Published OnlineFirst September 21, 2014; DOI: 10.1158/1055-9965.EPI-14-0182

Cancer Epidemiology, Biomarkers & Prevention

Research Article

The Expression of Four Genes as a Prognostic Classifier for Stage I Lung Adenocarcinoma in 12 Independent Cohorts

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Abstract

Background: We previously developed a prognostic classifier using the expression levels of *BRCA1*, *HIF1A*, *DLC1*, and *XPO1* that identified stage I lung adenocarcinoma patients with a high risk of relapse. That study evaluated patients in five independent cohorts from various regions of the world. In an attempt to further validate the classifier, we have used a meta-analysis–based approach to study 12 cohorts consisting of 1,069 tumor–node–metastasis stage I lung adenocarcinoma patients from every suitable, publically available dataset.

Methods: Cohorts were obtained through a systematic search of public gene expression datasets. These data were used to calculate the risk score using the previously published 4-gene risk model. A fixed effect meta-analysis model was used to generate a pooled estimate for all cohorts.

Results: The classifier was associated with prognosis in 10 of the 12 cohorts ($P < 0.05$). This association was highly consistent regardless of the ethnic diversity or microarray platform. The pooled estimate demonstrated that patients classified as high risk had worse overall survival for all stage I [HR, 2.66; 95% confidence interval (CI), 1.93–3.67; $P < 0.0001$] patients and in stratified analyses of stage IA (HR, 2.69; 95% CI, 1.66–4.35; $P < 0.0001$) and stage IB (HR, 2.69; 95% CI, 1.74–4.16; $P < 0.0001$) patients.

Conclusions: The 4-gene classifier provides independent prognostic stratification of stage IA and stage IB patients beyond conventional clinical factors.

Impact: Our results suggest that the 4-gene classifier may assist clinicians in decisions about the postoperative management of early-stage lung adenocarcinoma patients. *Cancer Epidemiol Biomarkers Prev; 23(12); 2884–94. ©2014 AACR.*

Introduction

Lung cancer is the leading cause of cancer-related death in the world, accounting for more than one-fourth of all cancer deaths (1). Approximately 85% of lung cancers are non–small cell lung cancer (NSCLC). The most common histology for NSCLC is adenocarcinoma, followed by squamous cell carcinoma (SQC) and large cell carcinoma. Despite therapeutic advances, prognosis remains considerably poor relative to other solid cancers, even in early-stage patients (1). Thus, more refined treatment strategies are needed.

Tumor–node–metastasis (TNM) staging is the best prognostic factor for NSCLC. TNM staging is used by clinicians to guide treatment options for NSCLC. Early-stage patients, including TNM stage I and II, are typically approached with curative surgery as the optimal treatment. Among such patients with completely resected NSCLC, adjuvant chemotherapy is recommended only for stage II patients based on several randomized trials that demonstrated survival benefit of platinum-based chemotherapy (2–4). In contrast, clinical trials have revealed no survival advantage and potential deleterious side-effects of adjuvant chemotherapy for stage IA patients (2, 5). With regard to stage IB patients, the evidence supporting the routine use of adjuvant chemotherapy is controversial (2, 6, 7). A more detailed histologic subtyping of lung cancer may improve on TNM classification system. For example, the presence of the micropapillary histologic subtype has been found to be associated with cancer recurrence after limited resection of peripheral lung adenocarcinoma and may help guide treatment strategies (8).

Approximately 30% of stage I lung cancer patients will relapse and ultimately die of this disease. The majority of these patients are being treated by surgery alone because

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Note: Supplementary data for this article are available at Cancer Epidemiology, Biomarkers & Prevention Online (http://cebp.aacrjournals.org/).

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doi: 10.1158/1055-9965.EPI-14-0182

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of the lack of clear evidence of benefit from adjuvant chemotherapy. Consequently, 5-year overall survival rates for pathologic stage IA and IB are 73% and 58%, respectively, based on the recently revised, 7th edition of TNM staging (9). One simple and critical question is how clinicians can distinguish the approximately 30% of stage I patients who have higher risk of relapse from the other 70% of patients who have excellent prognosis. High-risk patients might have undetectable micrometastases at the time of surgery. Hence, their outcome could potentially be improved by postoperative systemic therapy with the primary goal of eliminating residual occult metastases that lead to disease recurrence. There is a substantial need to identify stage IB patients who are unlikely to benefit from adjuvant chemotherapy and/or immunotherapy as well as stage IA patients who have the highest risk of relapse. In view of that, it is vital to develop prognostic biomarkers that can help clinicians determine appropriate postoperative management for each individual patient. The demand for such clinical prognostic tests is now undoubtedly increasing, as the extensive use of CT screening becomes widely accepted, in which the majority of patients are diagnosed at stage I (10).

Numerous studies have identified prognostic biomarkers for NSCLC based on multigene expression by using qRT-PCR and/or microarray technology (11–26). However, associations reported in single studies often failed to provide sufficient validation in additional populations (12, 26, 27). A recent review criticized prognostic gene signatures for their unspecified clinical utility as well as the lack of reproducibility, and suggested that no lung cancer signatures are ready for clinical application (27). Taking into account the guidelines suggested in that review, we started to develop a gene expression-based prognostic signature that was intended to be used for early-stage lung adenocarcinomas, especially for stage I patients. Our goal was to make a classifier based on a few key genes that would be a simple and robust classifier for prognosis of stage I lung cancer patients. A similar strategy based on analyzing important 31 cell proliferation genes has shown to be a robust prognostic classifier for lung cancer (28). Our resulting signature, namely the 4-gene classifier, was found to be highly robust in all five cohorts that we analyzed. Its prognostic significance was independent of other clinical factors, including age, gender, TNM stage, and smoking status (29). These results suggest that the 4-gene classifier may be useful in guiding therapeutic decisions for early-stage lung cancer patients. We have now set out to test the 4-gene classifier in as many independent populations as we could identify from publically available gene expression data. As a result, we have evaluated more than 1,000 stage I adenocarcinoma patients from 12 independent cohorts using different gene expression platforms. We use a meta-analytic approach to measure the association of the 4-gene classifier with prognosis and evaluate its reproducibility across those cohorts. We focus on stage IA and IB patients separately to further evaluate the clinical usefulness of this classifier.

