ORIGINAL RESEARCH

The 40-Gene Expression Profile Test Identifies Patients with National Comprehensive Cancer Network High-Risk Cutaneous Squamous Cell Carcinoma at High Risk of Poor Outcomes to Inform Management Decisions

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ABSTRACT

Introduction: Validate use of the 40-gene expression profile (GEP) to identify patients with National Comprehensive Cancer Network (NCCN) high-risk (HR) cutaneous squamous cell carcinoma (cSCC) who are at increased risk for local recurrence (LR) and metastasis, despite negative margins after surgical resection.
Methods: NCCN HR cSCC patients with definitive negative margin Mohs surgery (n=414) from a previously published cohort were analyzed for risk prediction of local recurrence-free survival (LRFS) and metastasis-free survival (MFS) using Kaplan-Meier analysis with log-rank test. Multivariable Cox regression models were used to assess the effects of 40-GEP and NCCN HR clinicopathologic risk factors on LRFS.
Results: The 40-GEP stratified NCCN HR patients, with low risk Class 1 patients having a higher 3-year LRFS and MFS than Class 2A or Class 2B patients (LRFS: 95.3% vs. 85.5% vs. 71.4%, P=0.001; MFS: 97.1% vs. 89.3% vs. 57.1%, P<0.001). BWH and AJCC staging systems were unable to stratify LRFS and MFS. Class 2A, Class 2B, PNI, and immunosuppression were identified as significant predictors of LR risk.
Conclusions: In NCCN HR patients, 40-GEP testing stratifies LRFS and MFS and is therefore a significant predictor for both LR and metastasis above actionable pathway thresholds, enabling improved treatment decision-making for a patient subgroup who were previously challenging to reliably identify.

INTRODUCTION

Cutaneous squamous cell carcinoma (cSCC) has a very high rate of cure if diagnosed early and treated completely. While most primary tumors are successfully managed with surgical excision, cSCC still causes approximately 15,000 deaths annually, with up to 12,600 patients per year developing nodal metastasis, and poor outcomes tending to occur within the first three years

after treatment of the primary tumor.[1-3] The rate of cSCC local recurrence (LR) varies, with incidence rates ranging between 2% and 8% in lower-stage cSCC patients and exceeding 20% in patients with higher-stage disease.[4-9] Another high-risk subset will develop regional and distant metastasis, with the risk of nodal spread ranging from 1.2% to 5.8% in broad cohort and tumor registry studies.[2,7–11] Significant efforts have been made to identify cSCC patients at high risk for poor outcomes by developing risk stratification and staging systems to guide risk-based patient management decisions. However, more granular stratification of LR and metastasis in patients with cSCC is needed to improve risk-aligned treatment decisions within the existing population-based treatment pathways.

The National Comprehensive Cancer Network (NCCN) assigns patients with cSCC to NCCN low-risk, high-risk (HR), or very-high-risk (VHR) groups based on clinicopathological features associated with poor outcomes and provides practical management guidelines.[5,12,13] Compared to the NCCN low-risk group, NCCN HR and VHR cSCC patients have an elevated risk of both LR and metastasis, with NCCN HR patients having a greater risk of LR than metastasis. It has become clear that NCCN HR patients are actually a heterogenous group, within which is a subset of patients who possess a level of risk of progression typically seen in NCCN VHR patients.[14] Such patients are more likely to be undermanaged, with a rate of progression higher than that of the typical NCCN HR group.

Guideline-driven adjuvant treatment options for NCCN HR and VHR patients with negative surgical margins, who are broadly defined as having an increased individual likelihood of “high risk for regional or distant metastasis”, a “poor prognosis”, “significant risk of extensive local recurrence, nodal or in transit metastasis”, or specific perineural invasion (PNI), include adjuvant radiation therapy (ART) and surveillance imaging. Surveillance imaging is recommended for consideration if “clinical exam is insufficient for following disease” or “there is appreciable risk of subclinical local or nodal recurrence” and for the latter, determination is made based on the “suspected extent of disease”.[12] The broad, population-based descriptions used in the currently available guidelines pose a challenge for clinicians whose patients require more accurate risk-based management decisions.

Postoperative ART has been demonstrated to provide a 50% reduction in the risks of LR and metastasis in a heterogenous high-risk cSCC patient population.[15] However, ART is also associated with a high adverse event rate, particularly on the head and neck, that can include acute or severe radiation-induced dermatitis and carries an estimated direct Medicare cost of roughly $61,000 per course of treatment.[16] Similarly, on a population basis, use of surveillance imaging in cSCC identifies subclinical disease progression in 20-42% of imaged patients, leading to changes in patient management and a resultant 50% reduction in disease-related poor outcomes.[17-20]

