Validation of a 40-gene expression profile test to predict metastatic risk in localized high-risk cutaneous squamous cell carcinoma

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Background: Current staging systems for cutaneous squamous cell carcinoma (cSCC) have limited positive predictive value for identifying patients who will experience metastasis.

Objective: To develop and validate a gene expression profile (GEP) test for predicting risk for metastasis in localized, high-risk cSCC with the goal of improving risk-directed patient management.

Methods: Archival formalin-fixed paraffin-embedded primary cSCC tissue and clinicopathologic data (n = 586) were collected from 23 independent centers in a prospectively designed study. A GEP signature

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Funding sources: Supported by Castle Biosciences, Inc, which provided funding for tissue and clinical data retrieval to contributing centers.

Disclosure: Drs Wysong (steering committee), Newman (steering committee), Schmults (steering committee), Arron (steering committee), Ibrahim, Farberg, Bar, Cleaver, Somani, Brodland, Toyohara, Maher, Xia, Bibee, Griego, and Rigel are principal investigators for the study. Dr Wysong is an unpaid board member of the American College of Mohs Surgeons and World Congress of Dermatology. Drs Covington, Kurley, Meldi Plasseraud, Estrada, and Cook, and authors Scholl and Johnson are employees and options holders at Castle Biosciences. Dr Schmults is an investigator for Novartis, Genentech and Merck, a consultant for Sanofi and Regeneron (steering committee), and chair for the National Comprehensive Cancer Network. Dr Arron is an investigator for Pfizer, Regeneron and PellePharm, and a consultant for Enspectra Health, Rakuten Medical, and Gerson Lehrman Group. Drs Panther and Zitelli have no disclosures.

Presented at the 2019 American Society for Dermatologic Surgery Annual Meeting, Chicago, IL, October 24-27, 2019.

IRB approval status: Approved by IRBs before initiation. IRB granted a waiver of consent because of the nature of the disease under study.

Accepted for publication April 15, 2020.

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Published online April 25, 2020.

0190-9622

https://doi.org/10.1016/j.jaad.2020.04.088

was developed using a discovery cohort (n = 202) and validated in a separate, nonoverlapping, independent cohort (n = 324).

Results: A prognostic 40-GEP test was developed and validated, stratifying patients with high-risk cSCC into classes based on metastasis risk: class 1 (low risk), class 2A (high risk), and class 2B (highest risk). For the validation cohort, 3-year metastasis-free survival rates were 91.4%, 80.6%, and 44.0%, respectively. A positive predictive value of 60% was achieved for the highest-risk group (class 2B), an improvement over staging systems, and negative predictive value, sensitivity, and specificity were comparable to staging systems.

Limitations: Potential understaging of cases could affect metastasis rate accuracy.

Conclusion: The 40-GEP test is an independent predictor of metastatic risk that can complement current staging systems for patients with high-risk cSCC. (J Am Acad Dermatol 2021;84:361-9.)

Key words: cutaneous squamous cell carcinoma; gene expression profile; metastasis; prognostication; risk.

CAPSULE SUMMARY

- Development and independent validation of a 40-gene expression profile (40-GEP) test showed improved metastasis risk stratification of patients with high-risk cutaneous squamous cell carcinoma.
- Incorporation of 40-GEP prognostication into clinical practice could support risk-aligned patient management decisions by complementing current staging systems.

The incidence of cutaneous squamous cell carcinoma (cSCC) has increased substantially in recent decades,[1,2] with concurrent increases in morbidity and mortality. Currently, estimated cSCC incidence ranges from 1 to 2.5 million cases annually in the United States,[2-5] and deaths from cSCC are estimated to exceed deaths from melanoma.[2,4-11] The rates of metastasis of tumors with high-risk features can surpass 20%.[3,10,12-19] Once metastasis is detected, 5-year survival rates drop to 50% to 83% and to less than 40% for patients with regional and distant metastasis, respectively.[16,20-22] Because early detection of metastasis is correlated with better outcomes, accurate identification of patients at high risk for metastasis is critical, potentially allowing for early adjuvant therapy, while also avoiding overtreatment of low-risk tumors.

Clinicopathologic staging and national guidelines are used to risk-stratify and treat patients. The National Comprehensive Cancer Network (NCCN) guidelines assign patients with local disease to low- and high-risk groups using clinicopathologic features associated with recurrence, providing broad recommendations for surgical and therapeutic interventions.[3] The American Joint Committee on Cancer (AJCC) *Cancer Staging Manual* uses clinicopathologic features of the primary tumor with 4 T stages grouped into binary risk groups (T1/T2 vs T3/T4).[23] Positive predictive value (PPV) is low for NCCN and AJCC (14%-17%),[24-27] because many patients categorized as high risk do not develop advanced disease.[28,29] The Brigham and Women's Hospital (BWH) staging system includes 4 T stages (T1, T2a, T2b, and T3), categorizing tumors by number of high-risk features observed. For BWH, T2b and T3 tumors are generally combined to identify high-risk disease. Sensitivity is comparable between BWH and AJCC, whereas PPV for BWH (24%-38%) is superior to AJCC.[24-27]

To improve identification of patients with primary cSCC at high risk for metastatic disease, a 40-gene expression profile (40-GEP) test was developed. The GEP of primary cSCC tumors with known outcomes was used to develop a prognostic molecular algorithm. We report validation of this 40-GEP test, which identifies 3 classes (classes 1, 2A, and 2B) of patients with cSCC with different likelihoods of developing metastasis within 3 years of diagnosis. The 40-GEP test is an independent predictor of outcomes and improves on risk prediction with staging systems, supporting its potential clinical use in conjunction with standard staging and patient management criteria.

