistochemical (IHC) analysis serves as a cornerstone technique in the pathologic evaluation of site of tumor origin, especially in cases of poorly differentiated or undifferentiated tumors; however, few studies have evaluated the accuracy of IHC analysis in identifying the site of origin, particularly in poorly differentiated metastatic tumors. Individual IHC stains have varied sensitivity and specificity and are not applied in an objective and standardized manner in routine practice. Several research groups have developed and evaluated IHC diagnostic algorithms using panels of IHC markers.[12–16] A meta-analysis of these studies reported that IHC analysis had an accuracy of 66%

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in the characterization of metastatic tumors.[17] Given that optimal therapeutic approaches often depend on a definitive diagnosis, this relative lack of diagnostic accuracy in this setting represents an important unmet clinical need.

Recently, gene expression–based tests have been used as diagnostic complements to standard clinicopathologic evaluation. These molecular classifiers have been clinically validated and have demonstrated overall sensitivities ranging from 83% to 89%[18–21]; however, their performance has never been directly compared with that of IHC analysis. This study is the first, to our knowledge, to directly compare the diagnostic accuracy of IHC analysis versus molecular classification for determination of the primary tumor site and tumor subtype in a series of difficult-to-diagnose high-grade metastatic tumors.

Materials and Methods

The primary objective of this prospectively defined, blinded study was to compare the accuracy of a 92-gene molecular classifier (CancerTYPE ID; bioTheranostics Inc., San Diego, CA) with that of the standard morphologic examination with the aid of IHC analysis in determining the primary site of cancer origin and tumor subtype in poorly differentiated to undifferentiated neoplasms. Study approval was obtained from the institutional review board at City of Hope (Duarte, CA).

Tumor Specimen Selection

Tumor tissue was collected from 131 formalin-fixed, paraffin-embedded archived tumor samples at City of Hope. Cases were selected based on being challenges to determine the primary site and were generally poorly differentiated neoplasms without obvious histologic clues as to site of origin. Well-differentiated tumors in which the primary site could easily be identified or suspected histologically (eg, metastatic papillary carcinoma of the thyroid) were not included. The cases were primarily metastatic; however, some primary tumors were included such that primary tumors in common areas of metastatic presentation (eg, lung or liver) could not be ruled out based on inclusion criteria alone. Cases were reviewed (by L.M.W. or P.C.) and reference diagnoses for the primary site of origin were established by clinical correlation using patient history and clinical, pathologic, and imaging information. The gold standard for the study was usually the clinical information supplemented by previous pathologic studies; indeed, some of these patients had an accurate cancer diagnosis for many years and underwent biopsy to rule out the possibility of a second primary site or another disease.

The inclusion criteria for sample selection were i) formalin-fixed, paraffin-embedded tissue; ii) primary tumor site of origin verified by strong clinical or other correlates; iii) tissue from excisional or core needle biopsies with sufficient tissue for processing at both study sites (see later herein); iv) poorly differentiated to undifferentiated; v) and biopsied and processed <6 years before the study. The exclusion criteria included no tumor on pathology slide review and RNA quality did not pass the quality control cutoff point for the 92-gene assay.

Study Conduct and Assay Protocols

Tissue sections were collected, deidentified, coded, and sent in a blinded manner to two study sites for analysis: 92-gene RT-PCR assay (bioTheranostics Inc.) and IHC analysis (Clarient Inc., Aliso Viejo, CA). Only sex and biopsy site information were provided for each case. The 92-gene assay was performed on isolated total RNA as previously described.[19] Cases exceeding the PCR analytical cutoff point for internal controls were considered quality control failures. Briefly, a prespecified computational algorithm generates probabilities for primary tumor site of origin based on degree of similarity of the expression of the 92 genes to a reference database with gene expression data from >2000 tumors of known origin.[18] IHC analysis predictions were based on a Board-certified pathologist's review and interpretation of morphologic features (on H&E-stained slides) and on up to 15 blank slides for the performance of IHC stains of the pathologist's choosing for each case. Each pathologist had access to a catalog of >100 standard antibodies for their use. Primary site predictions were made after consensus review of each case by two pathologists. For both study sites, predictions were scored within a standardized, clinically relevant categorization system designed by City of Hope including a main type based on organ system and a subtype when applicable (Table 1).