Current cSCC staging systems, including the American Joint Committee on Cancer (AJCC) version 8 staging system, specific to the head and neck, and the Brigham and Women’s Hospital (BWH) T-staging system are based on clinicopathologic factors and are focused on metastatic risk prediction.[21,22] While studies of the AJCC and BWH staging systems suggest that increasing LR incidence is associated with increased tumor stage, these staging systems provide only a general, population-based prediction of poor outcomes and lack accurate discriminative

ability in lower stage tumors, especially since certain cSCCs can have additional high-risk clinicopathologic factors which have not been formally incorporated into staging systems (e.g., immunosuppression or moderate differentiation).[23-26] Additionally, as noted in other tumors, cSCC tumor biology is not fully captured by clinicopathologic factors alone.[14,27,28] As a result, over 30% of poor outcomes occur in lower stage BWH T1/T2a tumors, including 44% of LR and 30% of nodal metastases.[24] Many of these cSCCs are classified as NCCN HR tumors rather than VHR tumors. Determining which patients should receive escalated versus de-escalated management planning is traditionally based upon estimated likelihood of progression. The limitations of the existing staging systems in accurately stratifying this heterogenous patient population highlights a clinical need to improve identification of patients whose tumors have an elevated risk of LR and metastasis, which would in turn reduce the likelihood of over- and under-treatment.[22,23,26,29] Thus, identification of patients within the NCCN HR group whose LR risk is great enough to recommend ART or surveillance imaging is of significant clinical importance, as this is likely to improve outcomes in patients who are at a higher likelihood of disease progression. Similarly, there is a significant need to avoid over-treatment in patients who have a lower individual likelihood of progression.

Previously, the 40-gene expression profile (40-GEP) test was validated to stratify metastatic risk in cSCC patients with one or more NCCN HR or VHR factors into low risk (Class 1), higher risk (Class 2A), or highest risk (Class 2B) groups, independent of clinicopathologic factors, and to improve risk-aligned treatment pathway decisions.[14,30,31] Separately, the 40-GEP test was also shown to predict benefit from ART in Class 2B patients.[32,33] The current study validates the ability of 40-GEP testing to stratify LR risk in cSCC patients with NCCN HR tumors, further improving physician's ability to make risk-aligned treatment pathway recommendations.

METHODS

Patient enrollment

Overall study enrollment and data acquisition have been previously described.[14,31] Briefly, archival formalin-fixed, paraffin-embedded (FFPE) cSCC tumor tissue with clinicopathologic factors and outcome data was obtained for patients under an Institutional Review Board (IRB) approved (Western IRB; 20162697) study protocol with waiver of patient consent. Study inclusion criteria included patients with a documented event of either LR, regional or distant metastasis (defined together as metastasis), or documented follow-up of at least three years post-diagnosis of the primary tumor without a local or metastatic event, the period during which almost all events occur.[3] Cases with prior history of cSCC, cutaneous basal cell carcinoma or melanoma in situ were permitted if prior malignancies were considered cured by the treating physician. The large, comprehensive study cohort that met clinical testing criteria excluded patients receiving ART for the purpose of removing any bias of treatment effect on patient outcomes, and only included radiation-treated patients if treatment occurred after a local or metastatic event.[14,31] For this study, only patients (i) classified as NCCN HR, (ii) who underwent Mohs surgery with (iii) negative surgical margins were included for analysis (n=414; **Figure 1**). A secondary analysis including NCCN HR patients who underwent either Mohs or WLE and had negative margins (n=523) was also

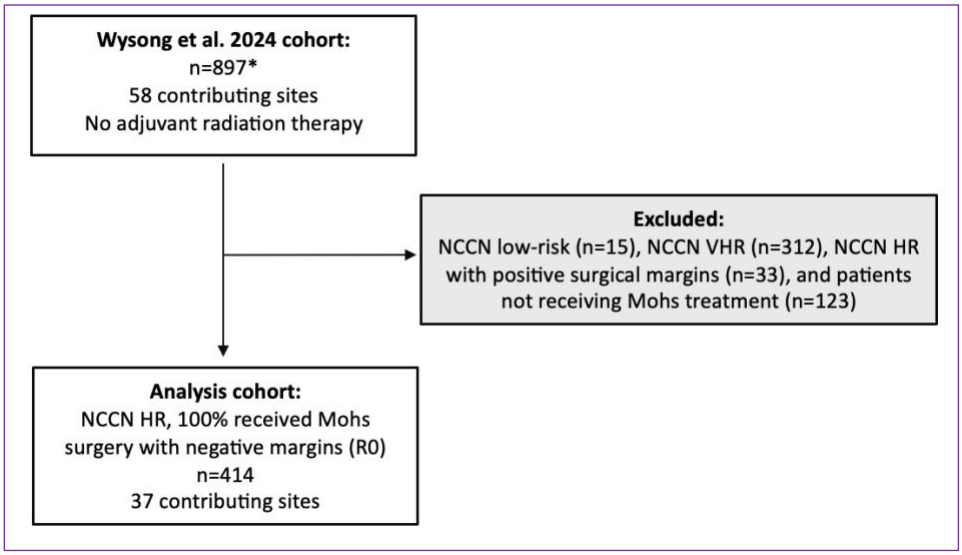

Figure 1. Consort diagram describing derivation of the final patient NCCN HR study cohort, from a previously published study [14,31], that was used for validation of 40-GEP to predict the risk of local recurrence. The subset used for utility analysis was subset to NCCN HR patients who received Mohs surgery and obtained negative margins. NCCN HR: National Comprehensive Cancer Network high-risk group; 40-GEP: 40-gene expression profile; VHR: very-high-risk.