METHODS

Study design

A prospectively designed biomarker study was conducted by using archival primary cSCC formalin-fixed paraffin-embedded tissue. The primary

Abbreviations used:

AJCC:	American Joint Committee on Cancer
BWH:	Brigham and Women's Hospital
cSCC:	cutaneous squamous cell carcinoma
GEP:	gene-expression profile
HR:	hazard ratio
LR:	likelihood ratio
MFS:	metastasis-free survival
NCCN:	National Comprehensive Cancer Network
NPV:	negative predictive value
PPV:	positive predictive value

endpoint was 3-year metastasis-free survival (MFS), including regional and distant metastatic events. Regional metastasis was defined as metastasis within the regional nodal basin, including satellite or in-transit metastasis, but excluding local recurrence. Distant metastasis was defined as metastasis beyond the regional lymph node basin. Disease-specific death, a secondary endpoint, was defined as documented death from cSCC. All samples from patients included in the study were from primary cSCC tumors (Fig 1). Patients with local recurrence only were not considered as having a metastatic event.

Expression levels of 140 candidate genes, identified by discovery efforts or literature review,[30-36] were determined for samples in the discovery and development cases (cohort 1, n = 202). Deep machine learning was applied to expression data from 122 genes passing initial expression thresholds to select genes for further signature training; see the supplemental materials (available via Mendeley at https://doi.org/10.17632/f33w9wmng4.1) for detailed methods of discovery/development. The algorithm encompassing the 40-GEP assay was selected based on prognostic performance in the training cases (n = 122). Coefficients for each gene in the algorithm were locked before validation. Power calculations indicated that the validation cohort (cohort 2; samples passing quality control, n = 321) could detect a hazard ratio (HR) of 2.1 for metastasis (90% power; alpha, 0.05). After validation of the algorithm using cohort 2, clinically actionable cutpoints for probability scores were set to optimize negative predictive value (NPV), PPV, and sensitivity for metastasis risk groups (class 1: low risk; class 2A, high risk; class 2B, highest risk).

Patient enrollment and specimen acquisition

Primary cSCC tissue and associated deidentified clinical data were obtained from 23 independent centers after institutional review board approval. Clinicopathologic and outcomes data were entered into a secure case report form. All reported patient

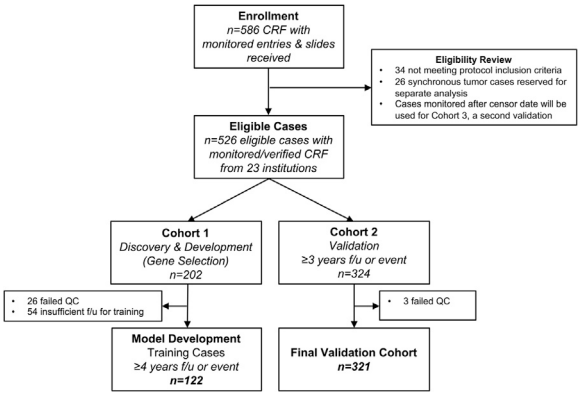


Fig 1. Study cohorts: tissue samples and associated data acquisition. Protocol and monitoring are ongoing; assessment was performed October 1, 2019. To ensure proper classification, the training set was restricted to cases with a documented metastatic event or at least 4 years of follow-up. Cases not included in this report will be used for a second validation cohort. QC criteria were different between the discovery and validation assays. *CRF,* Case report form; *event,* regional or distant metastasis; *f/u,* follow-up; *QC,* quality control.

data were monitored onsite, including review of all available pathology reports and medical records. Per the ongoing study protocol, 586 archival cSCC cases were received between the study onset (September 3, 2016) and October 1, 2019 (Fig 1). The complete protocol inclusion/exclusion criteria are summarized in the supplemental materials. The protocol targeted enrollment of cases with at least 1 high-risk feature as defined by NCCN guidelines or by AJCC or BWH staging as greater than T1, either at the patient or tumor level, to model the high-risk cSCC patient population for whom the 40-GEP assay was developed. For the validation cohort, monitors reviewed 98.4% (314/319) of all definitive surgery pathology reports. Staging incorporated all available data in the medical record and centralized pathology review by a board-certified dermatopathologist.

Assay methods and statistical analyses

Tissue sections (5 μm) were freshly cut at contributing institutions and collected at a central College of American Pathologists-accredited laboratory. Tumor tissue, including tumor stroma, was macrodissected from slides and processed to generate RNA and complementary DNA, as previously described.[37] Complementary DNA underwent a 14-cycle preamplification step before dilution and then was mixed 1:1 with 2× TaqMan Gene Expression Master Mix (Thermo Fisher Scientific, Waltham, MA).

Table I. Demographics and clinical characteristics of the validation cohort (n = 321)

