Enhanced metastatic risk assessment in cutaneous squamous cell carcinoma with the 40-gene expression profile test

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Aim: To clinically validate the 40-gene expression profile (40-GEP) test for cutaneous squamous cell carcinoma patients and evaluate coupling the test with individual clinicopathologic risk factor-based assessment methods. **Patients & methods:** In a 33-site study, primary tumors with known patient outcomes were assessed under clinical testing conditions (n = 420). The 40-GEP results were integrated with clinicopathologic risk factors. Kaplan–Meier and Cox regression analyses were performed for metastasis. **Results:** The 40-GEP test demonstrated significant prognostic value. Risk classification was improved via integration of 40-GEP results with clinicopathologic risk factor-based assessment, with metastasis rates near the general cutaneous squamous cell carcinoma population for Class 1 and $\geq$50% for Class 2B. **Conclusion:** Combining molecular profiling with clinicopathologic risk factor assessment enhances stratification of cutaneous squamous cell carcinoma patients and may inform decision-making for risk-appropriate management strategies.

Plain language summary: Cutaneous squamous cell carcinoma is a common skin cancer, with approximately 2 million cases diagnosed each year in the USA. Because substantial numbers of patients experience metastasis, which can result in death, accurate metastatic risk assessment is important. Clinicians use clinicopathologic factors to determine risk for disease progression. However, traditional methods miss pinpointing many patients who experience metastasis and sometimes categorize patients as at risk who do not develop metastasis, indicating that additional tools are needed. A molecular test, the 40-gene expression profile (40-GEP), was developed to predict metastatic risk based on the biology of the tumor. This study demonstrates that the 40-GEP, either as an independent tool or together with traditional methods, accurately identifies patients' risk of metastasis. Using the 40-GEP could improve patient management to improve patient outcomes.

An estimated 2–6% of cutaneous squamous cell carcinoma (cSCC) patients develop regional or distant metastasis, and approximately 2% die from the disease annually in the USA. [1–6] Although the fatality rate is low, the incidence of cSCC is high (∼2 million diagnosed cases/year) and continues to grow, resulting in a substantial number of patients with poor outcomes [7–11]. As the distribution of nonmelanoma skin cancer is shifting from a historical 1:4 toward a 1:1 ratio of cSCC to basal cell carcinoma, the estimated mortality rate of cSCC will likely surpass that for melanoma [8,9].

Development of metastatic disease has a profound impact on cSCC patient survival, as most cSCC deaths are due to progressive locoregional disease (e.g., regional lymph node metastasis or in-transit metastasis) [1,12]. This underscores the need for effective identification of patients at risk of metastasis. While the 5-year disease-specific survival rate is >90% for localized disease [6,13], this rate drops to 50–83% and below 40% for patients with regional and distant metastases, respectively. Thus, although interventions are available for treatment of locally advanced cSCC [14,15], accurate and early identification of cSCC tumors with higher metastatic potential is essential for optimizing patient management to reduce and prevent metastasis and death from an otherwise curable disease. Once metastasis is detected, more intense treatment is often warranted [8].

Many patients with cSCC are broadly classified as having high-risk disease based on clinicopathologic factors associated with increased likelihood of poor outcomes [6,8]. The most current National Comprehensive Cancer Network (NCCN) guidelines categorize a patient as being high risk or very high risk by the presence of specific high-risk factors, indicating that the patient is a candidate for more intensive management [6]. Tumors with diameter ≥2 cm have demonstrated two- and threefold greater risk for recurrence and metastasis, respectively, relative to smaller tumors [8,16]. Likewise, tumors invading beyond subcutaneous fat, with perineural invasion (PNI) of large-caliber nerves, or with poor histologic differentiation have been linked to a twofold to 23-fold increased risk for recurrence and/or metastasis in univariate analyses [8,16]. At the patient level, immunosuppressed individuals are at greater risk for developing cSCC and often present with more aggressive tumors [8,17]. While these and other factors are used to stratify patient risk, low accuracy, histopathologic discordance and a lack of standardized reporting limit the clinical utility of this clinicopathologic factor-based approach.

Although methods for cSCC risk stratification continue to be refined, a single universally accepted system has not yet been adopted [18]. Current tumor (T) staging systems include the American Joint Committee on Cancer (AJCC) Cancer Staging Manual, Eighth Edition (AJCC8) [19] and the Brigham and Women's Hospital (BWH) system [20]. AJCC8 has improved upon previous editions for cSCC, albeit specifically noted for use in head and neck tumors only, and the BWH tumor classification system has been reported to provide better prognostication of localized cSCC relative to AJCC8 [18,21]. Both systems utilize clinicopathologic factors of the primary tumor to categorize risk into T stages. For stratifying metastatic risk in cSCC, these systems have demonstrated positive predictive value (PPV) ranges of 14–17 (AJCC8) and 24–38% (BWH) [20–24]. Staging systems do not take into account all risk factors known to be associated with metastasis, and discordance in assessment and reporting has been reported [3,20,23,25,26]. Published reports demonstrate that approximately 30% of cases having metastatic outcomes are misclassified as low-risk T stage (e.g., BWH T1/T2a), and approximately 75% of those classified as high-risk T stage (e.g., BWH T2b/T3) do not develop metastasis [20,21,24,27–30]. This suggests that, while staging has improved, there remains a need for refinement of risk assessment in high-risk cSCC. Given these clinical limitations, physicians may rely on professional experience and institution-specific approaches, rather than staging systems, to drive patient management decisions [31]. Despite ongoing efforts to improve risk assessment [32], a standardized and accurate stratification system remains a clinically unmet need in the management of patients with high-risk cSCC.