Statistical Analysis

Sample size powering calculations assumed an α of 0.05 and a 2-sided test, and estimated sensitivities of 83% for the 92-gene assay[18,22] and 65% for IHC analysis.[17] One hundred twenty-five samples were required to provide 80% power to detect a difference in accuracy. Although powering calculations were based on a conservative independent sample assumption,[23] statistical significance was determined using a matched-pairs design (McNemar test, see later herein), with the P value based on the proportion of discordant predictions.

Study unblinding and data analysis were conducted by an independent third party not involved in any aspect of the sample processing (Powered 4 Significance LLC, Bloomsbury, NJ). The primary end point was overall accuracy (assay sensitivity), defined as the number of correct predictions divided by the total number of evaluable cases. The Cohen kappa statistic was calculated to assess the agreement between the prediction and the reference diagnosis of tumor type, which adjusted for agreement due to

chance alone.[24] Sensitivity calculations were performed at the main type (organ system) and subtype levels. A comparison of the overall sensitivities between the 92-gene assay and IHC analysis was performed using the McNemar test on paired proportions.[25] Specificity was defined as the proportion of true-negatives correctly identified. Overall specificity was calculated as the weighted average of the specificities for all tumor types, weighted by the sample size used in the calculation of specificity for each tumor type. Although the study was not powered to compare accuracy within individual tumor classes, sensitivity and specificity within tumor classes were calculated for each study arm.

Table 1 Evaluable Main Types (Organ System) and Subtypes; Correct Predictions by IHC/Morphology Analysis and 92-Gene Assay

Tumor main type and subtype	Evaluable	IHC/morphology analysis correct	92-gene assay correct
Lymphoma	0		
Melanoma	2	0	0
Sarcoma	7	6	7
Gastrointestinal stromal	1	1	1
Other	6	4	6
Mesothelioma	1	0	1
Brain	0		
Germ cell	0		
Lung	24	16	18
Adeno	13	8	11
Squamous cell	7	4	4
Small cell	0		
Other	4	1	0
Gynecologic	8	7	7
Surface ovary	5	5	5
Uterus	3	1	0
Cervix	0		
Other	0		
Gastrointestinal	26	24	24
Colon/appendix	17	16	16
Small intestine	0		
Stomach/ esophageal adeno	5	3	3
Pancreaticobiliary	4	3	2
Other	0		
Urinary bladder	11	5	9
Urothelial	11	5	9
Other	0		
11. Kidney	13	10	10
12. Endocrine	9	5	5
Ovarian stromal	0		
Adrenocortical	0		
Panganglioma/ pheochromocytoma	0		
Thyroid	4	1	2
Carcinoid/islet cell	3	1	3
Nonlung, small cell	0		
Other	2	1	0
Hepatocellular	1	1	1
Head and neck/ esophageal squamous	3	2	2
Salivary gland	1	0	0
Prostate	4	2	4
Breast	11	6	8
Thymus	0		
Meningioma	0		
Skin Basal Cell	1	0	0
Total: main type (subtype)	122	84 (75)	96 (88)

Results

One hundred thirty-one cases were originally identified for study. One case was eliminated from the study because no tumor was present in the residual paraffin block, and eight cases were eliminated from the study because of inadequate RNA (three of these latter specimens were derived from bone specimens that had been decalcified before paraffin embedding). The primary sites of the evaluable 122 cases are summarized in Table 1. The most common primary sites included the lung ($n = 24$), colon ($n = 17$), kidney ($n = 13$), urinary bladder ($n = 11$), and breast ($n = 11$). The means $\pm$ SD age of the evaluable patients was 61.0 $\pm$ 14.3 years. Men composed 48% of the population; 90% of the cases were metastatic.