Materials and Methods

Selection of studies

We searched Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) in June 2013 with the search terms "lung cancer," "non–small cell lung cancer," "lung adenocarcinoma," "lung adenocarcinomas," and "NSCLC." The retrieved GEO series were filtered by Organism (Homo Sapiens) and Series Type (Expression Profiling by Array) as well as sorted by the number of samples (series that have at least 30 samples). Ninety-two GEO series identified by the initial GEO search were screened on the basis of their Title, Summary, and Overall Design as described in GEO Accession Display. Datasets were excluded if they analyzed only cell lines/xenograft samples, only nontumor specimens (e.g., bronchial epithelial cells, blood, fluid), or contained no primary adenocarcinoma tumors. Also, several superseries that consisted of one or more subseries were excluded (due to duplicate data) and the corresponding subseries with gene expression data were retrieved, leaving 46 GEO datasets of lung cancer-related clinical studies. In parallel with this search, we used ONCOMINE (Compendia Bioscience; http://www.oncomine.com) to identify public microarray datasets that had patients with adenocarcinoma with survival status. ONCOMINE search identified 10 datasets, five of which were not deposited in GEO. The resulting 51 datasets containing primary adenocarcinoma samples were further reviewed on the basis of the Sample Characteristics in Series Matrix File, or the Dataset Detail in ONCOMINE. Selection criteria for all publicly available datasets required each dataset to include survival information for more than 30 TNM stage I patients of adenocarcinoma and have expression data for BRCA1, HIF1A, DLC1, and XPO1. After removing 40 datasets that did not fit our criteria, we found 11 independent microarray datasets, including the Botling (GSE37745; ref. 30), Tang (GSE42127; ref. 24), Rousseaux (GSE30219; ref. 31), Matsuyama (GSE11969; ref. 32), Wilkerson (GSE26939; ref. 33), Lee (GSE8894/ONCOMINE; ref. 17), Bild (GSE3141/ONCOMINE; ref. 34) cohorts as well as the Bhattacharjee (ONCOMINE; ref. 35), Directors (ONCOMINE; ref. 12), Japan (GSE31210; ref. 36), and Tomida (GSE13213; ref. 16) cohorts. Among them, the former seven cohorts were newly obtained from GEO or ONCOMINE (if available) for this present study, whereas the latter four datasets were the original cohorts that we analyzed in our initial study (29). The selection flowchart and the list of retrieved datasets are presented in Fig. 1 and Supplementary Table S1. All of the data from the stage I lung adenocarcinoma cohorts are available in the Supplementary Data.

For the 4-coding gene analyses in patients with SQC, we used multiple cohorts of stage I SQC. Six cohorts, including the Botling, Rousseaux, Tang, Matsuyama, Lee, and Bild datasets among the adenocarcinoma datasets mentioned above, were included, as these cohorts also contained expression data for patients with SQC with survival information. We obtained one SQC dataset from

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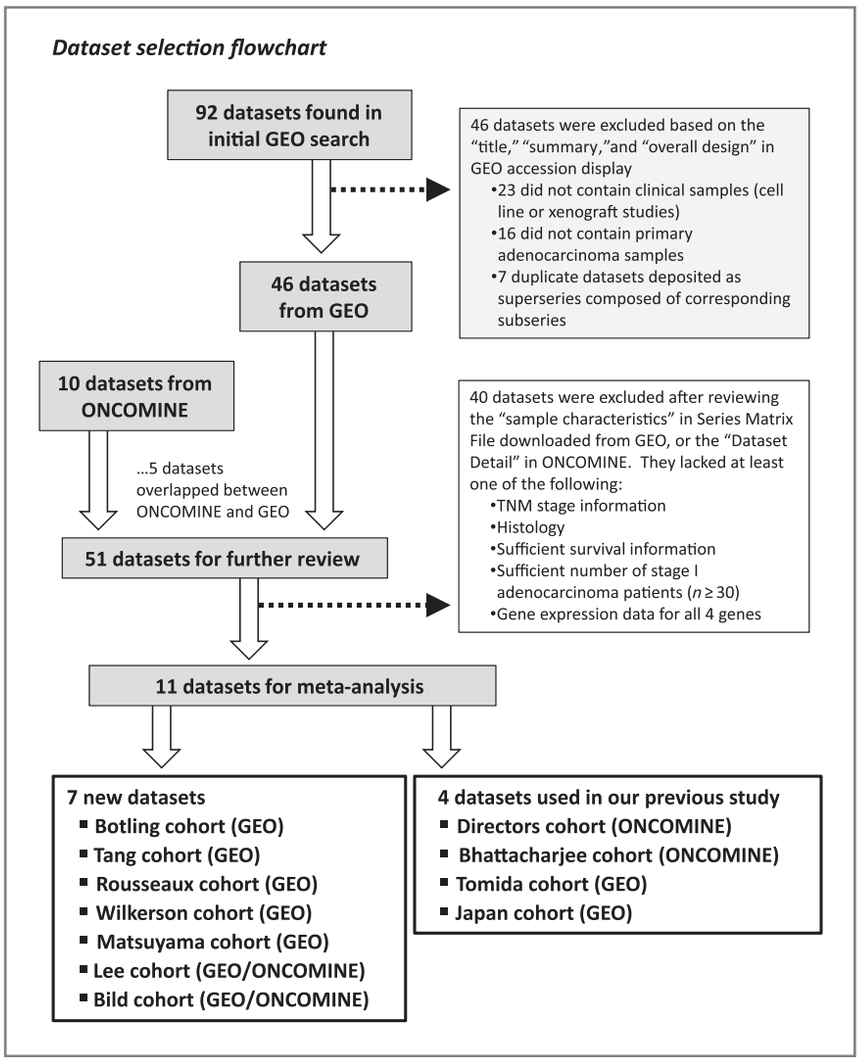