performed to validate the test in all cases of negative margins and was similarly derived from the previously published cohort of 897 patients.[14,31]

Clinicopathologic factors, staging, procedures, and outcomes data were collected and fully monitored. Participant age ≥90 years old was reported as 90 to protect patient-identifying information. Additionally, a board-certified dermatopathologist who was blinded both to outcomes and 40-GEP results independently reviewed tissue samples for tumor and histologic factors. The definition of LR for data capture of all LR events was that previously used by Leitenberger et al.[34] Briefly, LR was defined as recurrent tumor adjacent to or contiguous with the scar at the primary tumor site but not associated with any residual/persistent tumor. The collected and collated clinicopathologic data were used to generate risk classification conforming to each staging or risk stratification system's criteria (AJCC, BWH, NCCN).[21,22,35] Due to a low number of AJCC stage T3 tumors (n=3), these patients were combined with AJCC stage T2 patients (n=137) for analyses. The AJCC T3 cases were confirmed to meet NCCN HR criteria.

Gene expression analysis

In a Clinical Laboratory Improvement Amendment (CLIA) certified, College of American Pathologist (CAP) accredited, New York State Department of Health permitted laboratory, samples were analyzed using 40-GEP clinical testing standard operating procedures as previously described.[30,31] Samples with at least 40% tumor content were processed for real-time PCR. All laboratory personnel were blinded to patient outcomes.[36]

The Chi-square test was used to compare categorical variables and the Wilcoxon signed-rank test was used to compare continuous variables. The endpoints of local recurrence-free survival (LRFS) and metastasis-free survival (MFS) were used to analyze risk stratification by the 40-GEP test in this Mohs-surgery- treated NCCN HR

patient population. Survival was estimated using Kaplan-Meier analysis with the log-rank test used to compare survival between groups. Univariate Cox regression was used to assess the individual contributions of 40-GEP class and clinicopathologic factors potentially influencing LR risk. General clinicopathologic variables included age (continuous) and biological sex (male/female), along with risk factors from the NCCN HR group such as immune status (immunocompetent/immunosuppressed), tumor location (head and neck/special site – acral, anogenital, pretibial/trunk and extremities), PNI (not present or not reported/PNI of <0.1mm or unspecified nerve diameter), tumor diameter (<2cm or unknown /≥2cm), and tumor thickness (<2mm or unknown/≥2mm). Multivariable Cox regression analyses were used to assess risk classification systems. Multivariable modeling to understand the effects of 40-GEP and clinicopathologic risk factors were assessed in two ways. First, multivariable Cox regression was performed using only the factors identified as significant in the univariate analysis (P<0.05). Second, a forward-backwards stepwise variable selection procedure was used to determine which combination of factors resulted in the strongest predictive model (using Akaike Information Criterion, AIC). Likelihood ratios were calculated for each model to capture the relative predictive power over a null model without predictors. In each case, the multivariable model based only on clinicopathological risk factors was compared to the same model including the 40-GEP test result using analysis of deviance (model ANOVA) to determine whether including the test added significant predictive accuracy to the model. Statistical analysis was performed using R Statistical Software v.4.3.1 (https://www.r-project.org/https://www.r-project.org/; survival v3.7-0, MASS v7.3-60), and P<0.05 was considered statistically significant.

RESULTS

Patient characteristics

The median follow-up time for patients was 4.2 years (range: 0.7-11.6). Patient demographics and tumor characteristics are provided in **Table 1**. The overall median study age was 72 (range 32-90) and 73.0% (302/414) of the patients were male. The subset included 29.0% (120/414) immunosuppressed patients, with 66.7% (276/414) of tumors occurring in the head and neck region.

Association of metastasis with local recurrence

Out of the 414 patients, 37 (8.9%) experienced LR and 25 (6.0%) experienced regional and/or distant metastases. Of the 37 patients that experienced a LR, 14 (37.8%) developed regional metastasis with all metastatic events occurring after or concurrently with LR. This contrasted sharply with the 2.9% (11/377) metastatic rate of patients who did not experience a LR. A significant relationship between LR and regional metastasis was observed, whereby patients experiencing a LR were more likely than nonrecurrent patients to also experience regional metastasis (X^2= 72.41, P<0.001). These observations support an association between LR and metastasis; an association that highlights the need to identify patients at an increased risk of LR due to the high rate of progression to metastasis after LR in these NCCN HR patients.

40-GEP Class 2A and 2B results showed higher local recurrence risk relative to tumor stage

Table 1. NCCN HR study cohort patient and tumor characteristics by 40-GEP class result (n=414).