Feature	All (n = 321)	No metastasis (n = 269)	Regional/distant metastasis (n = 52)	*P* value
Age, y, median (range)	70 (34-95)	70 (34-95)	72 (44-90)	.84
Male sex, n (%)	235 (73.2)	191 (71.0)	44 (84.6)	.042
White, n (%)	320 (99.7)	269 (100)	51 (98.1)	.16
Non-Hispanic, n (%)*	312 (97.2)	262 (97.4)	50 (96.2)	.62
Immune deficient, n (%)†	76 (23.7)	59 (21.9)	17 (32.7)	.10
Prior Hx of SCC, n (%)	135 (42.1)	109 (40.5)	26 (50.0)	.22
Located on head and neck, n (%)	214 (66.7)	171 (63.6)	43 (82.7)	.007
Tumor diameter, cm, mean ± SD‡	1.8 ± 1.9	1.6 ± 1.8	2.8 ± 2.4	<.0001
Tumor thickness, mm, mean ± SD§	3.9 ± 6.4	3.4 ± 6.6	7.2 ± 3.6	<.0001
Poorly differentiated, n (%)	36 (11.2)	22 (8.2)	14 (26.9)	<.0001
Clark level IV/V, n (%)	62 (19.3)	49 (18.2)	13 (25.0)	<.0001
PNI, n (%)‖				<.0001
Present (≥0.1 mm)	7 (2.2)	5 (1.9)	2 (3.9)	
Present (<0.1 mm or unknown caliber)	29 (9.0)	16 (6.0)	13 (25)	
Not present	285 (88.8)	248 (92.2)	37 (71.2)	
Invasion into fat, n (%)	43 (13.4)	28 (10.4)	15 (28.9)	.0004
Definitive surgery MMS, n (%)¶	256 (79.8)	222 (82.5)	34 (65.4)	.032
AJCC8 T stage, n (%)				
T1	201 (62.6)	175 (65.1)	26 (50)	.001
T2	59 (18.4)	53 (19.7)	6 (11.5)	
T3	54 (16.8)	36 (13.4)	18 (34.6)	
T4	7 (2.2)	5 (1.9)	2 (3.9)	
BWH T stage, n (%)				
T1	186 (57.9)	166 (61.7)	20 (38.5)	.0004
T2a	98 (30.5)	79 (29.4)	19 (36.5)	
T2b	30 (9.4)	19 (7.1)	11 (21.2)	
T3	7 (2.2)	5 (1.9)	2 (3.9)	
NCCN high risk, n (%)	300 (93.5)	250 (92.9)	50 (96.2)	.39

Data were analyzed using chi-square test or Kruskal-Wallis *F* test. *AJCC8,* American Joint Committee on Cancer *Cancer Staging Manual*, eighth edition; *BWH,* Brigham and Women's Hospital; *Hx,* history; *MMS,* Mohs micrographic surgery; *NCCN,* National Comprehensive Cancer Network; *PNI,* perineural invasion; *SCC,* squamous cell carcinoma; *SD,* standard deviation.

*One patient did not report ethnicity.

†Of 76 immune-deficient patients, 67 were organ transplant recipients.

‡Tumor diameter reported (n = 295).

§Tumor thickness reported (n = 115).

‖PNI with nerve caliber of 0.1 mm or greater or in nerve deeper than the dermis are upstaging factors for AJCC. Only nerve caliber of 0.1 mm or greater is an upstaging factor for BWH. One of 7 cases met AJCC upstaging but not BWH upstaging.

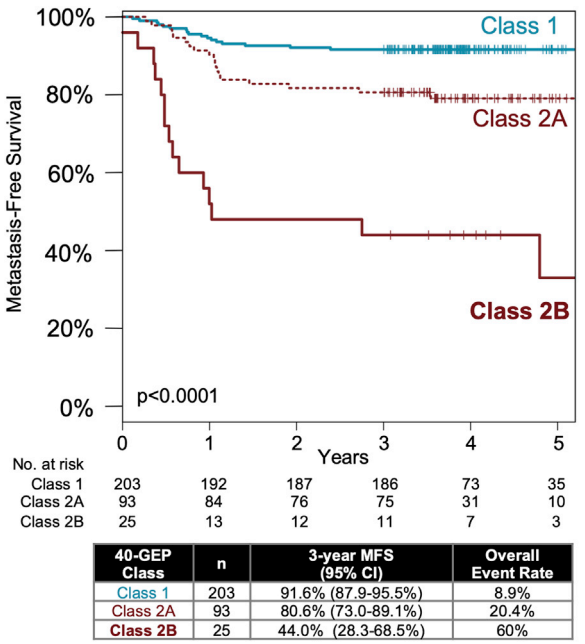
¶Mohs or wide local excision (n = 319), with 2 patients not having additional surgery beyond biopsy.

Quantitative polymerase chain reaction was then performed by using high-throughput microfluidics gene cards containing primers specific to the genes of interest and the QuantStudio 12K Flex Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA). Each sample was run in triplicate with randomization onto plates to distribute metastatic and nonmetastatic cases. Laboratory personnel and clinical monitoring staff were blinded to GEP results during data capture. Statistical analysis was performed as previously described using standard methods for Kaplan-Meier analysis, multivariable Cox regression analysis, accuracy metrics, and sensitivity analysis (see the supplemental materials).

RESULTS

Development of the prognostic signature

To identify a prognostic signature capable of patient stratification by risk for regional or distant metastasis from primary cSCC tumors, deep machine learning was applied to training cohort gene expression data (n = 122) (Supplemental Table I; available via Mendeley at https://doi.org/10.17632/f33w9wmng4.1). The algorithm selected for validation comprised 2 gene expression signatures, inclusive of 6 control and 34 discriminant genes, with modeling performed using neural networks. This 40-GEP algorithm generated linear scores for probability of metastasis from each signature.



40-GEP Class	n	3-year MFS (95% CI)	Overall Event Rate
Class 1	203	91.6% (87.9-95.5%)	8.9%
Class 2A	93	80.6% (73.0-89.1%)	20.4%
Class 2B	25	44.0% (28.3-68.5%)	60%

Fig 2. Kaplan-Meier analysis of the 40-GEP prognostic test and outcomes from independent validation of cSCC cases (n = 321). *CI,* Confidence interval; *cSCC,* cutaneous squamous cell carcinoma; *GEP,* gene-expression profile; *MFS,* metastasis-free survival.