We previously reported validation of a gene expression profiling-based algorithm (the 40-gene expression profile [40-GEP]) that stratifies cSCC tumor risk of metastasis [23]. In this study we demonstrate the clinical validity of the 40-GEP test for classifying low (Class 1), moderate (Class 2A) and high (Class 2B) metastatic risk in high-risk cSCC, along with its ability to further stratify risk within the newly defined NCCN risk groups; we also combine 40-GEP test results with clinicopathologic factor-based risk assessment. In an expanded cohort of cSCC patients (n = 420) with high-risk factors, we show significant prognostic value of the 40-GEP test performed using clinical laboratory-developed and implemented standard operating procedures (SOPs) on primary tumor specimens from biopsy or surgical excision. Combining novel molecular prognostication with clinicopathologic risk assessment

approaches can complement current systems and support overall metastatic risk stratification. This, in turn, could facilitate management decisions for high-risk cSCC patients.

Patients & methods

Study cohort

Using an ongoing, institutional review board-approved protocol, formalin-fixed paraffin-embedded (FFPE) samples from primary cSCC lesions (either biopsy or wide local excision specimens) and corresponding clinicopathologic and outcomes data were collected (Supplementary Figure 1). The study design targeted enrollment of patients with ≥1 high-risk feature as defined by NCCN guidelines or by AJCC or BWH staging greater than T1. The primary end point of the study was 3-year metastasis-free survival (MFS). Study inclusion/exclusion criteria were as previously reported [23]. Cases had a documented regional or distant metastasis, or documented follow-up of at least 3 years post-diagnosis of the primary tumor without a metastatic event, the time by which almost all metastases develop in cSCC [13,33]. Cases with prior history of cSCC, cutaneous basal cell carcinoma or melanoma *in situ* were allowed if deemed cured by the investigator. All cases, when available, underwent review of biopsy pathology reports, definitive surgery reports and medical records. Clinicopathologic factors were deemed positive/present if identified during any review step, resulting in comprehensive staging.

As shown in Supplementary Figure 1, 420 cases (307 previously assessed prior to implementation of clinical SOPs [23] and 113 novel) made up the clinical validation cohort, from which tissue samples were independently reviewed by a board-certified dermatopathologist for tumor content and high-risk factors [6,34,35]. Cases with synchronous tumors were not included in the 420-case clinical validation cohort, as they were reserved for future intrapatient heterogeneity analysis. Also, cases lacking sufficient material to be run under clinical SOPs or not meeting clinical testing criteria were excluded from the study. Seven risk factors were assessed to generate an overall risk factor count: tumor size and location, immunosuppression, PNI, depth of invasion, degree of differentiation, histologic subtype and lymphovascular invasion (Supplementary Table 1). Also, based on comprehensive review (as described above), each case was staged according to AJCC8 and BWH tumor classification systems.

Gene expression analysis

All samples were tested under 40-GEP clinical SOPs in a central College of American Pathologists-accredited laboratory, with personnel blinded to patient outcomes. Briefly, FFPE tumor tissue was macrodissected, processed for real-time PCR and run in triplicate, as previously described [23]. Duplicate sample runs were used to generate clinical 40-GEP class scores.

Statistics

Statistical analyses were performed in R (v3.6.3) as previously described [23]. MFS analyses were performed using Kaplan–Meier methods and log-rank test. Univariate and multivariate Cox regression analyses were performed using standard methods, and assumptions of proportional hazards were confirmed using the zph test of the fitted model for Cox regression, as previously described [23]. Binary T stages (consistent with previous reports) [20–24] as well as individual T stages, determined by comprehensive review as described above, were included in the analyses. The McNemar test was used for comparing accuracy metrics [20].

Results

Cohort characteristics & risk assessment by molecular prognostication

To validate the 40-GEP test for use in the clinical setting (when at least one high-risk factor is present at time of biopsy or surgical excision), FFPE samples from primary cSCC tumors meeting protocol and clinical testing inclusion criteria were assessed using the previously published 40-GEP algorithm [23] and unreported clinical SOPs. The clinical validation cohort (n = 420) included 63 cases with regional and/or distant metastases, and 357 cases without an event and with ≥3 years of follow-up; cohort characteristics are shown in Table 1. All 63 metastatic cases had regional disease (which included satellite, in-transit, nodal or parotid metastases), 17 of which also developed distant metastases. Median time to metastasis was 0.9 years (95th percentile: 2.7 years). The following characteristics had significantly different rates for metastatic cases: male sex, head and neck location, tumor diameter, tumor thickness, poor differentiation, PNI, invasion beyond subcutaneous fat, cases undergoing Mohs micrographic surgery and cases deemed very high risk by NCCN (Table 1).

The 40-GEP test classified patients based on risk for regional and/or distant metastasis (Figure 1A). Of the 420

Table 1. Demographics and clinical characteristics of the study cohort (n = 420).

Characteristic	All (n = 420)	Nonmetastatic (n = 357)	Regional/distant metastasis (n = 63)	p-value
Age (years):median (range)	71 (34–95)	71 (34–95)	70 (44–90)	ns
Male sex	308 (73.3%)	253 (70.9%)	55 (87.3%)	0.007
Caucasian	417 (99.3%)	355 (99.4%)	62 (98.4%)	ns
Immunosuppression†	103 (24.5%)	83 (23.2%)	20 (31.7%)	ns
Located on H&N	278 (66.2%)	224 (62.7%)	54 (85.7%)	0.0002
– Ear	64 (15.2%)	53 (14.8%)	11 (17.5%)	
– Lip	25 (6.1%)	17 (4.8%)	8 (12.7%)	
– Scalp	56 (13.3%)	40 (11.2%)	16 (25.4%)	
Tumor diameter (cm):mean (SD)‡	2.01 (±1.86)	1.84 (±1.67)	3.11 (±2.52)	<0.0001
Tumor thickness (mm):mean (SD)§	4.34 (±6.45)	3.72 (±6.63)	7.71 (±4.07)	<0.0001
Poorly differentiated	58 (13.8%)	36 (10.1%)	22 (34.9%)	<0.0001
PNI present (≥0.1 mm)	7 (1.7%)	5 (1.4%)	2 (3.2%)	<0.0001
– Present (<0.1 mm)	22 (5.2%)	12 (3.4%)	10 (15.9%)	
– Present (unknown caliber)	24 (5.7%)	17 (4.8%)	7 (11.1%)	
– Not present	367 (87.4%)	323 (90.5%)	44 (69.8%)	
Invasion beyond subcutaneous fat	51 (12.1%)	34 (9.5%)	17 (27.0%)	<0.0001
Definitive surgery MMS¶	333 (79.3%)	291 (81.5%)	42 (66.7%)	0.023
NCCN v2.2021 high risk	255 (60.7%)	230 (64.4%)	25 (39.7%)	<0.001
– Very high risk	165 (39.3%)	127 (35.6%)	38 (60.3%)	