Evaluable cases and number of correct predictions for each main type category are shown in Table 1. Mean IHC use was 7.9 stains per case (median, 8; range, 2 to 15); only one case required use of all 15 slides. In the IHC/morphology analysis arm, the primary site of origin was correctly identified in 84 of 122 cases (69%), including 16 of 24 lung (67%), 24 of 26 gastrointestinal (92%), 10 of 13 kidney (77%), 5 of 11 urinary bladder (45%), and 6 of 11 breast (55%) cases. Overall sensitivity and specificity at the main type/organ system level were 69% (95% CI, 60% to 76%) and 99% (95% CI, 98% to 99%), respectively (see Table 2 for sensitivity and specificity by tumor type). Agreement between the IHC analysis prediction and the adjudicated diagnosis, as measured by the Cohen kappa statistic, was 0.65. At the subtype level, correct predictions were made in 75 of 122 cases (61%), including 16 of 17 colorectal (94%) and 8 of 13 lung (62%) adenocarcinomas. Overall sensitivity and specificity at the subtype level were 61% (95% CI, 53% to 70%) and 99% (95% CI, 99% to 99%), respectively. Fourteen cases were classified as unknown carcinoma. The most common tumor types classified as unknown were breast ($n = 4$), lung ($n = 3$), kidney ($n = 2$), and prostate ($n = 2$).

The 92-gene classifier correctly identified the primary site of origin in 96 of 122 cases (79%), including 18 of 24 lung (75%), 24 of 26 gastrointestinal (92%), 10 of 13 kidney (77%), 9 of 11 urinary bladder (82%), and 8 of 11 breast (73%) cases. The overall sensitivity and specificity were 79% (95% CI, 71% to 85%) and 99% (95% CI, 98% to 99%), respectively. The prediction of the 92-gene classifier had a kappa statistic of 0.76 for agreement with the adjudicated diagnosis. At the subtype level, correct predictions were made in 88 of 122 cases (72%), including 16 of 17 colorectal (94%) and 11 of 13 lung (85%) adenocarcinomas.

Table 2 Sensitivity and Specificity of the 92-Gene Assay and IHC/Morphology Analysis at the Main Type Level and for the Colon/Appendix Subtype

		IHC/morphology analysis		92-gene assay	
Main type	No.	Sensitivity (%)	Specificity (%)	Sensitivity (%)	Specificity (%)
Melanoma	2	0 (0–66)	100 (97–100)	0 (0–66)	99 (95–100)
Sarcoma	7	86 (49–97)	98 (94–100)	100 (65–100)	98 (94–100)
Mesothelioma	1	0 (0–79)	100 (97–100)	100 (21–100)	99 (95–100)
Lung	24	67 (47–82)	94 (87–97)	75 (55–88)	95 (89–98)
Gynecologic	8	88 (53–98)	98 (94–100)	88 (53–98)	96 (91–99)
Gastrointestinal	26	92 (76–98)	93 (86–96)	92 (76–98)	97 (91–99)
Colon/appendix subtype	17	94 (73–99)	97 (92–99)	94 (73–99)	99 (95–100)
Urinary bladder	11	45 (21–72)	99 (95–100)	82 (52–95)	99 (95–100)
Kidney	13	77 (50–92)	100 (97–100)	77 (50–92)	99 (95–100)
Endocrine	9	56 (27–81)	99 (95–100)	56 (27–81)	99 (95–100)
Hepatocellular	1	100 (21–100)	100 (97–100)	100 (21–100)	100 (97–100)
Head and neck/esophageal squamous	3	67 (21–94)	98 (94–100)	67 (21–94)	97 (92–99)
Salivary gland	1	0 (0–79)	100 (97–100)	0 (0–79)	98 (93–99)
Prostate	4	50 (15–85)	100 (97–100)	100 (51–100)	100 (97–100)
Breast	11	55 (28–79)	97 (92–99)	73 (43–90)	100 (97–100)
Skin basal cell	1	0 (0–79)	100 (97–100)	0 (0–79)	100 (97–100)
Total main type	122	69 (60–76)	99 (98–99)	79 (71–85)	99 (98–99)

The 95% CIs are provided in parentheses.