Figure 1. Dataset selection flow chart. A total of 92 datasets from GEO and 10 datasets from ONCOMINE were evaluated. A total of 11 datasets were selected to be included in this meta-analysis.

GEO (GSE17710) deposited by Wilkerson and colleagues (37), separated from their adenocarcinoma data (GSE26939, the Wilkerson adenocarcinoma cohort; ref. 33). In addition, among three SQC datasets with survival information which were found in ONCOMINE, including the Raponi (SQC only; ref. 38), Larsen (SQC only; ref. 39), and Zhu (adenocarcinoma and SQC; ref. 11) cohort, the Raponi and the Zhu cohorts were included in SQC analyses. For the Zhu cohort, only patients with SQC were analyzed, whereas patients with adenocarcinoma ($n = 14$, stage I) were disregarded, since considerable number of patients with adenocarcinoma were already used within (CAN/DF) of the Directors cohort (11). The Larsen cohort was excluded because BRCA1 gene was not available in their platform.

Gene expression data analysis

In this study, we focused only on stage I patients. The 4-coding gene analysis of five original cohorts used American Joint Committee on Cancer (AJCC) TNM 6th edition as described previously (29). Concerning seven new cohorts, although the TNM edition was not specified as either 6th or 7th in each of original papers, we assumed that they were based on AJCC TNM 6th edition because most tumors were collected before the development of TNM 7th edition in 2009. For the Rousseaux cohort, T1N0 tumors were defined as stage IA, whereas T2N0 tumors were defined as stage IB, according to the provided TNM classification for each patient. Among all available stage I cases obtained from the public datasets, two adenocarcinomas and one SQC in the Tang cohort, three adenocarcinomas and four SQCs in the Lee cohort were excluded from the analysis because survival information was not provided for those cases.

For all analyses, the normalized expression values were obtained from each dataset and were not processed further. We then generated criteria to select the most reliable, informative probes. In brief, if there were multiple probes to a single gene, pairwise correlation of each probe was analyzed in each cohort. Probes were removed if they

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showed no correlation (R<0.5) between any of the other probes for that gene. If there were only two probes to a single gene that did not correlate with each other, the probe with the highest expression was selected. Probes that were selected are shown in Supplementary Table S2. If more than one probe was selected, they were averaged and no further processing was performed. The 4-coding gene classifier [(0.104 × *BRCA1*) + (0.133 × *HIF1A*) + (−0.246 × *DLC1*) + (0.378 × *XPO1*)] was applied to all newly obtained cohorts using microarray expression data, and the resulting classifier score was categorized as low, medium, or high based on tertiles within each cohort separately. The within cohort categorization was performed to standardize risk scores across all cohorts. This will compensate for the fact that each study used different methodologies to measure the expression of each of the four genes and these expression values are not directly comparable with one another across cohorts. The association between the 4-gene classifier and survival was assessed by the Kaplan–Meier log-rank test for trend using Graphpad Prism v5.0 (Graphpad Software Inc). Cox regression analyses were carried out using SPSS 11.0 (SPSS Inc), and all univariable and multivariable models were adjusted for cohort membership where appropriate. Forest plot analyses and calculations for the nomograms were performed using Stata 11.2 (Stata-Corp LP). Heterogeneity test for the combined HR was carried out using the I^2 statistics (40). Nomograms were developed based on coefficients from multivariable Cox regression models on 5-year overall survival using all variables that were significantly associated with patient outcome.

Results

Identification of eligible studies

Because the purpose of this gene expression-based classifier is to identify high-risk, stage I adenocarcinoma patients who may benefit from additional intervention after surgery, we limited all the analyses in this study to stage I primary adenocarcinoma tumors. The systematic search identified 11 microarray datasets consisting each of more than 30 cases of stage I adenocarcinoma patients with sufficient survival information with gene expression data for all four genes, including *BRCA1, HIF1A, DLC1*, and *XPO1* as described in Fig. 1. Four of the 11 datasets were previously analyzed for our initial paper describing the signature, in which five independent cohorts of stage I adenocarcinomas were each analyzed by qRT-PCR and/or microarrays (29). Hence, seven independent cohorts were newly obtained through this systematic search and a total of 12 cohorts were included in this study. The characteristics of the 12 cohorts are summarized in Table 1. This analysis includes 1,069 patients in total, consisting of 546 stage IA and 518 stage IB cases (five cases were not specified as stage IA or IB). These cohorts were derived from six different countries, including Japan, Norway, Sweden, France, South Korea, as well as at least eight different institutions in the United States. Nine of 12 cohorts reported overall survival information, two cohorts reported relapse-free survival and one cohort reported cancer-specific survival. In each cohort, RNA samples were isolated from frozen tumor specimens and were subjected to gene expression analysis based on various platforms, including qRT-PCR and Affymetrix, Illumina, or Agilent microarrays (Table 1).