Data analyzed using χ-square test or Kruskal–Wallis F test as appropriate for variable type.
†86 of 103 immunosuppressed patients were transplant patients.
‡Tumor diameter reported (n = 393).
§Tumor thickness reported (n = 123).
¶MMS or wide local excision (n = 415), with two cases not having additional surgery beyond biopsy and three cases with unknown definitive surgery.
H&N: Head and neck; MMS: Mohs micrographic surgery; NCCN: National Comprehensive Cancer Network; ns: Not statistically significant; PNI: Perineural invasion; SD: Standard deviation.
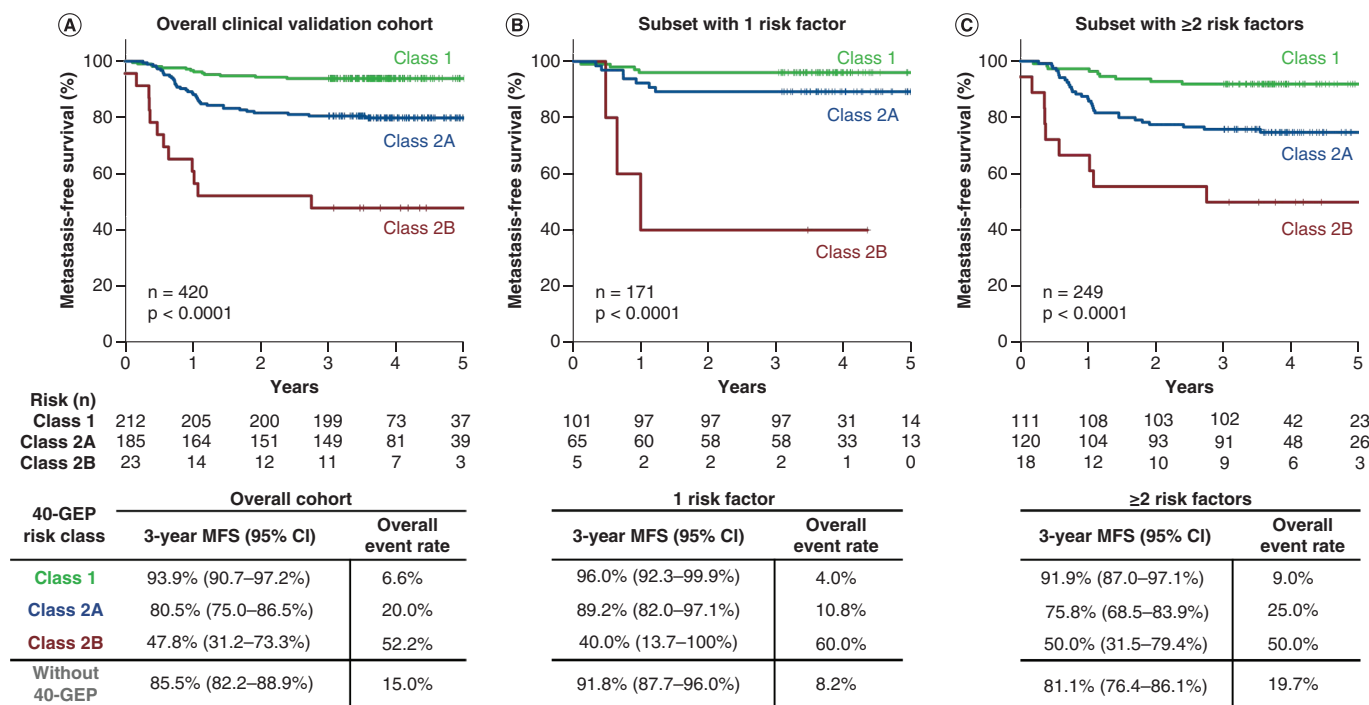

Figure 1. Kaplan–Meier analysis of prognostication by 40-gene expression profile results alone and after combining with risk factor counts. (A) Prognostication by 40-GEP results only. **(B)** Subset with one risk factor. **(C)** Subset with two or more risk factors.
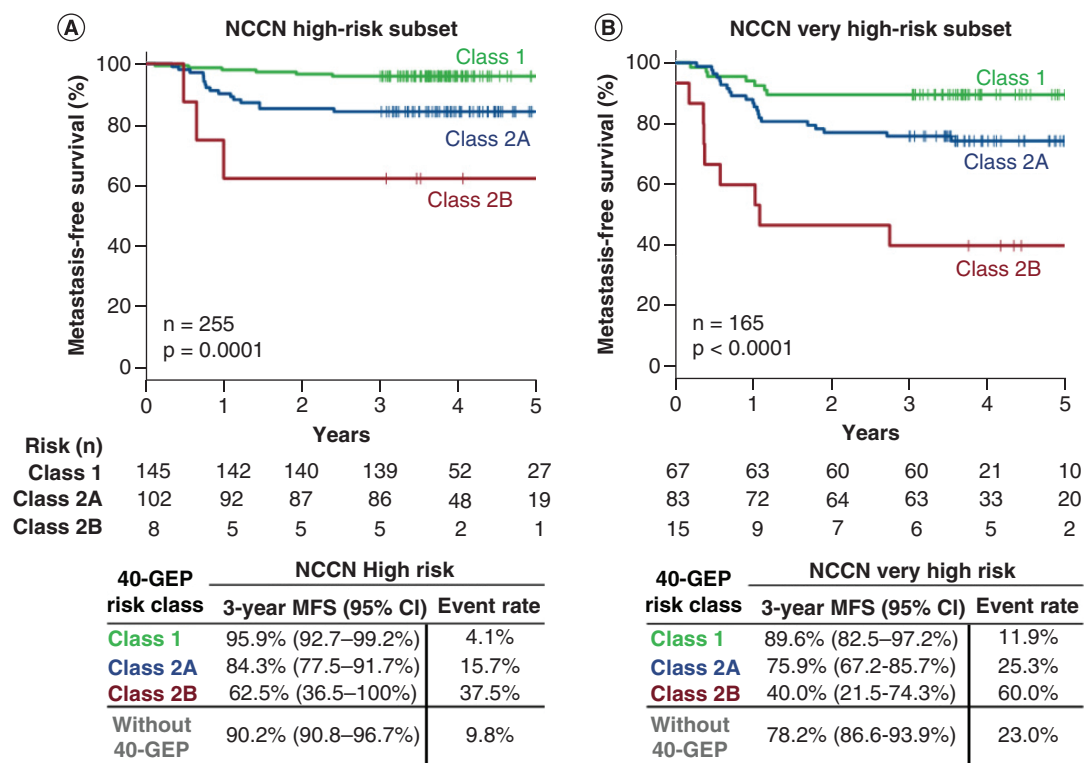40-GEP: 40-gene expression profile; MFS: Metastasis-free survival.