Table II. Multivariate Cox regression analyses of risk for metastasis in 40-GEP validation cases (n = 321) with binary AJCC and BWH T stage

	Multivariate cox regression	
n = 321 (52 events)	**HR (95% CI)**	***P* value**
40-GEP		
Class 1	1.0	—
Class 2A	2.15 (1.12-4.12)	.021
Class 2B	9.55 (4.79-19.06)	<.0001
AJCC8		
T1/T2	1.0	—
T3/T4	2.68 (1.52-4.72)	<.001
40-GEP		
Class 1	1.0	—
Class 2A	2.27 (1.19-4.35)	.013
Class 2B	8.72 (4.30-17.71)	<.0001
BWH		
T1/T2a	1.0	—
T2b/T3	2.03 (1.07-3.88)	.032

An event was regional or distant metastasis. *AJCC8,* American Joint Committee on Cancer *Cancer Staging Manual,* eighth edition; *BWH,* Brigham and Women's Hospital; *CI,* confidence interval; *GEP,* gene expression profile; *HR,* hazard ratio.

Independent validation of the 40-GEP prognostic signature

To validate the prognostic capability of the 40-GEP, the algorithm was applied to an independent validation cohort made up of 321 primary cSCC cases (52 with documented metastasis and 269 cases without an event) (Table I). The algorithm showed a statistically significant ability to stratify metastatic risk. The validated 40-GEP was then used to define risk groups with increasing metastasis risk: class 1 (low risk, n = 203), class 2A (high risk, n = 93), and class 2B (highest risk, n = 25). Significantly different 3-year MFS rates were observed for class 1 (91.6%), class 2A (80.6%), and Class 2B (44.0%) groups by Kaplan-Meier survival analysis (Fig 2) (log rank test, $P < .0001$). Higher 40-GEP class was associated with a statistically significant increase in risk for metastasis and disease-specific death. HRs for metastasis for class 2A and class 2B were 2.44 and 10.15 ($P < .01$, $P < .0001$), and for disease-specific death were 5.4 and 8.8 ($P < .05$, $P < .01$), respectively. Of the 13 reported deaths due to cSCC, 10 were classified as class 2.

Prognostic accuracy of the 40-GEP test compared to staging systems

The 40-GEP signature was an independent predictor of risk when analyzed in a bivariable model with AJCC (class 2A: HR, 2.15; $P = .021$; class 2B: HR, 9.55; $P < .0001$) or BWH (class 2A: HR, 2.27, $P = .013$; class 2B: HR, 8.72; $P < .0001$) T stage (Table II, Supplemental Table II; available via Mendeley at https://doi.org/10.17632/f33w9wmng4.1). Multivariable analysis with individual clinicopathologic features also showed independent prognostic value of the 40-GEP signature (Supplemental Table III; available via Mendeley at https://doi.org/10.17632/f33w9wmng4.1). Supplemental Table IV (available via Mendeley at https://doi.org/10.17632/f33w9wmng4.1) reports the number of cases by metastatic outcome, 40-GEP class, and NCCN risk group or T-stage. Cases with missing clinicopathologic data (n = 168, most missing tumor thickness) were staged in the bivariable analysis with assumption of null values for missing data. Because this may have resulted in understaging by T stage or binary T stage in 34 or 6 cases, respectively, via BWH and 164 cases via AJCC, post hoc sensitivity analyses were performed. These analyses yielded similar effect sizes and significance, showing the robustness of the primary analysis despite the assumption of null values for missing data (Supplemental Table V; available via Mendeley at https://doi.org/10.17632/f33w9wmng4.1).

Overall, accuracy metrics for AJCC (low T1/T2 vs high T3/T4) and BWH (low T1/T2a vs high T2b/T3) staging aligned with previously published data (Table III), although the percentages of metastases occurring in low T stages were higher than

Table III. Accuracy of risk prediction of the 40-GEP and risk assessment methods (n = 321)

Accuracy metric	40-GEP (class 2B vs 1/2A)	40-GEP (class 2 vs 1)	AJCC 8* (T3/T4 vs T1/T2)	BWH* (T2b/T3 vs T1/T2a)	NCCN* (high vs low)
Sensitivity, %	28.8	65.4	38.5	25.0	96.2
Specificity, %	96.3	68.8	84.8	91.1	7.1
+LR	7.78	2.10	2.53	2.81	1.04
-LR	0.74	0.50	0.73	0.82	0.54
PPV, %	60.0	28.8	32.8	35.1	16.7
NPV, %	87.5	91.1	87.7	86.3	90.5

AJCC8, American Joint Committee on Cancer *Cancer Staging Manual*, eighth edition; *BWH,* Brigham and Women's Hospital; *GEP,* gene expression profile; *LR,* likelihood ratio; *NCCN,* National Comprehensive Cancer Network; *NPV,* negative predictive value; *PPV,* positive predictive value.

*Missing histopathologic information was treated as negative.

previously reported (62% and 75% for AJCC and BWH stages, respectively).[24-27] The 40-GEP class 2B group showed a PPV of 60% compared to 32.8%, 35.1%, and 16.7% for the AJCC, BWH, and NCCN high-risk groups, respectively (Table III). The class 1 group was associated with a 91.1% NPV compared with 87.7%, 86.3%, and 90.5% NPVs for the AJCC, BWH, and NCCN, respectively. Likelihood ratios (LRs), combining sensitivity and specificity to indicate the probability that metastasis will (+LR) or will not (-LR) occur based on class result, are reported in Table III. Importantly, 63.0% (n=203) of the high-risk NCCN cases were identified as low-risk class 1 by the 40-GEP.