Overall sensitivity and specificity at the subtype level were 72% (95% CI, 64% to 79%) and 99% (95% CI, 99% to 99%), respectively.

The difference in sensitivity between the two methods was significant ($P = 0.019$ and $P = 0.031$ at the main type and subtype levels, respectively; McNemar test). Table 3 shows the comparison between correct and incorrect predictions of IHC/morphology analysis and the 92-gene classifier according to main tumor types and subtypes. At the main type level, both methods identified the same correct site of origin in 79 cases (65%). The 92-gene classifier correctly identified the major site of origin in which IHC/morphology analysis was incorrect in 17 cases (14%): the most common tumor types included bladder (4 of 11 cases), lung (4 of 24 cases, all adenocarcinomas), breast (2 of 11 cases), and prostate (2 of 4 cases). IHC/morphology analysis correctly identified the major site of origin in five cases in which the 92-gene classifier was incorrect (4%): the most common tumor type was lung (2 of 24 cases, both squamous cell carcinomas). In 21 cases (17%), both methods were unable to determine the correct primary site, at least as determined by the gold standard. In 10 of these cases, both methods identified the same incorrect site. In six of these cases, the lung was either the correct site of origin or was the incorrectly predicted site of origin.

Of the eight cases excluded owing to insufficient RNA, IHC/morphology analysis had identified the correct primary site in seven (overall sensitivity including these cases, 70%).

The number of IHC stains used per case was not specified per protocol; however, a post hoc analysis examined the accuracy of IHC/morphology analysis stratified by number of IHC stains used (Table 4). When 1 to 6 ($n = 42$) or 7 to 9 ($n = 41$) IHC stains were used, the correct primary site was predicted by IHC/morphology analysis in 79% and 80% of cases, respectively. In cases requiring $\geq$10 IHC stains ($n = 39$), IHC/morphology analysis accuracy was 46%. In the same subsets, correct predictions were made by the 92-gene classifier in 81%, 85%, and 69% of cases, respectively.

Discussion

IHC/morphology analysis determined the primary site in a series of poorly differentiated neoplasms in 69% of cases. It is difficult to assess the literature for the efficacy of IHC analysis in determining the primary site of metastatic tumors, as controlled, blinded studies are rare. A reported meta-analysis suggested a value of approximately 66%

Table 3 Comparisons between Correct and Incorrect Predictions

	IHC/morphology analysis prediction		
92-gene assay prediction	Incorrect	Correct	Subtotal
Main type (organ system) level*			
Incorrect	21	5	26
Correct	17	79	96
Subtotal	38	84	122
Subtype level†			
Incorrect	25	9	34
Correct	22	66	88
Subtotal	47	75	122

*IHC analysis: sensitivity, 69% (95% CI, 60% to 76%), and specificity, 99% (95% CI, 98% to 99%); 92-gene assay: sensitivity, 79% (95% CI, 71% to 85%), and specificity, 99% (95% CI, 98% to 99%); $P = 0.019$.

†IHC analysis: sensitivity, 61% (95% CI, 53% to 70%), and specificity, 99% (95% CI, 99% to 99%); 92-gene assay: sensitivity, 72% (95% CI, 64% to 79%), and specificity, 99% (95% CI, 99% to 99%); $P = 0.031$.