Figure 2.   Kaplan–Meier analysis of prognostication by 40-gene expression profile results in combination with National Comprehensive Cancer Network (v2.2021) risk classification. (A) NCCN high risk. **(B)** NCCN very high risk. 40-GEP: 40-gene expression profile; MFS: Metastasis-free survival; NCCN: National Comprehensive Cancer Network.

cases included in the study, 212 were identified as Class 1 (low risk), 185 as Class 2A (moderate risk) and 23 as Class 2B (high risk), with metastasis rates of 6.6, 20.0 and 52.2%, respectively. The 3-year MFS rates were 93.9, 80.5 and 47.8% for Class 1, Class 2A & B, respectively (log-rank, p < 0.001).

Combination of the 40-GEP with clinicopathologic factor-based risk assessment

To determine the impact of molecular prognostication on existing risk-assessment strategies, subsets composed of molecular class and risk factors were interrogated by Kaplan–Meier and regression analysis. First, cases were assessed for total count of risk factors, as determined by NCCN risk criteria [6] or Mohs micrographic surgery appropriate use criteria [34,35] (as described in the methods; Supplementary Table 1), and then binned into two subsets: those with one risk factor (n = 171) and those with two or more risk factors (n = 249; Figure 1B & C). A direct relationship between risk factor count and metastasis rate was demonstrated. The metastasis rate for cases with a single risk factor was 8.2%, while it was 19.7% for cases with two or more risk factors (compared with 15.0% for the whole cohort). Incorporating 40-GEP test results identified Class 1 cases with rates of 4.0 and 9.0%, respectively, for one and two or more risk factors (Figure 1B & C). Combining Class 2A results with risk factors identified cases with higher metastasis rates relative to pre-40-GEP testing (10.8 and 25.0% for one and two or more factors, respectively). Regardless of risk factor count, Class 2B metastasis rates were 50–60% (>twofold above the rate for each subset without 40-GEP prognostication), which represent statistically equivalent findings within CIs. These findings were supported by significantly different 3-year MFS rates for Class 1, 2A & B cases in the cohort and corresponding rate changes for each subset (log-rank p < 0.0001; Figure 1).

When criteria from the newly developed NCCN guidelines were applied [6], all 420 patients of the cohort were defined as either high or very high risk (with 3-year MFS rates of 90.2 and 78.2%, respectively; p = 0.0002), confirming the cohort's high-risk designation (Figure 2). The 40-GEP test further stratified risk within each of the NCCN groups (Figure 2A & B). For the high-risk subset, MFS rates were 95.9, 84.3 and 62.5% for Class 1, Class 2A & B, respectively (p = 0.0001); for the very high-risk subset, MFS rates were 89.6, 75.9 and 40.0% for Class 1, Class 2A & B, respectively (p < 0.001).

Table 2. Accuracy metrics for the cutaneous squamous cell carcinoma validation cohort (n = 420) per 40-gene expression profile class and Brigham and Women's Hospital or American Joint Committee on Cancer Cancer Staging Manual, Eighth Edition binary T stage.

Metric	Accuracy (%)			
	40-GEP (Class 2)	40-GEP (Class 2B)	BWH (T2b/T3)	AJCC8 (T3/T4)
Sensitivity†	77.8	19.0	30.2	38.1
Specificity‡	55.5	96.9	89.6	84.3
PPV	23.6	52.2	33.9	30.0
NPV	93.4	87.2	87.9	88.5

†p < 0.0001 for a 40-GEP Class 2 result vs BWH T2b/T3 stage or AJCC8 T3/T4 stage; p < 0.02 for a 40-GEP Class 2B result vs AJCC8 T3/T4 stage.
‡p < 0.0001 for a 40-GEP Class 2B result vs BWH T2b/T3 stage or AJCC8 T3/T4 stage, and for a 40-GEP Class 2 result vs BWH T2b/T3 stage or AJCC8 T3/T4 stage; based on the McNemar test.
Note: p-values were not estimated for PPV and NPV, as these values are based on prevalence of metastasis.
40-GEP: 40-gene expression profile; AJCC8: American Joint Committee on Cancer Cancer Staging Manual, Eighth Edition; BWH: Brigham and Women's Hospital; NPV: Negative predictive value; PPV: Positive predictive value.