DISCUSSION

This study reports the discovery, development, and validation of a 40-GEP test that classifies patients with cSCC into prognostic groups; low risk for metastasis (class 1, 91.6% 3-year MFS), and high and highest risk for metastasis (class 2A, 80.6%; and class 2B, 44.0% 3-year MFS, respectively). The study was designed to include cases with at least 1 NCCN high-risk feature to model a high-risk cSCC population (93.5%). This is reflected in the overall 16.2% rate of regional or distant metastasis, compared with previously reported rates of <6% for the general population of patients with cSCC.[5,10,15]

Clinical decision-making has benefitted from the development of multianalyte algorithmic GEP tests that report metastasis risk independently of clinicopathologic features. GEP tests currently offered for breast cancer,[38-40] prostate cancer,[41,42] uveal melanoma,[43,44] and cutaneous melanoma[45-47] have been shown to help guide treatment. The NCCN guidelines for cSCC recommend that patients with certain high-risk features consider preoperative nodal staging, elective nodal surgery, Mohs micrographic surgery or standard excision with wider margins, adjuvant radiation, or clinical trial enrollment.[3,48-51] One challenge with clinicopathologic-based guidelines is that high-risk features are often undetected through initial biopsy and, therefore, often cannot be used for surgical planning. The 40-GEP can be performed on superficial biopsies, thus enabling improved surgical decision making using molecular risk refinement before full capture of histopathologic features on excisional specimens. In addition, because the 40-GEP class results showed prognostic value independent from staging, this risk assessment may help guide postoperative decision making.[52]

Contemporary staging systems are limited in accuracy for identifying patients who are at high risk for developing metastatic disease, because only 24% to 38% of patients with BWH stage T2b/T3 tumors and 14% to 17% of AJCC T3/T4 patients develop metastasis.[24-27] NCCN's expansive definition of high-risk cSCC has a still lower PPV and risks overtreating patients. Although cSCC guidelines recommend considering specific interventions for patients with high-risk tumors, lack of accurate assessment of metastatic risk prevents some physicians from confidently selecting nodal staging, adjuvant therapy, clinical trials, or increased surveillance. Prognostic tools that improve the ability to identify both low- and high-risk patients within the high-risk cSCC spectrum would facilitate risk-appropriate reductions in the intensity of surveillance and treatment for patients with low-risk biology and improved allocation of health care resources to patients at higher risk.

The 40-GEP test achieved a PPV of 60% for class 2B tumors, exceeding the PPV observed for the BWH and AJCC systems in this study (35.1% and 32.8%, respectively), while maintaining comparable accuracy metrics for NPV, sensitivity, and specificity. The NPV for the 40-GEP test was 91.1% for class 1 versus class 2 tumors, which was comparable to NCCN and approximately 5% higher than BWH and AJCC. Likelihood ratios show that a class 2B result is associated with significantly increased probability

for metastasis and a class 1 result is associated with lower probability. Thus, incorporation of a class 1 result for clinically defined high-risk tumors could identify a substantial group of patients with biologically low-risk tumors who could be considered for de-escalation of management, potentially ruling out adjuvant treatment plans and nodal surgical staging. On the other hand, a class 2B result could identify a group of patients who may benefit from adjuvant interventions and surveillance.

Descriptive molecular characterization of cSCC has previously identified genes involved in disease pathogenesis.[53-56] Studies comparing specimens from various stages of progression (eg, *in situ* to invasive cSCC) have reported differential expression of various genes and microRNAs.[30,57-67] However, few studies of prognostic biomarkers from primary tumors have been reported.[68,69] Many of the discriminant genes comprising the 40-GEP algorithm (Supplemental Table VI[70]; available via Mendeley at https://doi.org/10.17632/f33w9wmng4.1) have been previously reported in cSCC and/or have known functions in cancer-relevant pathways. Some genes in the 40-GEP signature do not have an established role in cSCC biology, but future studies have the potential to identify how these genes promote cSCC metastasis.

As with all archival studies, there is possible bias in specimen collection based on the availability of tissue and adherence to protocol inclusion/exclusion criteria. This may account for the high fraction of metastases occurring in cases that were low-stage by BWH and AJCC criteria. Because not all histologic features used for staging are consistently reported in pathology and Mohs reports, cases may be understaged. To address this problem, all specimens underwent central pathology review and restaging according to contemporary staging criteria, with medical records reviewed for any additional high-risk features. Because cases excised via Mohs generally have no tissue available for review other than the shave biopsy, underreporting of high-stage features and understaging may result if features were not reported in surgical notes or if a surgical report was not available for review. The low sensitivities of AJCC and BWH staging reported here relative to other cohorts (39% and 25%, respectively, vs 78% and 73% recently reported[24]) are reflective of the high fraction of metastases occurring in low-stage cases in the present cohort, potentially a result of understaging. However, sensitivity analysis supported that missing features had negligible impact on the prognostic capacity of the 40-GEP. Additional multicenter cohort studies in target populations for 40-GEP testing should be undertaken to confirm the PPVs and NPVs reported here, and to determine to what degree they are reflective of the high-risk cSCC population. However, the 16% metastasis rate of the present NCCN high-risk validation cohort, as well as AJCC and BWH PPVs that were comparable to prior studies, indicate a likelihood of high reliability for the 40-GEP.

Because cSCC poses a significant burden on the health care system with increasing morbidity and mortality, it is essential to identify which patients warrant additional surveillance and therapeutic interventions and which are low risk and, thus, could avoid unnecessary procedures. Staging systems based on clinicopathologic features alone are limited in their ability to accurately stratify patients, primarily because of low PPV. The 40-GEP showed a PPV of 60% in the present study, the highest reported to date for cSCC, thus identifying a patient group with a 60% risk for metastasis. Coupling clinicopathologic features with tumor-intrinsic risk, as per the 40-GEP prognostic test developed and validated here, has potential to improve patient outcomes, quality of life, and appropriate allocation of health care resources for patients with cSCC.