The 4-gene classifier is tested in 12 independent cohorts

The 4-gene classifier was applied to each of the seven newly obtained cohorts using microarray expression data for four genes, and then cases were categorized as high, medium, or low, based on tertiles for each cohort separately. Similar to our previous results, highly concordant associations were found between the 4-gene classifier and prognosis in all seven newly obtained cohorts, including the Tang ($P = 0.046$), Rousseaux ($P = 0.044$), Wilkerson ($P = 0.014$), Matsuyama ($P = 0.028$), Lee ($P = 0.010$), Botling ($P = 0.058$), and Bild ($P = 0.120$) cohorts by the Kaplan–Meier analysis (Fig. 2). Overall, the 4-gene classifier was significantly associated with prognosis in 10 cohorts, whereas the remaining two cohorts had marginal associations in the proper direction.

The nine cohorts that had overall survival information were analyzed in a fixed effects meta-analysis model, which included 817 stage I cases. There was no evidence for heterogeneity or inconsistency across multiple cohorts ($I^2 = 0.0\%$, $P = 0.980$), suggesting that these results are representative of most lung adenocarcinomas and not a result of selection bias (Fig. 3). Patients that were classified as high risk had significantly worse overall survival (HR = 1.73; 95% CI, 1.47–2.02) in stage I analysis (Fig. 3A). The corresponding Kaplan–Meier analysis for the combined stage I patients demonstrated a significant association with overall survival and the 4-gene classifier ($P < 0.0001$; Fig. 3A). Furthermore, stratified analyses were performed for stage IA and IB separately, to address the prognostic impact of this classifier in these subgroups. Significant associations between the 4-gene classifier and overall survival were found in both stage IA (HR = 1.61; 95% CI, 1.27–2.06) and stage IB (HR = 1.76; 95% CI, 1.41–2.19) analyses, respectively (Fig. 3B and C).

The 4-gene classifier is an independent prognostic biomarker for stage IA as well as stage IB patients

Given that the classifier is significantly associated with survival in stage IA and IB subgroups, Cox regression analysis was conducted using the combined cohort with respect to each stage (Table 2). All univariable and multivariable Cox analyses were adjusted for cohort membership and multivariable models were adjusted for age, gender, and TNM stage. Because most of the public datasets did not provide complete clinical information, we could not apply other parameters, such as smoking status or adjuvant chemotherapy to the Cox analysis. In univariable analysis, older age, male gender, TNM stage IB, and the 4-gene classifier were each significantly associated with worse outcome. Multivariable models

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Table 1. Twelve independent cohorts of stage I lung adenocarcinoma patients

			TNM Stage			Age	Gender			Postoperative therapy					
Cohorts	**Country**	***n***	**IA**	**IB**	**IA or IB**	**Mean**	**M**	**F**	**Smoker (%)**	**CT/RT**	**None**	**Unknown**	**Outcome**	**Platform**	**GEO ID**
Five cohorts															
Japan	Japan	149	100	49	0	59.7	66	83	45.6	0	149	0	RFS	qRT-PCR	—
U.S./Norway	U.S. (UMD), Norway	67	29	38	0	64.6	37	30	96.9	4	43	20	CSS	qRT-PCR	—
Directors	U.S. (MSK,HLM,CAN/DF,UM)	276	114	162	0	64.4	131	145	NA	46	157	73	OS[a]	Affymetrix U133A	NA[b]
Bhattacharjee	U.S. (Harvard)	76	35	40	1	64.2	32	44	90.8	0	0	76	OS[a]	Affymetrix U95A	NA[b]
Tomida	Japan	79	42	37	0	61.4	41	38	50.6	0	79	0	OS[a]	Agilent 44K	GSE13213
Seven new cohorts															
Tang	U.S. (MD Anderson)	87	32	55	0	64.1	37	50	NA	22	65	0	OS[a]	Illumina WG6 V3	GSE42127
Rousseaux	France	81	73	8	0	61.8	65	16	NA	0	0	81	OS[a]	Affymetrix U133+2	GSE30219
Botling	Sweden	70	28	42	0	63.5	31	39	NA	5	31	34	OS[a]	Affymetrix U133+2	GSE37745
Wilkerson	U.S. (UNC)	62	31	31	0	65.6	26	36	88.0	0	0	62	OS[a]	Agilent 44K custom	GSE26939
Matsuyama	Japan	52	28	24	0	62.3	28	24	46.2	0	0	52	OS[a]	Agilent 21.6K custom	GSE11969
Lee	Korea	36	13	23	0	61.4	16	20	38.9	0	0	36	RFS	Affymetrix U133+2	GSE8894[b]
Bild	U.S. (Duke)	34	21	9	4	64.8	17	17	NA	0	0	34	OS[a]	Affymetrix U133+2	GSE3141[b]
	Total	1,069	546	518	5	63.1	527	542		77	524	468			

Abbreviations: CT/RT, chemotherapy and/or radiotherapy; NA, not available; RFS, relapse-free survival; CSS, cancer-specific survival; OS, overall survival.

[a]Nine cohorts with overall survival information were used in the combined analysis ($n = 817$).

[b]Data were obtained from ONCOMINE.