Descriptor	Class 1 (n=276)	Class 2A (n=131)	Class 2B (n=7)	Combined (n=414)
Patient characteristics				
Age, years, median (range)	72 (32-90)	73 (34-90)	78 (40-90)	72 (32-90)
Biological sex, male, n (%)	201 (72.8)	95 (72.5)	6 (85.7)	302 (73.0)
Immunosuppressed, n (%)	85 (30.8)	34 (26.0)	1 (14.3)	120 (29.0)
Tumor characteristics, n (%)				
Location: head and neck	169 (61.2)	101 (77.1)	6 (85.7)	276 (66.7)
Tumor diameter[a]				
<1cm	72 (26.1)	30 (22.9)	1 (14.3)	103 (24.9)
1-2cm	125 (45.3)	55 (42.0)	0 (0)	180 (43.5)
2-4cm	59 (21.4)	40 (30.5)	2 (28.6)	101 (24.4)
PNI[b]	3 (1.1)	5 (3.8)	1 (14.3)	9 (2.2)
Histological differentiation				
Well differentiated	213 (77.2)	79 (60.3)	3 (42.9)	295 (71.3)
Moderately differentiated	63 (22.8)	52 (39.7)	4 (57.1)	119 (28.7)
Disease status, n (%)				
Local recurrence	16 (5.8)	19 (14.5)	2 (28.6)	37 (8.9)
Nonlocal metastasis	8 (2.9)	14 (10.7)	3 (42.9)	25 (6.0)

[a]Tumor diameter was missing in 30 (7.3%) of the patients. [b]PNI <0.1mm or unspecified nerve diameter. *40-GEP*: 40-gene expression profile; *NCCN HR*: National Comprehensive Cancer Network high-risk group; *PNI*: perineural invasion.

LR rates based on risk stratification and staging systems are shown in **Table 2**. Overall, the LR rate for NCCN HR patients was 8.9% (37/414). The LR rate for patients with 40-GEP Class 2B results was 28.6% (2/7) and for patients with a 40-GEP Class 2A result was 14.5% (19/131) as compared to the 40-GEP Class 1 result rate of 5.8% (16/276) (P=0.003). LR rates were nominally higher in BWH T2a and AJCC T2/T3 (10.0%; 14/140) patients than in BWH T1 and AJCC T1 (8.4%; 23/274) (P=0.719). The results were comparable in BWH and AJCC staging systems as patients in the lower or higher T-stage groups completely overlapped between the two systems (i.e., BWH T2a and AJCC T2/T3 were composed of the same patients; **Table 2**). Additionally, the metastasis rates (regional or distant) for 40-GEP Class 2B (42.9%) and Class 2A (10.7%) were elevated compared to the low rate of 2.9% observed in 40-GEP Class 1 patients (P<0.001, **Table 1**), paralleling previous observations relating to LR risk and further supporting the linkage of risks between LR and metastasis.

Multivariable analysis was performed to assess the effects of 40-GEP class results and BWH or AJCC T-stage on predicting LR in the NCCN HR study cohort. 40-GEP Class 2A and Class 2B were the only significant predictors of LR with hazard ratios of 2.6 (P=0.005) and 6.5 (P=0.013), respectively (**Table 3**).

40-GEP risk class stratifies LR-free survival (LRFS) and metastasis-free survival (MFS) whereas currently used staging systems are unable to provide risk stratification in NCCN HR patients

Table 2. Local recurrence events in risk classification and staging systems for the NCCN HR study cohort (n=414).

Classification System	Combined (n=414)	No Recurrence (n=377)	Recurrence (n=37)	Local Recurrence Rate (%)	*P*-value
NCCN HR, n (%)	414 (100)	377 (100)	37 (100)	8.9	--
BWH[a], n (%)					
T1	274 (66.2)	251 (66.6)	23 (62.2)	8.4	0.719
T2a	140 (33.8)	126 (33.4)	14 (37.8)	10.0	
AJCC[a], n (%)					
T1	274 (66.2)	251 (66.6)	23 (62.2)	8.4	0.719
T2/T3	140 (33.8)	126 (33.4)	14 (37.8)	10.0	
40-GEP result, n (%)					
Class 1	276 (67.7)	260 (69.0)	16 (43.2)	5.8	0.003
Class 2A	131 (31.6)	112 (29.7)	19 (51.4)	14.5	
Class 2B	7 (1.7)	5 (1.3)	2 (5.4)	28.6	

[a]Results were the same for both staging systems as the patients had the same partitioning in lower and higher T-stage groups between the two staging systems. *NCCN HR*: National Comprehensive Cancer Network high-risk group; *BWH*: Brigham and Women's Hospital; *AJCC*: American Joint Committee on Cancer.

Table 3. Multivariable Cox regression analyses of local recurrence risk associated with 40-GEP class result and tumor stage in the NCCN HR study cohort (n=414).

Risk Classification System	Hazard ratio (95% CI)	*P*-value
40-GEP and BWH[a]		
40-GEP Class 1	Reference	--
40-GEP Class 2A	2.6 (1.3-5.1)	0.005*
40-GEP Class 2B	6.5 (1.5-28.3)	0.013*
BWH T1	Reference	--
BWH T2a	1.1 (0.6-2.1)	0.831
40-GEP and AJCC[a]		
40-GEP Class 1	Reference	--
40-GEP Class 2A	2.6 (1.3-5.1)	0.005*
40-GEP Class 2B	6.5 (1.5-28.3)	0.013*
AJCC T1	Reference	--
AJCC T2/T3	1.1 (0.6-2.1)	0.831

[a]Results were the same for both staging systems due to the same partitioning of patients in lower and higher T-stage groups between the two staging systems. *Statistically significant, (*P*<0.05). *40-GEP:* 40-gene expression profile; *NCCN HR*: National Comprehensive Cancer Network high-risk group; *BWH*: Brigham and Women's Hospital; *AJCC*: American Joint Committee on Cancer; *CI*: confidence interval.