Next, after T stages for comprehensively assessed tumors were determined (AJCC8 and BWH), accuracy metrics were calculated using previously published groupings [20–24], and metastatic risk rates within T stage-defined risk groups were stratified by 40-GEP class. When the accuracy metrics of the 40-GEP test were compared with those of AJCC8 and BWH T staging for this cohort, the PPV for a 40-GEP Class 2B result was 52.2% compared with 30.0 and 39.9% for high-stage AJCC8 (T3/T4) and BWH (T2b/T3), respectively, while maintaining a similar negative predictive value (87.2% compared with 88.5 and 87.9%; Table 2). The specificity of a 40-GEP Class 2B result (96.9%) was significantly higher (p < 0.0001) than that of high-stage AJCC8 (84.3%) and BWH (89.6%), demonstrating that a Class 2B result has a significantly reduced likelihood of yielding a false positive regarding metastasis relative to AJCC8 and BWH high-stage classifications. The sensitivity of a 40-GEP Class 2 (Class 2A or 2B) result (77.8%) was significantly higher (p < 0.0001) than that of high-stage AJCC8 (38.1%) and BWH (30.2%). This indicates that a Class 2 result is significantly more likely to successfully identify tumors at moderate to high risk of metastasis (i.e., it demonstrates fewer false negatives) relative to AJCC8 and BWH staging approaches.

Analyses of metastasis rates stratified by 40-GEP class and either binary T stage (a grouping used for clinical decision-making and accuracy metrics) [5,20] or individual T stage (as distinct risk groups) are reported in Figure 3, Supplementary Figure 2 & Supplementary Table 2. Addition of molecular testing results to binary T stage status identified subpopulations ranging from 5.7 to 71.4% (BWH) and from 5.6 to 83.3% (AJCC8), compared with 12.1–33.9% (BWH) and 11.5–30.0% (AJCC8) for binary staging alone, demonstrating that risk assessment can be refined by combining the two approaches. Further, when metastatic rates for individual BWH T stages were assessed per 40-GEP class, a similar trend of increasing rates of metastasis was observed from T1 to T3 within classes and from Class 1 to 2B. Two exceptions were a lower rate for T3 stage within Class 1 & 2B, which also had relatively low numbers of cases (Figure 3, Supplementary Figure 2 & Supplementary Table 2). Metastatic rates for individual AJCC8 T stages were also compared and found to show similar trends (Figure 3, Supplementary Figure 2 & Supplementary Table 2).

To determine the contributions of individual factors to metastatic risk, factors with best-supported evidence for association with metastasis [6,8] and molecular prognostication by the 40-GEP were assessed by Cox regression analyses (Table 3). By univariate analysis, the risk of metastasis for Class 2A & B results was 3.22- and 11.61-fold greater, respectively, than that for Class 1 results (p < 0.001). The presence of poor differentiation, PNI and deep invasion (i.e., beyond subcutaneous fat, depth >6 mm, or Clark level V) were significant risk factors for metastasis (hazard ratios [HRs]: 3.93, 3.28 and 3.11, respectively; p < 0.001). Tumor diameter was also significantly predictive of metastatic risk (HR: 1.15 per cm; p < 0.001). Despite prior support for immunosuppression as a prognostic risk factor [8,36], it was not statistically significant in this cohort. It should be noted that lymphovascular invasion and increased tumor thickness were associated with immunosuppression (data not shown); however, lymphovascular invasion was rare in this cohort. Univariate analysis aligned with NCCN risk grouping, as NCCN very high-risk cases had a 2.54-fold increased risk of metastasis over cases with a high-risk designation (p < 0.001).

When a multivariate model was generated using factors found to be significant in the univariate analysis, 40-GEP results, poor differentiation and deep invasion were independent factors significantly associated with metastatic risk (Table 3). Similar HRs were observed for a Class 2A result, poor differentiation and deep invasion (2.33, 2.29 and
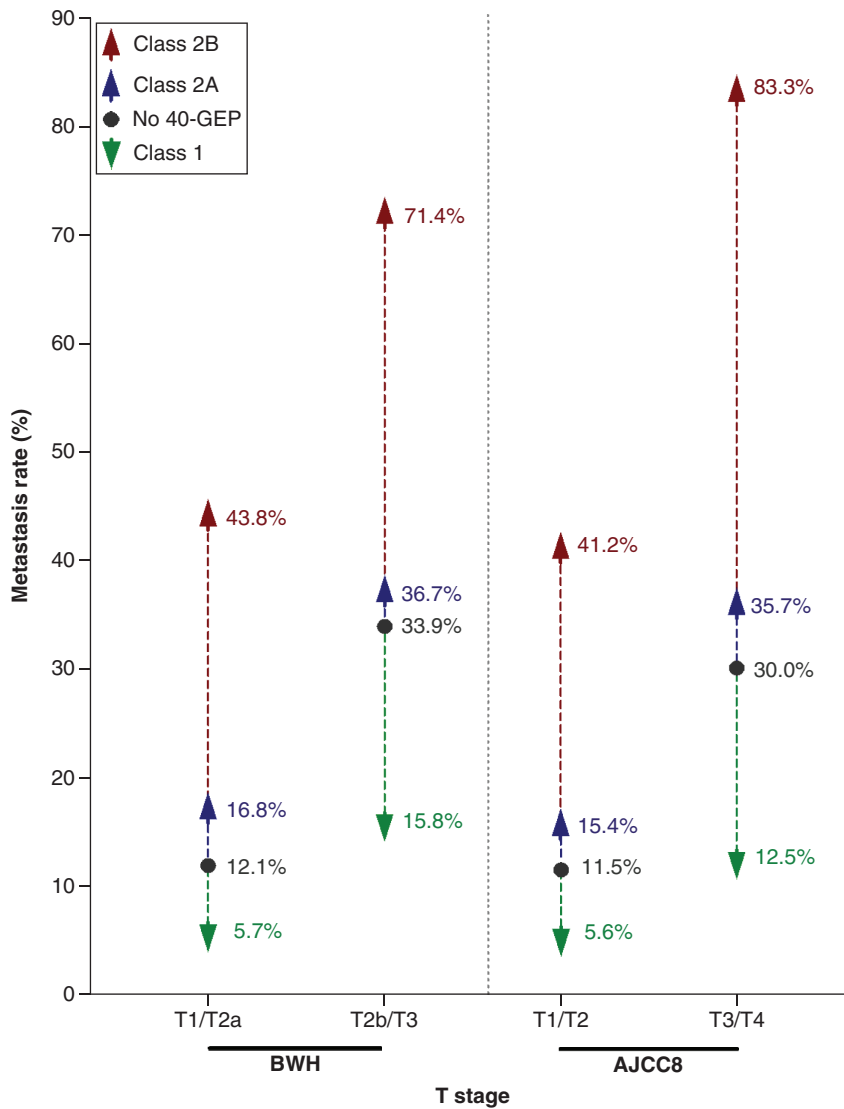
Figure 3.   Further stratification of metastatic risk by the 40-gene expression profile test per Brigham and Women's Hospital or American Joint Committee on Cancer Cancer Staging Manual, Eighth Edition binary tumor stage for the cutaneous squamous cell carcinoma validation cohort (n = 420).
40-GEP: 40-gene expression profile; AJCC8: American Joint Committee on Cancer Cancer Staging Manual, Eighth Edition; BWH: Brigham and Women's Hospital; cSCC: Cutaneous squamous cell carcinoma; T: Tumor.