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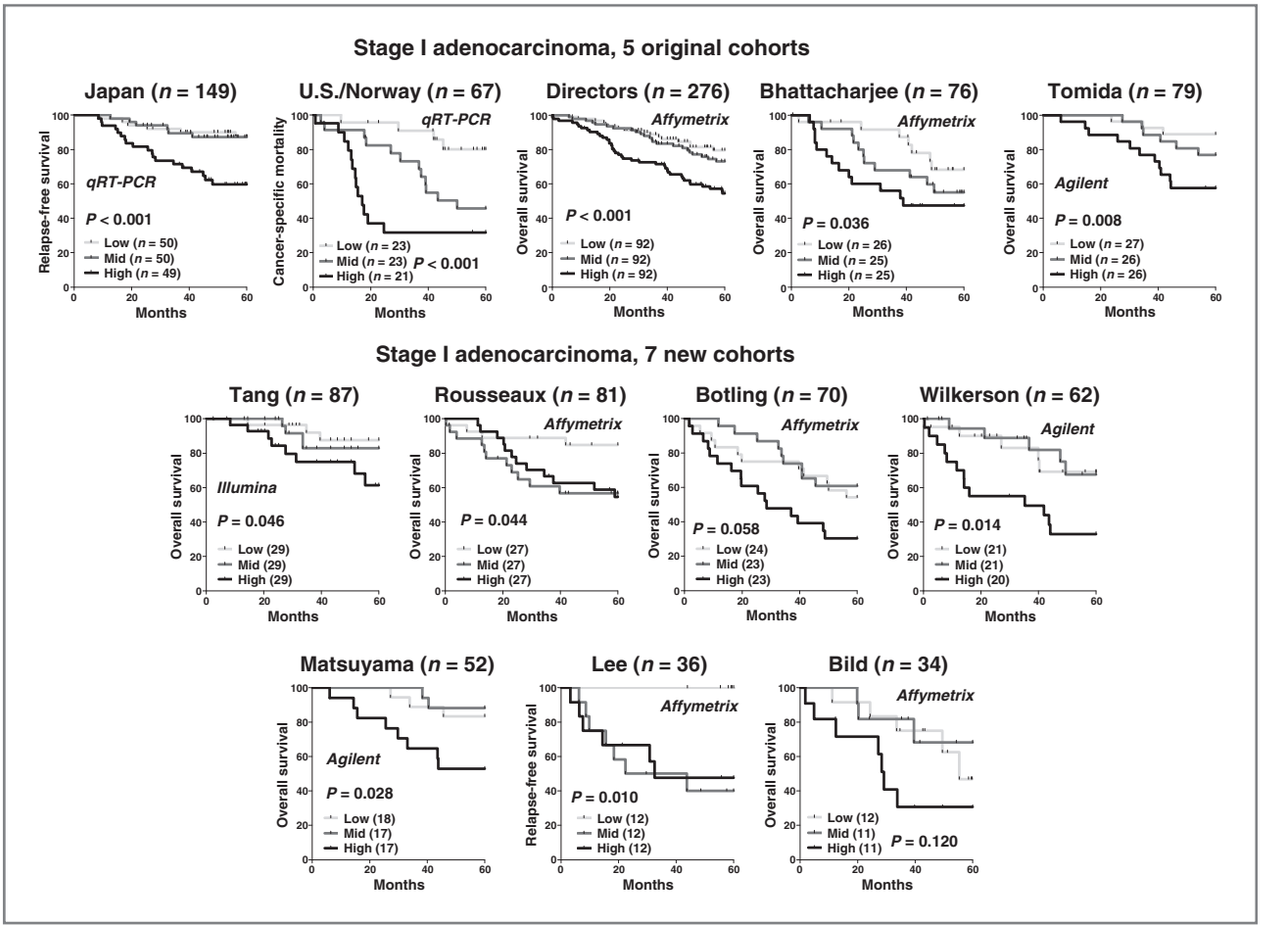

Figure 2. The performance of the 4-coding gene classifier in 12 independent cohorts of stage I lung adenocarcinoma patients. For each cohort, cases were categorized as high, medium, or low based on tertiles. *P* values were obtained by the log-rank test for trend.

revealed that the 4-gene classifier was significantly associated with poor overall survival, independent of other parameters, in stage I patients (HR = 2.66; 95% CI, 1.93–3.67; $P < 0.0001$) and in stratified analyses of stage IA (HR = 2.69; 95% CI, 1.66–4.35; $P < 0.0001$) and stage IB (HR = 2.69; 95% CI, 1.74–4.16; $P < 0.0001$) patients. We have also performed an analysis of the risk score as a linear variable (rather than an ordered categorical variable) to demonstrate these associations are highly robust and do not rely on using tertiles as cutpoints for the data (Supplementary Table S3)

The potential use of the 4-gene classifier to predict prognosis for stage I lung adenocarcinoma

In order for a prognostic classifier to be clinically useful, it has to provide actionable information to the physician. To demonstrate this potential for the 4-gene classifier, we developed a nomogram to predict 5-year survival rates in patients diagnosed with stage I lung adenocarcinoma (Fig. 4). This nomogram is based on the nine cohorts with overall survival data and includes all variables that were significantly associated with 5-year overall survival. The points assigned to each variable are weighted on the basis of Cox regression coefficients. This nomogram demonstrates that the 4-gene classifier could be used with clinical staging and other clinical parameters to predict the probability of 5-year survival. Subgroup analysis within stage is important to demonstrate to show clinical utility, therefore, we created nomograms stratified by TNM stage IA and IB separately as another example of how this classifier can be integrated with TNM staging to help determine patient prognosis (Supplementary Fig. S1).