The overall 3-year LRFS in the NCCN HR cohort was 91.8% (89.1-94.5), while MFS was 94.0% (91.7-96.3) (**Figure 2**). BWH (**Figure 2**) and AJCC (**Figure 3**) staging

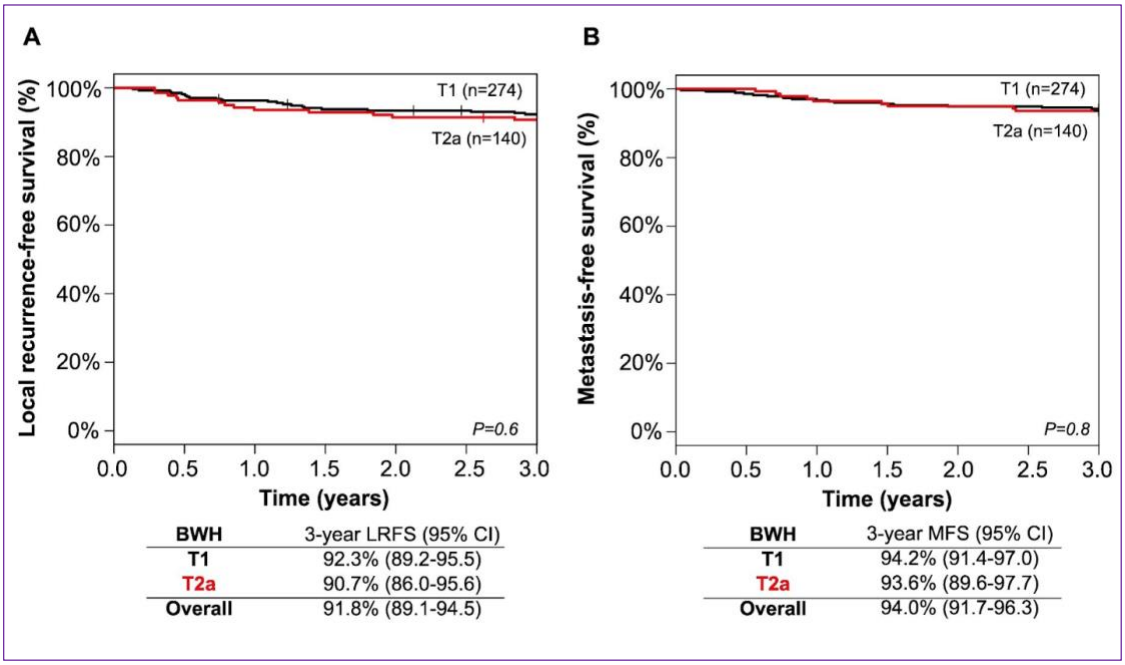

Figure 2. BWH (T1 vs. T2a) staging did not significantly stratify local recurrence or metastatic risk in the NCCN HR Mohs-treated cohort. Kaplan-Meier analysis of the BWH staging system based on 3-year local recurrence-free survival (LRFS) (**A**) and metastasis-free survival (MFS) (**B**) in NCCN HR study cohort (n=414) with Mohs surgery. BWH (T1 vs. T2a) staging did not significantly stratify LRFS or MFS in NCCN HR Mohs-treated patients. *LRFS*: local recurrence-free survival; *BWH*: Brigham and Women's Hospital; *NCCN HR*: National Comprehensive Cancer Network high-risk group; *CI*: confidence intervals.