Table 4 Post hoc Analysis of Performance Stratified by Number of IHC Stains Used

	No. correct (%) [95% CI]	
No. of IHC stains	IHC/morphology analysis	92-gene assay
1–6 (n = 42)	33 (79) [64–88]	34 (81) [67–90]
7–9 (n = 41)	33 (80) [66–90]	35 (85) [72–93]
10–15 (n = 39)	18 (46) [32–61]	27 (69) [54–81]

based on a small number of older series.[17] Given the increased number of antibodies with high specificity and good discriminatory ability currently available (TTF-1, CDX-2, PAX-8, etc), one might have expected stronger performance; however, an important consideration is that the study was not designed to examine neoplasms typically encountered in daily practice. These cases were specifically selected to challenge the two methods to their utmost. Cases that were missed by IHC/morphology analysis often lacked the organ-specific antibody expression that many pathologists have come to rely on (data not shown). Another category of case frequently missed by IHC/morphology analysis included bladder carcinoma, which lacks reliable organ-specific antibodies and frequently loses its distinctive keratin profile in poorly differentiated tumors.

Previous studies of the 92-gene assay have reported sensitivities of 83% to 87%.[18,22] In the present study, the sensitivity was similar, at 79%, suggesting that the RNA profile used by the 92-gene classifier is retained to a large extent even in poorly differentiated metastatic tumors. The overall sensitivity of the 92-gene classifier may be underestimated herein, compared with previous studies, owing to differences in tumor class representation between studies. For example, the 92-gene classifier has demonstrated high accuracy in several tumor types not represented in this study (eg, germ cell, lymphoma, and brain).

In this study, the accuracy of the 92-gene classifier was statistically significantly higher than that of IHC/morphology analysis, suggesting that poorly differentiated tumors may retain their RNA profile to a significantly greater extent than their morphologic and protein profile, as detected by a light microscope using currently available IHC reagents. Moreover, the 92-gene assay uses the collective expression of the biomarker panel to classify tumors rather than relying on one or a few tumor markers, which may have atypical expression or loss of expression in a poorly differentiated tumor. Neither method successfully identified the primary site in 21 of the 122 cases, at least as determined by the primarily clinically driven gold standard. It is possible that these neoplasms have lost all recognizable characteristics of their organ of origin; however, it was interesting to note that in half of these cases, both methods predicted the same incorrect site, and in most of these cases, the lung was either the presumed primary or metastatic site. It is well-known that it is often difficult to determine the primary site in patients with patterns of disease that include the lung versus another site in the differential diagnosis. It is possible that the IHC/morphology analysis and the 92-gene classifier actually found the true primary sites in some of these cases; thus, the sensitivity of both methods may be underestimated.

Accurate tumor classification is fundamental in personalizing cancer care, particularly in patients with metastatic disease. Site-specific chemotherapy regimens, choice of biomarker tests, and biomarker-driven molecular-targeted therapies all depend on definitive and accurate tumor classification. For example, an accurate diagnosis of metastatic lung adenocarcinoma indicates for biomarker testing for *EGFR* mutations, *ALK* gene rearrangement, and other biomarkers with emerging evidence and/or therapies in clinical development (eg, *KRAS*, *c-MET*, and *ROS1*). Similarly, the targeted RAF inhibitor vemurafenib is highly effective for metastatic melanoma with the BRAF V600E mutation but is ineffective against colorectal cancers harboring the same mutation.[9,26] These scenarios are only likely to increase in the future, as more targeted therapies and companion diagnostic biomarker tests are developed and become available for patient care. Recently published data directly support the clinical utility of molecular tumor classification in the most difficult-to-diagnose metastatic cases, ie, cancer of unknown primary site. In this prospective clinical trial of patients diagnosed as having cancer of unknown primary site, many of whom had poorly differentiated or undifferentiated tumors, those who were treated with site-specific therapy based on the 92-gene assay molecular diagnosis had overall survival of 12.5 months, a result that compared favorably with patients from previous prospective trials of patients with cancer of unknown primary site who were treated with empirical regimens (9.1 months) and in a subset of patients in this trial treated with empirical regimens (4.9 months).[27,28]