The 4-gene classifier is applicable only to patients with adenocarcinoma

Up to now we have focused only on patients with lung cancer adenocarcinoma histology. To determine whether this association could be observed across different histologies, we examined the 4-gene classifier in SQC, which is another major histologic type of NSCLC. Nine independent cohorts, consisting of 337 stage I SQC patients, were obtained and the 4-gene classifier was applied to each cohort (Supplementary Table S4). In a combined analysis,

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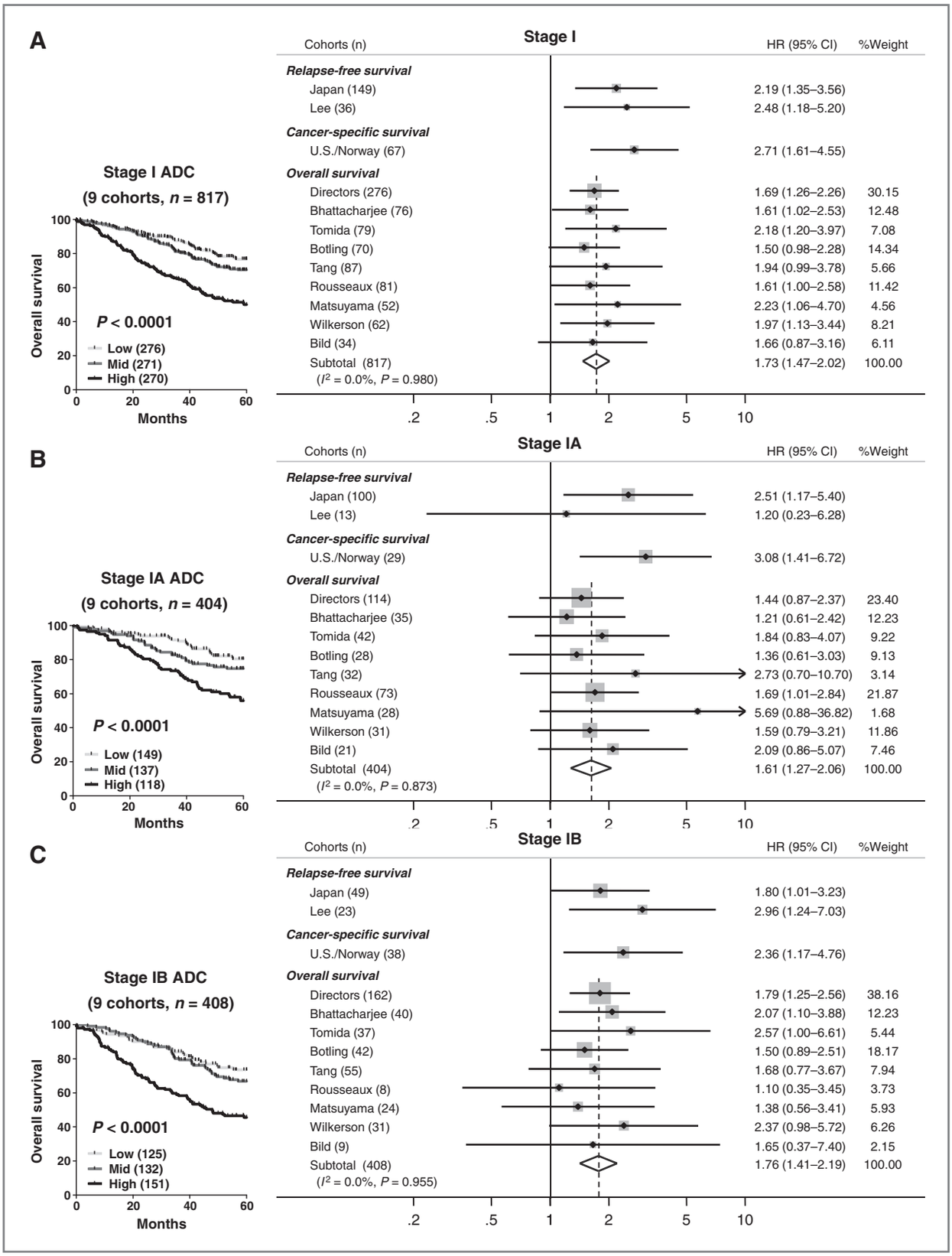

Figure 3. Forest plot of the prognostic impact of the 4-coding gene classifier in 12 independent cohorts of stage I lung adenocarcinoma. A, meta-analysis of all patients with TNM stage IA or IB lung adenocarcinoma. B, meta-analysis of all patients with TNM stage IA lung adenocarcinoma. C, meta-analysis of all patients with TNM stage IB lung adenocarcinoma.

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Table 2. Univariable and multivariable Cox regression of the 4-coding gene classifier in the combined cohort[a] of stage I adenocarcinoma patients

		Univariable analysis[b]		Multivariable analysis[b]	
Variable (*n*)		**HR (95% CI)**	***P***	**HR (95% CI)**	***P***
TNM stage I ($n = 817$)					
4-gene classifier[c]	Low (276)	Reference	NA	Reference	NA
	Medium (271)	1.34 (0.95–1.89)	0.101	1.27 (0.89–1.80)	0.183
	High (270)	**2.83 (2.07–3.86)**	**<0.0001**	**2.66 (1.93–3.67)**	**<0.0001**
		P_{trend} **<0.0001**		P_{trend} **< 0.0001**	
Stage[d]	IB (408)/IA (404)	**1.68 (1.29–2.19)**	**0.0001**	**1.55 (1.19–2.03)**	**0.001**
Age	Continuous	**1.03 (1.02–1.05)**	**<0.0001**	**1.04 (1.02–1.05)**	**<0.0001**
Gender	Female (409)/male (408)	**0.67 (0.52–0.87)**	**0.002**	0.78 (0.60–1.01)	0.062
TNM stage IA ($n = 404$)					
4-gene classifier[c]	Low (149)	Reference	NA	Reference	NA
	Medium (137)	1.47 (0.87–2.49)	0.151	1.42 (0.84–2.40)	0.191
	High (118)	**2.69 (1.67–4.34)**	**<0.0001**	**2.69 (1.66–4.35)**	**<0.0001**
		P_{trend} **< 0.0001**		P_{trend} **< 0.0001**	
Age	Continuous	**1.03 (1.01–1.06)**	**0.002**	**1.04 (1.02–1.06)**	**0.0007**
Gender	Female (205)/male (199)	**0.61 (0.40–0.91)**	**0.016**	**0.65 (0.43–0.99)**	**0.043**
TNM stage IB ($n = 408$)					
4-gene classifier[c]	Low (125)	Reference	NA	Reference	NA
	Medium (132)	1.20 (0.74–1.93)	0.456	1.14 (0.71–1.84)	0.586
	High (151)	**2.88 (1.88–4.43)**	**<0.0001**	**2.69 (1.74–4.16)**	**<0.0001**
		P_{trend} **< 0.0001**		P_{trend} **< 0.0001**	
Age	Continuous	**1.04 (1.02–1.05)**	**<0.0001**	**1.03 (1.02–1.05)**	**<0.0001**
Gender	Female (203)/male (205)	0.75 (0.54–1.06)	0.102	0.90 (0.64–1.26)	0.533