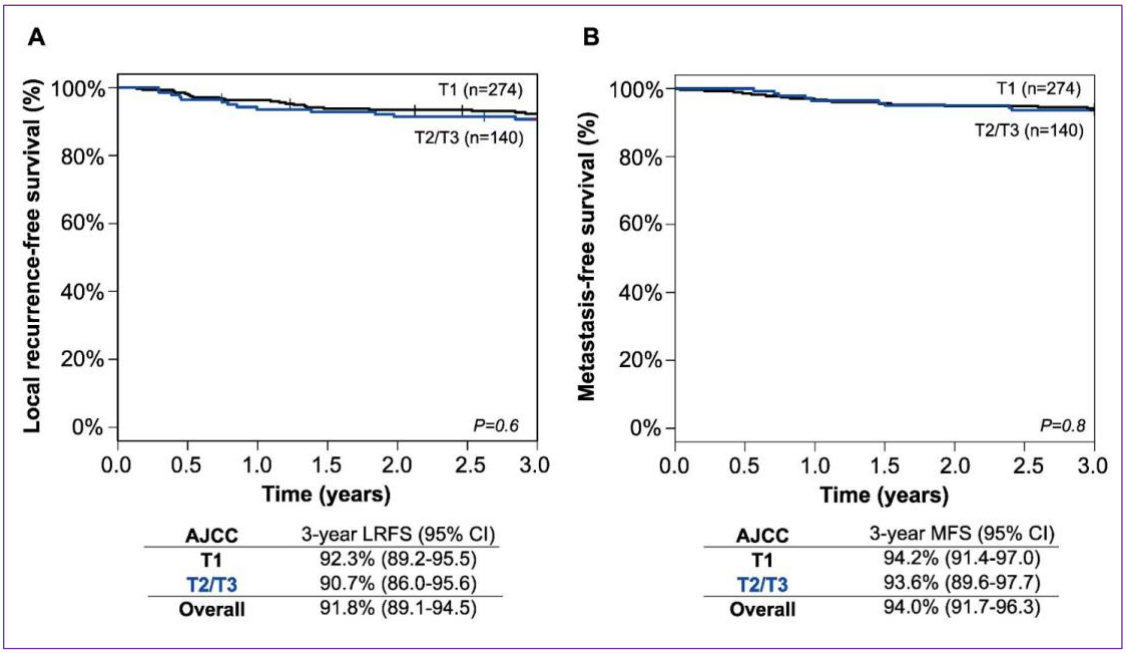

Figure 3. AJCC (T1 vs. T2/T3) staging did not significantly stratify local recurrence or metastatic risk in the NCCN HR Mohs-treated cohort. Kaplan-Meier analysis of the AJCC staging system based on 3-year local recurrence-free survival (LRFS) (**A**) and metastasis-free survival (MFS) (**B**) in NCCN HR study cohort (n=414) with Mohs surgery. AJCC (T1 vs. T2/T3) staging did not significantly stratify LRFS or MFS in NCCN HR Mohs-treated patients. *LRFS*: local recurrence-free survival; *AJCC*: American Joint Committee on Cancer; *NCCN HR*: National Comprehensive Cancer Network high-risk group; *CI*: confidence intervals.

systems failed to significantly stratify LRFS (log-rank, *P*=0.6) and MFS (log-rank, *P*=0.8). BWH and AJCC 3-year LRFS were 92.3% (89.2-95.5) for BWH T1 and AJCC T1 compared to 90.7% (86.0-95.6) for BWH T2a and AJCC T2/T3 (**Figures 2A and 3A**). BWH and AJCC 3-year MFS was 94.2% (91.4-97.0) for BWH T1 and AJCC T1 compared to 93.6% (89.6-97.7) for BWH T2a and AJCC T2/T3 (**Figures 2B and 3B**).

The 40-GEP demonstrated significant discriminatory capacity for LRFS (log-rank, *P*=0.001) and MFS (log-rank, *P*<0.001) (**Figure 4**). The 3-year LRFS for 40-GEP results were 95.3% (92.8-97.8) for Class 1, 85.5% (79.7-91.7) for Class 2A, and 71.4% (44.7-100) for Class 2B. The 3-year MFS for 40-GEP results were 97.1% (95.1-99.1) for Class 1, 89.3% (84.2-94.8) for Class 2A, and 57.1% (30.1-100) for Class 2B.

Because Mohs surgery is reported to provide the most accurate assessment of tumor margin clearance and the highest cSCC cure rates, we focused the primary analysis on NCCN HR patients receiving surgical excision with Mohs.[4,7] However, NCCN HR patients are also treated with other forms of surgical excision (e.g., WLE). Therefore, we performed a secondary analysis to assess the performance of the 40-GEP in predicting LR risk in patients with negative surgical margins after a definitive surgical approach. Patient demographics of the overall NCCN HR cohort (n=523) are summarized in **Table 4.** The 40-GEP test also significantly stratified LRFS (log-rank, *P*=0.003) and MFS (log-rank, *P*<0.001) in this expanded cohort with negative reported surgical margins (**Figure 5**). The 3-year LRFS for Class 1 was 94.9% (92.6-97.3) and decreased to 87.9% (83.2-92.9) in Class 2A and 75.0% (54.1-100.0) in Class 2B. Similarly, the 3-year MFS was 97.0% (95.2-98.9) in Class 1 and decreased to 89.1% (84.6-93.8) in Class 2A and 66.7% (44.7-99.5) in Class 2B.

40-GEP significantly predicts local recurrence risk and provides additional prognostic accuracy even when included with clinicopathologic risk factors

Univariate analysis identified 40-GEP Class 2A, 40-GEP Class 2B, immunosuppressed patient status, and small caliber PNI as individual risk factors significantly associated with LR (all *P*<0.05), while age, gender, tumor thickness, tumor diameter, and tumor location were not significantly associated (**Table 5**). The two clinicopathologic factors identified as significant individual contributors to LR in the univariate analysis were then included in testing multivariable Cox proportional hazard models in a focused comparison between these two factors with and without 40-GEP. In multivariable analysis including 40-GEP, Class 2A and Class 2B, immunosuppression, and small caliber PNI, all variables remained significant risk predictors, with hazard ratios of 2.6, 5.3, 2.3, and 3.7, respectively (all *P*<0.5; **Table 6)**. We then tested whether the addition of 40-GEP results to these two clinicopathological factors significantly increased prediction accuracy for LR. Modeled likelihood ratios were compared for the clinicopathologic-only (12.27) and clinicopathologic plus 40-GEP (21.65) models. The higher likelihood ratio of the model that included 40-GEP for prediction reflects a significant increase in prognostic accuracy (ANOVA, *P*=0.009), indicating that the inclusion of 40-GEP results provides additional predictive information in addition to relevant clinicopathological factors.