2.05, respectively; $p < 0.05$), indicative of independent and additive risk associated with each factor. Overall, a 40-GEP Class 2B result had the greatest independent prognostic value (HR: 6.86; $p < 0.001$). Multivariate analysis of 40-GEP risk assessment and NCCN risk grouping demonstrated statistical significance of Class 2A & B, with HRs of 2.92 and 9.50, respectively ($p < 0.001$), while the NCCN very high-risk category had a significant HR of 1.99 ($p = 0.009$; Table 3).

When univariate analysis was performed for BWH and AJCC8 T stage (binary or individual), HRs were statistically significant for binary high-stage BWH (T2b/T3, 2.55; $p = 0.009$) and AJCC8 (T3/T4, 2.14; $p = 0.022$; Table 3), as well as for individual BWH T2b or T3 (3.97 or 4.68; $p \leq 0.005$) and AJCC8 T3 or T4 (2.47 or 3.46; $p \leq 0.020$; Supplementary Table 3). Also, when compared with BWH or AJCC8 T staging (binary or individual) in univariate or multivariate analyses, a 40-GEP Class 2B result demonstrated more than twofold greater independent prognostic value ($p < 0.001$; Table 3 & Supplementary Table 3).

Table 3. Univariate and multivariate Cox regression analyses of risk of metastasis per 40-gene expression profile class, common risk factors for poor outcomes, National Comprehensive Cancer Network; risk group and binary Brigham and Women's Hospital and American Joint Committee on Cancer Cancer Staging Manual, Eighth EditionT stages in cutaneous squamous cell carcinoma validation cases.

Risk factor	n	Univariate Cox regression		Multivariate Cox regression[†]	
		Hazard ratio (95% CI)	p-value	Hazard ratio (95% CI)	p-value
40-GEP result					
Class 1	212	1.00 (–)	–	1.00 (–)	–
Class 2A	185	3.22 (1.74–5.95)	<0.001	2.33 (1.20–4.53)	0.013
Class 2B	23	11.61 (5.36–25.15)	<0.001	6.86 (2.73–17.22)	< 0.001
Clinicopathologic risk factors					
Poor differentiation	58	3.93 (2.34–6.60)	<0.001	2.29 (1.21–4.33)	0.011
Perineural invasion[‡]	53	3.28 (1.41–14.36)	<0.001	1.22 (0.58–2.59)	0.601
Deep invasion[§]	72	3.11 (1.86–5.20)	<0.001	2.05 (1.04–4.04)	0.039
Tumor diameter[¶]	N/A	1.15 (1.08–1.22)	<0.001	1.07 (0.97–1.17)	0.188
Immunosuppression	103	1.46 (0.86–2.49)	0.161	–	–
40-GEP result					
Class 1	212	1.00 (–)	–	1.00 (–)	–
Class 2A	185	3.22 (1.74–5.95)	<0.001	2.92 (1.57–5.44)	< 0.001
Class 2B	23	11.61 (5.36–25.15)	<0.001	9.50 (4.33–20.9)	< 0.001
NCCN risk group					
High	255	1.00 (–)	–	1.00 (–)	–
Very high	165	2.54 (0.26–3.62)	<0.001	1.99 (1.19–3.33)	0.009
40-GEP result					
Class 1	212	1.00 (–)	–	1.00 (–)	–
Class 2A	185	3.22 (1.74–5.95)	<0.001	2.98 (1.61–5.53)	< 0.001
Class 2B	23	11.61 (5.36–25.15)	<0.001	9.42 (4.28–20.7)	< 0.001
BWH T stage					
T1/T2a	364	1.00 (–)	–	1.00 (–)	–
T2b/T3	56	2.55 (1.26–5.17)	0.009	2.38 (1.38–4.13)	0.002
40-GEP result					
Class 1	212	1.00 (–)	–	1.00 (–)	–
Class 2A	185	3.22 (1.74–5.95)	<0.001	2.97 (1.60–5.51)	< 0.001
Class 2B	23	11.61 (5.36–25.15)	<0.001	11.40 (5.26–24.69)	< 0.001
AJCC8 T stage					
T1/T2	340	1.00 (–)	–	1.00 (–)	–
T3/T4	80	2.14 (1.11–4.09)	0.022	2.69 (1.61–4.48)	< 0.001

[†]n = 393, 54 events, excluding cases without tumor diameter reported.
[‡]Perineural invasion was considered positive regardless of nerve caliber.
[§]Deep invasion: beyond the subcutaneous fat, depth >6 mm or Clark level V.
[¶]Tumor diameter: continuous variable per cm.
40-GEP: 40-gene expression profile; AJCC8: American Joint Committee on Cancer Cancer Staging Manual, Eighth Edition; BWH: Brigham and Women's Hospital; cSCC: Cutaneous squamous cell carcinoma; N/A: Not applicable; NCCN: National Comprehensive Cancer Network; T: Tumor.