[a]The combined cohort consists of 9 publicly available, independent microarray datasets of stage I patients with overall survival information, including the Directors (276), Bhattacharjee (76), Tomida (79), Botling (70), Tang (87), Rousseaux (81), Matsuyama (52), Wilkerson (62), and Bild (34) cohorts.

[b]The univariable model was adjusted for cohort membership and the multivariable model included the 4-gene classifier, cohort membership, age, gender, and TMN staging. Statistically significant ($P < 0.05$) associations are in bold.

[c]The 4-coding gene classifier was categorized based on tertiles of stage I patients for each cohort.

[d]There were a total of five stage I cases in the Bhattacharjee (1) and Bild (4) cohorts for which stage IB/IA information is not available. These are included in univariable analyses and excluded in multivariable analyses.

the eight cohorts with overall survival information were combined ($n = 292$). However, no significant association was found in any of the SQC analyses (Supplementary Fig. S2), indicating that the 4-gene classifier is specific to adenocarcinoma. This was not completely unexpected because we built this classifier using adenocarcinoma gene expression data only. SQC and adenocarcinoma are also considered to be molecularly distinct entities (41). Therefore, this classifier seems to be only useful for lung adenocarcinoma and not SQC. Sufficient numbers were not available to examine other histologies.

Discussion

Translating prognostic gene signatures into clinical use is a major challenge in the field of lung cancer research. There is little doubt that prognostic tests are necessary for stage I lung cancer patients after complete resection. In breast cancer, it is striking to note that several multigene assays are already commercially available and are currently used by clinical oncologists and supported by the NCCN and other guidelines (42, 43). As for lung cancer, no prognostic biomarkers have been incorporated into the current guidelines despite a large number of published gene signatures. This may be at least in part due to insufficient reproducibility as well as the lack of large-scale validation (27). Also, it has been suggested that many signatures were developed without clear focus on specific clinical contexts (27). To address those issues, our study has set out to test whether the 4-gene classifier that we previously identified is a robust prognostic classifier for stage I lung adenocarcinoma using every publically available dataset. The 4-gene classifier was a robust classifier for over 1,000 TNM stage I lung adenocarcinoma cases from 12 cohorts regardless of ethnic difference in the genetic background of the patients. When each cohort was

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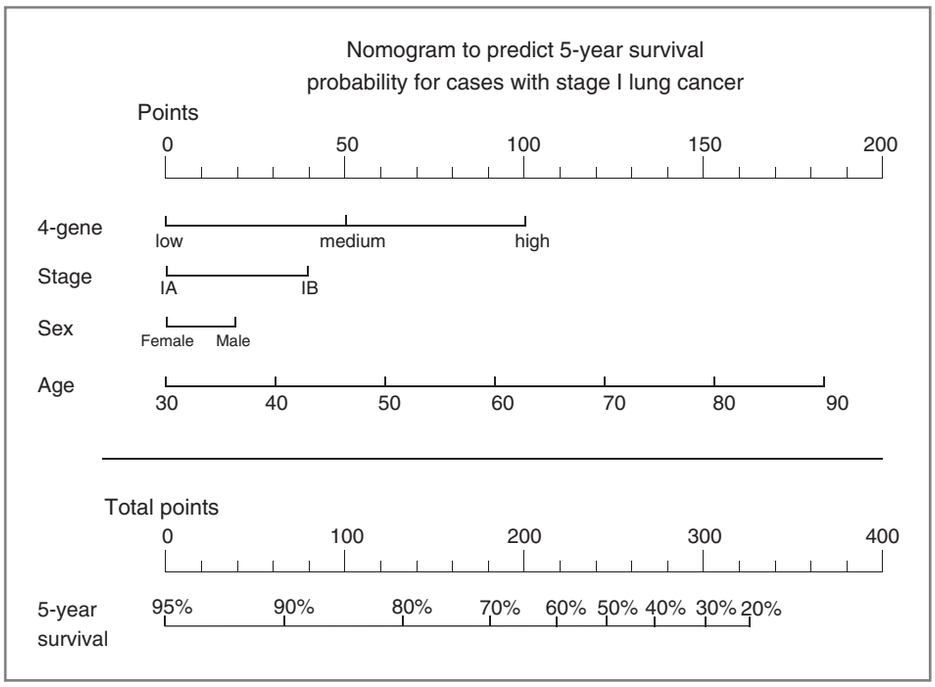

Figure 4. Nomogram to predict 5-year survival rates for stage I lung adenocarcinoma. Each clinical variable (4-gene score, TNM stage, sex, and age) is assigned a point value. The sum of those points can then be used to estimate probability of survival for 5 years. For example, if the sum of the points is 300, a patient has approximately a 30% 5-year survival probability.

separately analyzed, the 4-gene classifier showed highly consistent results for its association with survival. The classifier was highly reproducible across multiple platforms for gene expression measurement, including qRT-PCR and commercial/custom microarrays from Affymetrix, Agilent, and Illumina. There is no evidence of selection bias in any of the 12 cohorts, which suggests these results presented here are representative of stage I lung adenocarcinoma. However, the classifier had no prognostic impact on patients with SQC, indicating its limited utility only to lung adenocarcinomas.