As a second approach, NCCN HR factors were included in model development, and a stepwise process was used to determine the best set of clinicopathological factors to

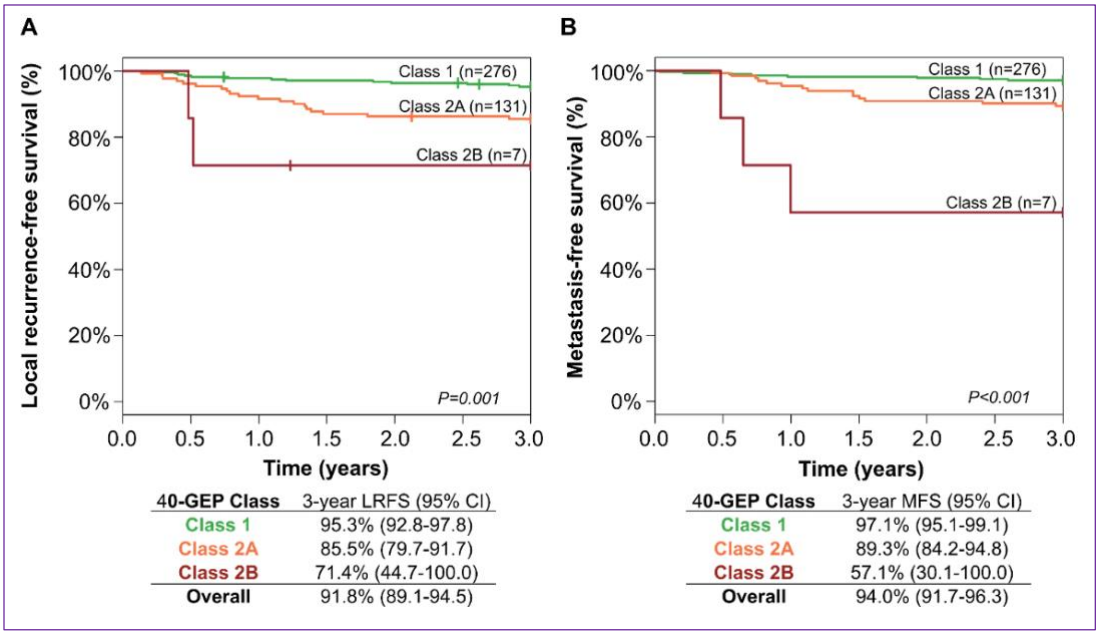

Figure 4. The 40-GEP test significantly stratifies local recurrence and metastatic risk in the NCCN HR Mohs-treated cohort. Kaplan-Meier analysis of the 40-GEP prognostic test based on 3-year local recurrence-free survival (LRFS) (**A**) and metastasis-free survival (MFS) (**B**) in NCCN HR study cohort (n=414) with Mohs surgery. LRFS and MFS are significantly stratified by 40-GEP Class results. *LRFS*: local recurrence-free survival; *MFS*: metastasis-free survival; *40-GEP*: 40-gene expression profile; *NCCN HR*: National Comprehensive Cancer Network high-risk group; *CI*: confidence interval.

Table 4. Overall NCCN HR cohort (Mohs and WLE) demographics stratified by 40-GEP class result (n=523).

Descriptor	Class 1 (n=337)	Class 2A (n=174)	Class 2B (n=12)	Combined (n=523)
Patient characteristics				
Age, years, median (range)	71 (32-90)	73 (34-90)	80 (40-90)	72 (32-90)
Biological sex, male, n (%)	240 (71.2)	128 (73.6)	10 (83.3)	378 (72.3)
Immunosuppressed, n (%)	107 (31.8)	43 (24.7)	3 (25.0)	153 (29.3)
Definitive surgery type, n (%)				
Mohs surgery	276 (81.9)	131 (75.3)	7 (58.3)	414 (79.2)
Wide local excision	61 (18.1)	43 (24.7)	5 (41.7)	109 (20.8)
Tumor characteristics, n (%)				
Location: head and neck	191 (56.7)	126 (72.4)	10 (83.3)	327 (62.5)
Tumor diameter[a]				
<1cm	83 (24.6)	39 (22.4)	2 (16.7)	124 (23.7)
1-2cm	154 (45.7)	72 (41.4)	1 (8.3)	227 (43.4)
2-4cm	71 (21.1)	53 (30.5)	4 (33.3)	128 (24.5)
PNI[b]	9 (2.7)	10 (5.8)	2 (16.7)	21 (4.0)
Histological differentiation				
Well differentiated	261 (77.4)	102 (58.6)	4 (33.3)	367 (70.2)
Moderately differentiated	76 (22.6)	72 (41.4)	8 (66.7)	156 (29.8)
Tumor staging, n (%)				

BWH				
T1	235 (69.7)	106 (60.9)	7 (58.3)	348 (66.5)
T2a	102 (30.3)	68 (39.1)	5 (41.7)	175 (33.5)
AJCC				
T1	235 (69.7)	106 (60.9)	7 (58.3)	348 (66.5)
T2/T3	102 (30.3)	68 (39.1)	5 (41.7)	175 (33.5)
Disease status, n (%)				
Local recurrence	20 (5.9)	21 (12.1)	3 (25.0)	44 (8.4)
Nonlocal metastasis	10 (3.0)	19 (10.9)	4 (33.3)	33 (6.3)

[a]Tumor diameter was missing in 8.4% (44/523) of the patients. [b]PNI <0.1mm or unspecified nerve diameter. *40-GEP*: 40-gene expression profile; *NCCN HR*: National Comprehensive Cancer Network high-risk group; *PNI*: perineural invasion.