Discussion

We propose that molecular prognostication, in conjunction with patient and tumor characteristics, increases accuracy of risk assessment for patients with high-risk cSCC. Here we clinically validated the 40-GEP test to identify cSCC tumors at low (Class 1), moderate (Class 2A) and high (Class 2B) risk of metastasis within 3 years of diagnosis, the time by which most metastatic events occur [6,13]. This study follows the previously reported validation of the 40-GEP algorithm for determining metastatic risk [23], validates the test under SOPs implemented for clinical application with primary tumor specimens from biopsy or surgical excision, and demonstrates impactful incorporation with clinicopathologic risk factor-based assessment.

Clinicopathologic risk prediction methods have been improving, in particular with the Eighth Edition updates to AJCC staging and validation of the BWH T-staging system [20–22,24]. However, these systems face the following challenges: lack of standardized reporting; subjective nature of histopathologic assessment; [6] and failure to capture biological risk at the molecular level. For example, differentiation was removed from AJCC8 tumor staging as the definitions of good, moderate and poor differentiation were inconsistent, limiting its clinical application [19]. While tumor depth is a well-accepted risk factor for metastasis, how this variable should be captured/reported and what degree of invasion is considered high risk is debated [37]. Additionally, the rarity of large-nerve PNI [21] (1.7% in this study) may limit its widespread utility for identifying high-risk patients. These caveats likely contribute to the low PPV associated with traditional risk assessment, supporting the need for objective and consistent molecular tools to assess tumor biology.

Molecular biomarker assays and gene expression profiling have demonstrated clinical utility for patient management decision-making in many types of cancer [38–44], although the availability of a clinically validated molecular test to assess metastatic risk in cSCC has been lacking until recently. Descriptive molecular characterization of cSCC has previously identified genes involved in disease progression, and biomarkers have been proposed [45–49]. However, low availability of quality samples has contributed to limitations in the clinical validation and application of many molecular testing strategies for cSCC. The current study demonstrates clinical validation of the 40-GEP test using clinically implemented procedures.

Integration of molecular prognostication into risk assessment can mitigate the limitations of assessment based on clinicopathologic factors alone [20,25,26]. The findings from this study demonstrate that the 40-GEP test can complement clinicopathologic factor-based risk assessment by the use of either individual risk factors or tumor staging. In a multivariate model with commonly utilized high-risk factors, the 40-GEP test provided independent and additive prognostic value. Class 2A & B results were higher and equivalent indicators, respectively, of risk of metastasis relative to other significant factors (poor differentiation and deep invasion; Table 3), indicating that the 40-GEP can augment risk classification of cSCC. Both deep invasion and tumor diameter were significant risk factors in univariate analysis; however, tumor diameter was not significant in multivariate analysis, suggesting that the directionality of growth (i.e., invasive behavior vs surface spread) provides an important distinction. Furthermore, PNI was only statistically significant in univariate analysis, consistent with prior studies showing that PNI loses significance in analysis with other high-risk factors [27]. Also, immunosuppression did not reach statistical significance for association with metastasis, despite the fact that it has been strongly associated with risk for multiple cSCC lesions and poor outcomes [8,17,36]. Interestingly, immunosuppression is not indicated within risk criteria of BWH or AJCC8 tumor staging systems [20]. Relative to the general cSCC patient population, similar metastasis rates have been reported in organ transplant recipients who develop cSCC, although the aggressiveness of disease in immunosuppressed individuals underscores the importance of intensive patient surveillance and management [50].

In this study we demonstrate that the 40-GEP test can enhance clinicopathologic risk factor-based assessment and identify a group of cSCC patients within a high-risk cohort who have metastasis rates similar to those of the general cSCC population (40-GEP Class 1 with one risk factor). With two or more risk factors, the metastasis rate for patients with a Class 1 result was below 10% (≥50% lower than the rate for the whole cohort). Likewise, patients having an NCCN high-risk classification and a 40-GEP Class 1 result had metastasis rates similar to those of the general cSCC population; however, the rate was more than twofold higher when 40-GEP risk stratification was not considered. Patients identified by the 40-GEP test as at highest risk of metastasis (Class 2B) consistently had metastasis rates ≥50%, regardless of whether they had one, two or more risk factors. Similarly, the NCCN very high-risk subset of patients who were also identified as 40-GEP Class 2B had a metastatic event rate of 60.0%, although the NCCN high-risk subset with 40-GEP Class 2B results had a metastatic rate of 37.5%. While this is, indeed, a high rate of metastasis and the 40-GEP provides independent prognostication, this finding is an important reminder of the impact of baseline clinicopathologic risk on comprehensive risk assessment.

When formal tumor staging systems commonly used in the USA (AJCC8 and BWH) [21] were applied in this cohort, a similar refinement of risk stratification was demonstrated by 40-GEP class, regardless of which staging system was used. Also, statistically significant improvements were demonstrated with 40-GEP test results across several accuracy metrics. Specificity of a Class 2B (96.9%) and sensitivity of a Class 2 (77.8%) result were significantly greater than the corresponding metrics for high-stage BWH and AJCC8 tumors. Together, these metrics demonstrate the capability of the 40-GEP test to identify tumors at high risk of metastasis with improved accuracy relative to BWH and AJCC8 tumor classifications, while stratifying these cases separately from those with Class 1 tumors who have risk similar to that observed in the general cSCC patient population. Thus combining

the 40-GEP test with clinicopathologic factor-based risk assessment, regardless of whether it is based on risk factor count or T stage, can further stratify risk of metastasis in cSCC patients. These findings demonstrate the capability of molecular tools to provide enhancement of current risk assessment methods in cSCC.