We would like to thank the following individuals and centers for their contributions to this project: Drs Simon Yoo and Pedram Gerami (Northwestern University), Dr Travis Blalock (Emory University), Dr Rogerio Neves (Penn State University), Dr Michael Fazio (Michael J. Fazio, MD, Inc), Dr Jonathan Zager (Moffitt Cancer Center), Dr Michael Murphy (The Indiana Skin Cancer Center), Dr John Campana (Centura Health Research Center), Dr Julia Kasprzak (The Medical College of Wisconsin), Dr Pariser (Virginia Clinical Research), Dr James Lewis and Mr Andrew Ward (University of Tennessee Medical Center), Dr Diamondis Papadopoulos (MetroDerm PC), and Dr Federico A. Monzon, Dr Mary Hall, Dr Alison Fitzgerald, Dr Jeff Wilkinson, Ms Victoria Salas, and Ms Kelsey Carter (Castle Biosciences, Inc.).

REFERENCES

1. Muzic JG, Schmitt AR, Wright AC, et al. Incidence and trends of basal cell carcinoma and cutaneous squamous cell carcinoma: a population-based study in Olmsted County, Minnesota, 2000 to 2010. *Mayo Clin Proc*. 2017;92:890-898.
2. Waldman A, Schmults C. Cutaneous squamous cell carcinoma. *Hematol Oncol Clin North Am*. 2019;33:1-12.
3. National Comprehensive Cancer Network. Squamous cell skin cancer, NCCN guidelines version 1.2020, NCCN Clinical Practice Guidelines in Oncology. Available at: https://www.nccn.org/professionals/physician_gls/default.aspx. Accessed April 1, 2020.
4. Rogers HW, Weinstock MA, Feldman SR, et al. Incidence estimate of nonmelanoma skin cancer (keratinocyte carcinomas) in the US population, 2012. *JAMA Dermatol*. 2015;151:1081-1086.
5. Karia PS, Han J, Schmults CD. Cutaneous squamous cell carcinoma: estimated incidence of disease, nodal metastasis,