There are several limitations to this study. First, case selection was not representative of daily practice in that case selection specifically identified difficult-to-diagnose tumors; thus, the study is not reflective of—and likely underestimates—the overall accuracy of both methods. However, the study results highlight an important unmet need in standard of care: atypical expression of IHC protein markers may result in false-negative diagnoses [eg, TTF-1–negative lung adenocarcinomas, PSA-negative prostate carcinoma, and Pax-8–negative renal cell carcinoma, all examples that occurred in this study (data not shown)]. Second, this study was not powered to examine differences in specific tumor types; thus, the conclusions apply when considering the universe of all subtypes examined, as in daily practice, but may not be true for any one particular subtype. Finally, although the two study sites received sequential tumor sections, tumor heterogeneity could have played a role in different predictions in some cases.

The present study demonstrates a role for molecular studies in the diagnosis of carcinomas of unknown or uncertain origin, although their precise clinical indication requires further clarification. The selection of IHC analysis versus molecular studies should depend on multiple factors, including clinical suspicion of a specific primary site, pathologic

suspicion of a specific primary site based on the morphologic findings, type of specimen, amount of tissue for study, clinical need in the specific patient (eg, how useful would organ-specific therapies be in the specific patient), and the costs of each technology. Settings that might favor primary use of IHC analysis include scenarios where there is a strong suspicion of the primary site based on clinical or morphologic information (which might require a relatively small battery of directed stains), when the tumor is relatively well-differentiated such that appropriate organ-specific immunostains would be expected to be reactive, or in specimens requiring rigorous decalcification (which might degrade mRNA). An added advantage of IHC is that stains can be performed in an incremental manner. Settings that might favor primary use of molecular studies might be situations with no or few specific clinical or morphologic clues to the primary site, the most poorly differentiated neoplasms (in which organ-specific antibodies might be least useful), or specimens with insufficient tissue to apply a large battery of IHC studies. Either IHC or molecular studies may be potentially useful in the situation where the other method has been applied and was unsuccessful in determining the primary site or obtained a result that did not fit clinically. For example, in this study, when a larger number of IHC stains was required (more than nine), IHC/morphology analysis accuracy was just 46% compared with 69% with the 92-gene assay in the same cases. Although these data are limited, consideration should be given to referral for molecular studies in cases requiring more than nine IHC stains to establish the organ of origin, as IHC analysis had the lowest yield in this setting.

In conclusion, this study is the first, to our knowledge, to directly compare the diagnostic accuracy of IHC/morphology analysis with molecular classification for the determination of primary tumor site and tumor subtype. In clinical practice, identification of a primary site remains equivocal in many cases, particularly when the clinical presentation is atypical and/or IHC analysis results are equivocal, as is frequently the case in poorly differentiated and undifferentiated metastatic disease. Gene expression–based molecular classification provides a complementary approach to IHC analysis, with a standardized and objective protocol and high accuracy. The results of this study strongly support the diagnostic utility of molecular classification in difficult-to-diagnose metastatic cancer.