The 4-gene classifier has potential to be used as a prognostic biomarker for the management of stage IA and stage IB adenocarcinoma patients in the current clinical setting. The pooled estimate revealed significant association between the 4-gene classifier and prognosis in stage IA and IB lung adenocarcinoma subgroups, independent of other clinical variables. This suggests that the classifier can add additional discriminative value to identify high-risk patients beyond conventional clinical characteristics. Hence, "low-risk" stage IB patients identified by the classifier and predicted to have excellent survival probabilities may be recommended to forgo adjuvant therapy. Likewise, the classifier may also identify "high-risk" stage IA patients for whom intensive postoperative intervention is considered. Future work should explore and identify the optimal cutpoint for this assay that should distinguish "high-risk" and "low-risk patients." For convenience, we have used tertile as a cutpoint for our studies to distinguish between high/medium/low risk. It is likely that optimal cutpoints can be found that will improve the clinical utility of this classifier.

Many published multigene signatures for NSCLC had utilized dozens to hundreds or even thousands of genes along with complex classification models that are difficult to understand. We believe that this is an obstacle to the rapid development and feasibility of the clinical tests. In contrast, the 4-gene classifier uses the expression values of only four genes, potentially providing an opportunity to develop a simple and practical laboratory test. The classifier is composed of biologically relevant genes that are mechanistically important and are each significantly associated with prognosis in early-stage lung adenocarcinoma (29). We consider that our strategy of focusing on only biologically relevant genes have improved the chances of developing a robust classifier that will be generalizable to lung adenocarcinoma.

A potential limitation for this analysis is that there were incomplete data on smoking status and types of chemotherapy that were retrieved for several of the cohorts used for the meta-analysis. Therefore, we were not able to include these covariates in the models. Smoking history was considered as a covariate within the discovery cohorts and found to not contribute to the risk association model (29). Adjuvant chemotherapy is not recommended for stage I patients, thus the majority of the patients included in our analysis would not have received any. In fact, only 4% of patients in the Japan discovery cohort received adjuvant chemotherapy (29). Still, these factors are important for survival after lung cancer surgery and it is possible that their inclusion may modulate the association between the 4-gene classifier and prognosis.

We have shown that the classifier is robust and that using qRT-PCR data and microarray data from a variety of laboratories provides similar results. To turn the 4-gene

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Published OnlineFirst September 21, 2014; DOI: 10.1158/1055-9965.EPI-14-0182

classifier into a clinical test, future work should focus on developing a standardized assay that will include developing methods for measuring each of the four genes and recommending methods for tissue handling, processing, and RNA isolation. Assays will have to be designed to minimize the potential batch effects and interlaboratory differences. Another future possibility is the use of RNA samples extracted from formalin-fixed paraffin-embedded (FFPE) tissues. This could extend the practical utility of the classifier to readily available archived specimens. Furthermore, our previous study demonstrated an improved prognostic association by combining multiple, validated classifiers, namely the combination of the 4-coding gene classifier with non-coding miRNA-21 (miR21) classifier for stage I lung adenocarcinoma (29). In addition to the present results, recent meta-analysis studies demonstrating miR21 as a promising prognostic biomarker for NSCLC may be supportive to the potential combined use of the 4-gene and miR21 classifiers as validated biomarkers (44, 45). This demonstrates that the integration of multiple, independent classifiers can further improve prognostic predictions and suggests that these classifiers can be combined with additional biomarkers and histologic subtyping data to improve decision making capabilities for early-stage lung cancer.

In conclusion, the 4-gene classifier that we recently developed was rigorously validated in large-scale, multiple cohorts with a meta-analytic approach consisting of more than 1,000 stage I patients. To our knowledge, this is the first report of an RNA-based classifier in lung adenocarcinoma to be tested and validated this extensively. The reproducibility of its performance was clearly demonstrated in the intended clinical context based on unbiased approaches. Particularly, the classifier provides additional prognostic stratification beyond the current risk factors, namely, stage IA and stage IB subgroups, highlighting the potential of this classifier in personalized management for early-stage patients. These results support the development of standardized tests for the 4-gene assays and the incorporation of these assays into prospective studies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: H. Okayama, A.J. Schetter, C.C. Harris
Development of methodology: H. Okayama, A.J. Schetter
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): H. Okayama, T. Kohno, J. Yokota
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): H. Okayama, A.J. Schetter, T. Ishigame, A.I. Robles
Writing, review, and/or revision of the manuscript: H. Okayama, A.J. Schetter, A.I. Robles, C.C. Harris
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): H. Okayama, J. Yokota
Study supervision: J. Yokota, S. Takenoshita, C.C. Harris

Grant Support

This work was supported by the Intramural Research Program of the National Cancer Institute, NIH and Department of Defense Congressionally Directed Medical Research Program Grant PR093793.

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Received February 18, 2014; revised September 15, 2014; accepted September 16, 2014; published OnlineFirst September 21, 2014.

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Cancer Epidemiology, Biomarkers & Prevention

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Cancer Epidemiol Biomarkers Prev 2014;23:2884-2894. Published OnlineFirst September 21, 2014.

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