and deaths from disease in the United States, 2012. *J Am Acad Dermatol*. 2013;68:957-966.
6. Rogers HW, Weinstock MA, Harris AR, et al. Incidence estimate of nonmelanoma skin cancer in the United States, 2006. *Arch Dermatol*. 2010;146:283-287.
7. Howell JY, Ramsey ML. Cancer, squamous cell of the skin. StatPearls. Available from: http://www.ncbi.nlm.nih.gov/books/NBK441939/. Accessed April 1, 2020.
8. Mudigonda T, Pearce DJ, Yentzer BA, et al. The economic impact of non-melanoma skin cancer: a review. *J Natl Compr Canc Netw*. 2010;8:888-896.
9. Schmults CD, Karia PS, Carter JB, et al. Factors predictive of recurrence and death from cutaneous squamous cell carcinoma: a 10-year, single-institution cohort study. *JAMA Dermatol*. 2013;149:541-547.
10. Yom SS. Integrating the management of nodal metastasis into the treatment of nonmelanoma skin cancer. *Semin Radiat Oncol*. 2019;29:171-179.
11. Brantsch KD, Meisner C, Schönfisch B, et al. Analysis of risk factors determining prognosis of cutaneous squamous-cell carcinoma: a prospective study. *Lancet Oncol*. 2008;9:713-720.
12. Rowe DE, Carroll RJ, Day CL. Prognostic factors for local recurrence, metastasis, and survival rates in squamous cell carcinoma of the skin, ear, and lip. Implications for treatment modality selection. *J Am Acad Dermatol*. 1992;26:976-990.
13. Kwon S, Dong Z, Wu PC. Sentinel lymph node biopsy for high-risk cutaneous squamous cell carcinoma: clinical experience and review of literature. *World J Surg Oncol*. 2011;9:80.
14. Joseph MG, Zulueta WP, Kennedy PJ. Squamous cell carcinoma of the skin of the trunk and limbs: the incidence of metastases and their outcome. *Aust N Z J Surg*. 1992;62:697-701.
15. Thompson AK, Kelley BF, Prokop LJ, et al. Risk factors for cutaneous squamous cell carcinoma outcomes: a systematic review and meta-analysis. *JAMA Dermatol*. 2016;152:419-428.
16. Sahovaler A, Krishnan RJ, Yeh DH, et al. Outcomes of cutaneous squamous cell carcinoma in the head and neck region with regional lymph node metastasis: a systematic review and meta-analysis. *JAMA Otolaryngol Head Neck Surg*. 2019;145:352-360.
17. Porceddu SV, Bressel M, Poulsen MG, et al. Postoperative concurrent chemoradiotherapy versus postoperative radiotherapy in high-risk cutaneous squamous cell carcinoma of the head and neck: the randomized phase III TROG 05.01 Trial. *J Clin Oncol*. 2018;36:1275-1283.
18. Genders RE, Weijns ME, Dekkers OM, et al. Metastasis of cutaneous squamous cell carcinoma in organ transplant recipients and the immunocompetent population: is there a difference? A systematic review and meta-analysis. *J Eur Acad Dermatol Venereol*. 2019;33:828-841.
19. McLaughlin EJ, Miller L, Shin TM, et al. Rate of regional nodal metastases of cutaneous squamous cell carcinoma in the immunosuppressed patient. *Am J Otolaryngol*. 2017;38:325-328.
20. Ogata D, Tsuchida T. Systemic immunotherapy for advanced cutaneous squamous cell carcinoma. *Curr Treat Options Oncol*. 2019;20:30.
21. Feinstein S, Higgins S, Ahadiat O, et al. A retrospective cohort study of cutaneous squamous cell carcinoma with lymph node metastasis: risk factors and clinical course. *Dermatol Surg*. 2019;45:772-781.
22. Ahadiat O, Higgins S, Sutton A, et al. SLNB in cutaneous SCC: a review of the current state of literature and the direction for the future. *J Surg Oncol*. 2017;116:344-350.
23. Amin MB, Edge S, Greene F, et al., eds. *AJCC Cancer Staging Manual*. 8th ed. New York: Springer International Publishing; 2017.
24. Ruiz ES, Karia PS, Besaw R, et al. Performance of the American Joint Committee on Cancer Staging Manual, 8th edition vs the Brigham and Women's Hospital tumor classification system for cutaneous squamous cell carcinoma. *JAMA Dermatol*. 2019;155:819-825.
25. Karia PS, Morgan FC, Califano JA, et al. Comparison of tumor classifications for cutaneous squamous cell carcinoma of the head and neck in the 7th vs 8th edition of the AJCC Cancer Staging Manual. *JAMA Dermatol*. 2018;154:175-181.
26. Jambusaria-Pahlajani A, Kanetsky PA, Karia PS, et al. Evaluation of AJCC tumor staging for cutaneous squamous cell carcinoma and a proposed alternative tumor staging system. *JAMA Dermatol*. 2013;149:402-410.
27. Karia PS, Jambusaria-Pahlajani A, Harrington DP, et al. Evaluation of American Joint Committee on Cancer, International Union Against Cancer, and Brigham and Women's Hospital tumor staging for cutaneous squamous cell carcinoma. *J Clin Oncol*. 2014;32:327-334.
28. Amin MB, Greene FL, Edge SB, et al. The eighth edition AJCC Cancer Staging Manual: continuing to build a bridge from a population-based to a more "personalized" approach to cancer staging. *CA Cancer J Clin*. 2017;67:93-99.
29. Lydiatt WM, Patel SG, O'Sullivan B, et al. Head and neck cancers—major changes in the American Joint Committee on Cancer eighth edition Cancer Staging Manual. *CA Cancer J Clin*. 2017;67:122-137.
30. Mitsui H, Suárez-Fariñas M, Gulati N, et al. Gene expression profiling of the leading edge of cutaneous squamous cell carcinoma: IL-24-driven MMP-7. *J Invest Dermatol*. 2014;134:1418-1427.
31. Warren TA, Broit N, Simmons JL, et al. Expression profiling of cutaneous squamous cell carcinoma with perineural invasion implicates the p53 pathway in the process. *Sci Rep*. 2016;6:34081.
32. Farshchian M, Nissinen L, Siljamäki E, et al. EphB2 promotes progression of cutaneous squamous cell carcinoma. *J Invest Dermatol*. 2015;135:1882-1892.
33. Hameetman L, Commandeur S, Bavinck JNB, et al. Molecular profiling of cutaneous squamous cell carcinomas and actinic keratoses from organ transplant recipients. *BMC Cancer*. 2013;13:58.
34. Nagata M, Fujita H, Ida H, et al. Identification of potential biomarkers of lymph node metastasis in oral squamous cell carcinoma by cDNA microarray analysis. *Int J Cancer*. 2003;106:683-689.
35. Li X. TIPE2 regulates tumor-associated macrophages in skin squamous cell carcinoma. *Tumor Biol*. 2015;37:5585-5590.
36. Cyrus N, Mai-Anh Bui C, Yao X, et al. Density and polarization states of tumor-associated macrophages in human cutaneous squamous cell carcinomas arising in solid organ transplant recipients. *Dermatol Surg*. 2016;42:S18-S23.
37. Gerami P, Cook RW, Wilkinson J, et al. Development of a prognostic genetic signature to predict the metastatic risk associated with cutaneous melanoma. *Clin Cancer Res*. 2015;21:175-183.
38. Scope A, Essat M, Pandor A, et al. Gene expression profiling and expanded immunohistochemistry tests to guide selection of chemotherapy regimens in breast cancer management: a systematic review. *Int J Technol Assess Health Care*. 2017;33:32-45.
39. Ward S, Scope A, Rafia R, et al. Gene expression profiling and expanded immunohistochemistry tests to guide the use of