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References

1. Hurwitz H, Fehrenbacher L, Novotny W, Cartwright T, Hainsworth J, Heim W, Berlin J, Baron A, Griffing S, Holmgren E, Ferrara N, Fyfe G, Rogers B, Ross R, Kabbinavar F: Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. N Engl J Med 2004, 350:2335–2342
2. Hanna N, Shepherd FA, Fossella FV, Pereira JR, De Marinis F, von Pawel J, Gatzemeier U, Tsao TC, Pless M, Muller T, Lim HL, Desch C, Szondy K, Gervais R, Shaharyar, Manegold C, Paul S, Paoletti P, Einhorn L, Bunn PA Jr: Randomized phase III trial of pemetrexed versus docetaxel in patients with non-small-cell lung cancer previously treated with chemotherapy. J Clin Oncol 2004, 22:1589–1597
3. Burris HA III, Moore MJ, Andersen J, Green MR, Rothenberg ML, Modiano MR, Cripps MC, Portenoy RK, Storniolo AM, Tarassoff P, Nelson R, Dorr FA, Stephens CD, Von Hoff DD: Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial. J Clin Oncol 1997, 15:2403–2413
4. Keedy VL, Temin S, Somerfield MR, Beasley MB, Johnson DH, McShane LM, Milton DT, Strawn JR, Wakelee HA, Giaccone G: American Society of Clinical Oncology provisional clinical opinion: epidermal growth factor receptor (EGFR) mutation testing for patients with advanced non-small-cell lung cancer considering first-line EGFR tyrosine kinase inhibitor therapy. J Clin Oncol 2011, 29:2121–2127
5. Allegra CJ, Jessup JM, Somerfield MR, Hamilton SR, Hammond EH, Hayes DF, McAllister PK, Morton RF, Schilsky RL: American Society of Clinical Oncology provisional clinical opinion: testing for KRAS gene mutations in patients with metastatic colorectal carcinoma to predict response to anti-epidermal growth factor receptor monoclonal antibody therapy. J Clin Oncol 2009, 27:2091–2096
6. Shaw AT, Solomon B, Kenudson MM: Crizotinib and testing for ALK. J Natl Compr Canc Netw 2011, 9:1335–1341
7. Shepherd FA, Rodrigues Pereira J, Ciuleanu T, Tan EH, Hirsh V, Thongprasert S, Campos D, Maoleekoonpiroj S, Smylie M, Martins R, van Kooten M, Dediu M, Findlay B, Tu D, Johnston D, Bezjak A, Clark G, Santabarbara P, Seymour L: Erlotinib in previously treated non-small-cell lung cancer. N Engl J Med 2005, 353:123–132
8. Eiermann W: Trastuzumab combined with chemotherapy for the treatment of HER2-positive metastatic breast cancer: pivotal trial data. Ann Oncol 2001, 12(Suppl 1):S57–S62
9. Chapman PB, Hauschild A, Robert C, Haanen JB, Ascierto P, Larkin J, Dummer R, Garbe C, Testori A, Maio M, Hogg D, Lorigan P, Lebbe C, Jouary T, Schadendorf D, Ribas A, O'Day SJ, Sosman JA, Kirkwood JM, Eggermont AM, Dreno B, Nolop K, Li J, Nelson B, Hou J, Lee RJ, Flaherty KT, McArthur GA: Improved survival with vemurafenib in melanoma with BRAF V600E mutation. N Engl J Med 2011, 364:2507–2516
10. Greco FA, Hainsworth JD: Cancer of unknown primary site. DeVita, Hellman, and Rosenberg's Cancer: Principles and Practice of Oncology, ed 9, ch 137. Edited by DeVita VTJ, Lawrence TS, Rosenberg SA. Philadelphia, Lippincott Williams & Wilkins, 2011, pp 2033–2051
11. Hillen HF: Unknown primary tumours. Postgrad Med J 2000, 76: 690–693
12. Brown RW, Campagna LB, Dunn JK, Cagle PT: Immunohistochemical identification of tumor markers in metastatic adenocarcinoma: a diagnostic adjunct in the determination of primary site. Am J Clin Pathol 1997, 107:12–19
13. Dennis JL, Hvidsten TR, Wit EC, Komorowski J, Bell AK, Downie I, Mooney J, Verbeke C, Bellamy C, Keith WN, Oien KA: Markers of adenocarcinoma characteristic of the site of origin: development of a diagnostic algorithm. Clin Cancer Res 2005, 11:3766–3772
14. Gamble AR, Bell JA, Ronan JE, Pearson D, Ellis IO: Use of tumour marker immunoreactivity to identify primary site of metastatic cancer. BMJ 1993, 306:295–298
15. Park SY, Kim BH, Kim JH, Lee S, Kang GH: Panels of immunohistochemical markers help determine primary sites of metastatic adenocarcinoma. Arch Pathol Lab Med 2007, 131:1561–1567
16. DeYoung BR, Wick MR: Immunohistologic evaluation of metastatic carcinomas of unknown origin: an algorithmic approach. Semin Diagn Pathol 2000, 17:184–193