While the analysis herein focuses on clinical validity of the 40-GEP assay following clinical SOPs, incorporation of the test into the guidance of patient management (i.e., clinical utility) is actively being addressed. As previously suggested by Farberg *et al.*, the 40-GEP test can be integrated within NCCN guideline recommendations to help refine risk-directed cSCC patient management decisions [51]. Also, Litchman *et al.* recently demonstrated that knowledge from the 40-GEP test can significantly impact and lead to risk-stratified change in clinician decisions for patient management relative to decisions made without 40-GEP results. Clinicians indicated they would increase management intensity with a 40-GEP Class 2B result and decrease management intensity with a Class 1 result, while remaining within national guidelines [52]. National guidelines for patient management support that the intensity of intervention/therapy/management should align with risk for poor outcomes, as in the systems proposed by Farberg *et al.* and Baum *et al.* [51,53]. As the 40-GEP predicts metastatic risk, one paradigm change could be to define the intensity of nodal assessment based on molecular prognostication, including decisions around various imaging modalities (e.g., ultrasound, MRI or CT scanning) or nodal biopsy. Indeed, the role of sentinel lymph node biopsy in management of cSCC is widely debated [54–57]. This prognostic tool could provide guidance on who might warrant the procedure because of a high likelihood of positivity; conversely, those at low risk for poor outcomes may be able to avoid this procedure.

The current study demonstrates that inclusion of molecular prognostication can augment cSCC risk stratification via objective and standardized risk classification methods. The findings from this study also show that the 40-GEP test could be helpful in predicting risk of metastasis in patients categorized as high risk by the recently updated NCCN guidelines (in which this clinical characterization alone is considered high risk for only local recurrence) [6]. Specifically, the NCCN high-risk classification is denoted by the guidelines as elevated risk of local recurrence, while the NCCN very high-risk classification is denoted as elevated risk of local recurrence and metastasis. Nevertheless, the data presented herein show that within the NCCN high-risk group there was a 15.7% risk of metastasis for Class 2A and a 37.5% risk of metastasis for Class 2B, which has potential to be clinically meaningful. Thus the significantly different risk profiles identified by the 40-GEP test on biopsy or surgical excision specimens from primary high-risk cSCC tumors could be used along with clinicopathologic factors to guide patient management decisions (i.e., follow-up frequency, nodal assessment and adjuvant therapy) [58] and, with additional studies, help define management guidelines. In addition, while efficacious treatment options for metastatic cSCC are being investigated via clinical trials and other molecular studies [58,59], the 40-GEP test could be used to stratify patients at high risk of metastasis in order to implement timely, risk-appropriate surveillance to identify and treat metastasis early. Ultimately, incorporating molecular tools into high-risk cSCC patient risk assessment could lead to more personalized and risk-appropriate methods for improved patient management and, subsequently, outcomes.

Conclusion

This study demonstrates the clinical validation and significant prognostic value of the 40-GEP test. Combining metastatic risk assessment via the clinically available 40-GEP test with clinicopathologic risk factor-based assessment can further stratify patients with cSCC and, within national guideline recommendations, could inform risk-appropriate patient management decisions.

The archival nature of samples and possible under-reporting of high-risk factors are potential limitations of this study. However, the cohort represents a high-risk cSCC population (15% metastasis rate; 60.7 and 39.3% of the cohort were NCCN high risk and very high risk, respectively) [6] and reflects current clinical pathology practices. Comprehensive monitoring of pathology reports, surgical reports and medical records, coupled with independent dermatopathologist review, was implemented to mitigate under-reporting. Another potential limitation of the study is identification of the primary lesion among multiple cutaneous lesions. However, according to the institutional review board-approved study protocol inclusion/exclusion criteria, if there was more than one cSCC in the same region, either the physician was able to unequivocally attribute the cSCC to one primary tumor (which then could be included in the study) or the tumors were excluded from the current study and were made available for future testing (reserved for synchronous tumor studies in the future). While recurrence is another cSCC tumor behavior that, like metastasis, can result in poor outcomes, the 40-GEP test was developed to classify a tumor based on risk of metastasis and at this time has not been validated for risk of local recurrence.

Future perspective

Ongoing study enrollment will allow for additional reporting for this molecular test. Currently, tissue from recurrent tumors is not being clinically tested, nor has the 40-GEP test been validated to predict risk of local recurrence, although these represent areas of ongoing research. We expect that incorporation of molecular profiling of tumors with traditional clinicopathologic factor-based assessment in patients with cSCC will evolve as a critical enhancement to patient risk assessment that better informs clinical decision-making for an optimal management strategy for each patient. This clinical enhancement for the care of patients with high-risk cSCC should lead to improved health outcomes and better use of resources.

Summary points

- The incidence of cutaneous squamous cell carcinoma (cSCC) is high (∼2 million cases diagnosed/year) and continues to grow, with a substantial number of patients having poor outcomes, and an annual death toll that is likely to surpass that for melanoma.
- Most deaths from cSCC are due to progressive locoregional disease (e.g., regional lymph node metastasis), which underscores the clinical need for accurate identification of patients at risk of metastasis to inform decision-making for optimal patient management.
- While clinicopathologic factor-based risk assessment and formal staging systems are clinically helpful, they have limitations and a universally accepted system for risk stratification in cSCC has not yet been adopted.
- In a cohort (n = 420) of patients with high-risk cSCC, the intended use population, the 40-gene expression profile (40-GEP) test demonstrated significant prognostic value for stratifying metastatic risk and refined risk classification via integration of 40-GEP results with clinicopathologic factor-based assessment, identifying patients with metastasis rates near those of the general cSCC population (Class 1) and those with rates ≥50% (Class 2B).
- The 40-GEP test is clinically validated to classify risk of metastasis in patients with cSCC with one or more risk factors, and provides independent prognostic information that can enhance clinicopathologic risk factor-based assessment and established staging systems.
- Incorporating the 40-GEP test into risk assessment for patients with cSCC, within national guidelines, could facilitate better-informed decision-making for more personalized, risk-appropriate patient management and, subsequently, improved outcomes.

Ethical conduct of research

The authors have obtained appropriate institutional review board (IRB) approval via Western IRB and informed consent has been obtained from human study participants as required.

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