adjuvant chemotherapy in breast cancer management: a systematic review and cost-effectiveness analysis. *Health Technol Assess*. 2013;17:1-302.
40. McVeigh TP, Kerin MJ. Clinical use of the Oncotype DX genomic test to guide treatment decisions for patients with invasive breast cancer. *Breast Cancer (Dove Med Press)*. 2017;9:393-400.
41. Alford AV, Brito JM, Yadav KK, et al. The use of biomarkers in prostate cancer screening and treatment. *Rev Urol*. 2017;19:221-234.
42. Kristiansen G. Markers of clinical utility in the differential diagnosis and prognosis of prostate cancer. *Mod Pathol*. 2018;31:S143-S155.
43. Plasseraud KM, Cook RW, Tsai T, et al. Clinical performance and management outcomes with the DecisionDx-UM gene expression profile test in a prospective multicenter study. *J Oncol*. 2016;2016:5325762.
44. Aaberg TM, Cook RW, Oelschlager K, et al. Current clinical practice: differential management of uveal melanoma in the era of molecular tumor analyses. *Clin Ophthalmol*. 2014;8:2449-2460.
45. Berger AC, Davidson RS, Poitras JK, et al. Clinical impact of a 31-gene expression profile test for cutaneous melanoma in 156 prospectively and consecutively tested patients. *Curr Med Res Opin*. 2016;32:1599-1604.
46. Farberg AS, Glazer AM, White R, et al. Impact of a 31-gene expression profiling test for cutaneous melanoma on dermatologists' clinical management decisions. *J Drugs Dermatol*. 2017;16:428-431.
47. Dillon LD, Gadzia JE, Davidson RS, et al. Prospective, multicenter clinical impact evaluation of a 31-gene expression profile test for management of melanoma patients. *Skin J Cutan Med*. 2018;2:111-121.
48. Marrazzo G, Zitelli JA, Brodland D. Clinical outcomes in high-risk squamous cell carcinoma patients treated with Mohs micrographic surgery alone. *J Am Acad Dermatol*. 2019;80:633-638.
49. Tschetter AJ, Campoli MR, Zitelli JA, et al. Long-term clinical outcomes of patients with invasive cutaneous squamous cell carcinoma treated with Mohs surgery: a five-year, multicenter, prospective cohort study. *J Am Acad Dermatol*. 2019;82:139-148.
50. Motley R, Arron S. Mohs micrographic surgery for cutaneous squamous cell carcinoma. *Br J Dermatol*. 2019;181:233-234.
51. Belkin D, Carucci JA. Mohs surgery for squamous cell carcinoma. *Dermatol Clin*. 2011;29:161-174.
52. Teplitz R, Giselle P, Litchman GH, Rigel DS. Impact of gene expression profile testing on the management of squamous cell carcinoma by dermatologists. *J Drugs Dermatol*. 2019;18:980-984.
53. Harwood C, Proby C, Inman G, et al. The promise of genomics and the development of targeted therapies for cutaneous squamous cell carcinoma. *Acta Derm Venerol*. 2016;96:3-16.
54. Pickering CR, Zhou JH, Lee JJ, et al. Mutational landscape of aggressive cutaneous squamous cell carcinoma. *Clin Cancer Res*. 2014;20:6582-6592.
55. Yilmaz AS, Ozer HG, Gillespie JL, et al. Differential mutation frequencies in metastatic cutaneous squamous cell carcinomas versus primary tumors. *Cancer*. 2016;123:1184-1193.
56. South AP, Purdie KJ, Watt SA, et al. NOTCH1 mutations occur early during cutaneous squamous cell carcinogenesis. *J Invest Dermatol*. 2014;134:2630-2638.
57. Shin J-M, Chang I-K, Lee Y-H, et al. Potential role of S100A8 in cutaneous squamous cell carcinoma differentiation. *Ann Dermatol*. 2016;28:179-185.
58. Belkin DA, Mitsui H, Wang CQF, et al. CD200 upregulation in vascular endothelium surrounding cutaneous squamous cell carcinoma. *JAMA Dermatol*. 2013;149:178-186.
59. Zhang X, Wu J, Luo S, et al. FRA1 promotes squamous cell carcinoma growth and metastasis through distinct AKT and c-Jun dependent mechanisms. *Oncotarget*. 2016;7:34371-34383.
60. Maly CJ, Cumsky HJL, Costello CM, et al. Prognostic value of inositol polyphosphate-5-phosphatase expression in recurrent and metastatic cutaneous squamous cell carcinoma. *J Am Acad Dermatol*. 2020;82:846-853.
61. Chitsazzadeh V, Coarfa C, Drummond JA, et al. Cross-species identification of genomic drivers of squamous cell carcinoma development across preneoplastic intermediates. *Nat Commun*. 2016;7:12601.
62. Solus JF, Hassan K, Lee SJ, et al. Cutaneous squamous cell carcinoma progression is associated with decreased GATA-3 immunohistochemical staining. *J Cutan Pathol*. 2016;43:347-353.
63. Hernández-Pérez M, El-hajahmad M, Massaro J, et al. Expression of gelatinases (MMP-2, MMP-9) and gelatinase activator (MMP-14) in actinic keratosis and in in situ and invasive squamous cell carcinoma. *Am J Dermatopathol*. 2012;34:723-728.
64. Kai H, Kadono T, Kakinuma T, et al. CCR10 and CCL27 are overexpressed in cutaneous squamous cell carcinoma. *Pathol Res Pract*. 2011;207:43-48.
65. Choi KH, Kim GM, Kim SY. The keratin-14 expression in actinic keratosis and squamous cell carcinoma: is this a prognostic factor for tumor progression? *Cancer Res Treat*. 2010;42:107-114.
66. Gillespie J, Skeeles LE, Allain DC, et al. MicroRNA expression profiling in metastatic cutaneous squamous cell carcinoma. *J Eur Acad Dermatol Venereol*. 2016;30:1043-1045.
67. Toll A, Masferrer E, Hernández-Ruiz ME, et al. Epithelial to mesenchymal transition markers are associated with an increased metastatic risk in primary cutaneous squamous cell carcinomas but are attenuated in lymph node metastases. *J Dermatol Sci*. 2013;72:93-102.
68. Cumsky HJL, Costello CM, Zhang N, et al. The prognostic value of inositol polyphosphate 5-phosphatase in cutaneous squamous cell carcinoma. *J Am Acad Dermatol*. 2019;80:626-632.
69. Hernández-Ruiz E, Toll A, García-Diez I, et al. The polycomb proteins RING1B and EZH2 repress the tumoral pro-inflammatory function in metastasizing primary cutaneous squamous cell carcinoma. *Carcinogenesis*. 2018;39:503-513.
70. Sayed S, Nassef M, Badr A, et al. A nested genetic algorithm for feature selection in high-dimensional cancer microarray datasets. *Exp Syst Appl*. 2019;121:233-243.