17. Anderson GG, Weiss LM: Determining tissue of origin for metastatic cancers: meta-analysis and literature review of immunohistochemistry performance. Appl Immunohistochem Mol Morphol 2010, 18:3−8
18. Erlander MG, Ma XJ, Kesty NC, Bao L, Salunga R, Schnabel CA: Performance and clinical evaluation of the 92-gene real-time PCR assay for tumor classification. J Mol Diagn 2011, 13:493−503
19. Pillai R, Deeter R, Rigl CT, Nystrom JS, Miller MH, Buturovic L, Henner WD: Validation and reproducibility of a microarray-based gene expression test for tumor identification in formalin-fixed, paraffin-embedded specimens. J Mol Diagn 2011, 13:48−56
20. Rosenwald S, Gilad S, Benjamin S, Lebanony D, Dromi N, Faerman A, Benjamin H, Tamir R, Ezagouri M, Goren E, Barshack I, Nass D, Tobar A, Feinmesser M, Rosenfeld N, Leizerman I, Ashkenazi K, Spector Y, Chajut A, Aharonov R: Validation of a microRNA-based qRT-PCR test for accurate identification of tumor tissue origin. Mod Pathol 2010, 23:814−823
21. Kerr SE, Schnabel CA, Sullivan PS, Zhang Y, Singh V, Carey B, Erlander MG, Highsmith WE, Dry SM, Brachtel EF: Multisite validation study to determine performance characteristics of a 92-gene molecular cancer classifier. Clin Cancer Res 2012, 18:3952−3960
22. Ma XJ, Patel R, Wang X, Salunga R, Murage J, Desai R, Tuggle JT, Wang W, Chu S, Stecker K, Raja R, Robin H, Moore M, Baunoch D, Sgroi D, Erlander M: Molecular classification of human cancers using a 92-gene real-time quantitative polymerase chain reaction assay. Arch Pathol Lab Med 2006, 130:465−473
23. Connor RJ: Sample size for testing differences in proportions for the paired-sample design. Biometrics 1987, 43:207−211
24. Cohen J: A coefficient of agreement for nominal scales. Educ Psychol Measure 1960, 20:37−46
25. McNemar Q: Note on the sampling error of the difference between correlated proportions or percentages. Psychometrika 1947, 12: 153−157
26. Kopetz S, Desai J, Chan E, Hecht JR, O'Dwyer PJ, Lee RJ, Nolop KB, Saltz L: PLX4032 in metastatic colorectal cancer patients with mutant BRAF tumors. J Clin Oncol 2010, 28:15s
27. Hainsworth JD, Rubin MS, Spigel DR, Boccia RV, Raby S, Quinn R, Greco FA: Molecular gene expression profiling to predict the tissue of origin and direct site-specific therapy in patients with carcinoma of unknown primary site: a prospective trial of the Sarah Cannon Research Institute. J Clin Oncol 2013, 10:217−223
28. Greco FA, Rubin MS, Boccia RV, Scola MA, Spigel DR, Raby S, Chirwa TW, Quinn R, Schnabel CA, Erlander MG, Hainsworth JD: Molecular gene expression profiling to predict the tissue of origin and direct site-specific therapy in patients (pts) with carcinoma of unknown primary site (CUP): results of a prospective Sarah Cannon Research Institute (SCRI) trial. J Clin Oncol 2012, 30(suppl). abstract 10530