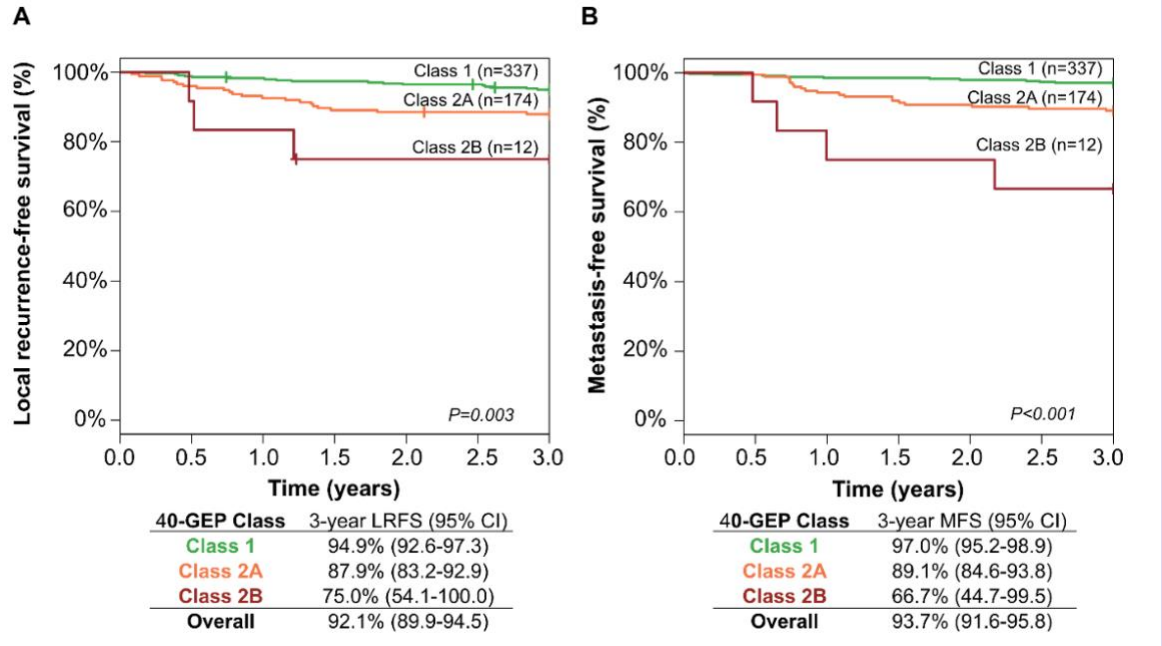

Figure 5. The 40-GEP test significantly stratifies local recurrence and metastatic risk in the overall NCCN HR cohort. Kaplan-Meier analysis of the 40-GEP prognostic test based on 3-year local recurrence-free survival (LRFS) (**A**) and metastasis-free survival (MFS) (**B**) in the overall NCCN HR study cohort (n=523), including patients treated with Mohs or WLE. LRFS and MFS are significantly stratified by 40-GEP Class results. *LRFS*: local recurrence-free survival; *MFS*: metastasis-free survival; *40-GEP*: 40-gene expression profile; *NCCN HR*: National Comprehensive Cancer Network high-risk group; *CI*: confidence interval.

Table 5. Univariate analysis of local recurrence risk associated with 40-GEP class result and clinicopathologic features in the NCCN HR study cohort (n=414).

Risk factor	Hazard Ratio (95% CI)	*P*-value
Age, continuous	1.0 (1.0-1.0)	0.906
Biological sex, female	Reference	--
Biological sex, male	2.5 (1.0-6.4)	0.057
Immunocompetent	Reference	--
Immunosuppressed	2.2 (1.2-4.2)	0.016*

Location: trunk and extremities	Reference	--
Location: special site[a]	1.5 (0.3-8.4)	0.617
Location: head and neck	3.5 (0.8-14.5)	0.088
PNI not present or not reported	Reference	--
PNI[b]	6.6 (2.3-18.7)	<0.001*
Tumor diameter, <2cm or unknown	Reference	--
Tumor diameter, ≥2cm	1.2 (0.6-2.3)	0.582
Tumor thickness, <2mm or unknown	Reference	--
Tumor thickness, ≥2mm	2.2 (0.5-9.1)	0.278
40-GEP Class 1	Reference	--
40-GEP Class 2A	2.7 (1.4-5.2)	0.004*
40-GEP Class 2B	6.5 (1.5-28.5)	0.012*

[a]Special site includes acral, anogenital, and pretibial. [b]PNI <0.1 mm or unspecified nerve diameter. *Statistically significant (P<0.05). *40-GEP*: 40-gene expression profile; *PNI*: perineural invasion; *NCCN HR*: National Comprehensive Cancer Network high-risk group; *CI*: confidence intervals.

Table 6. Multivariable Cox regression models using variables identified by univariate analysis as significant predictors of local recurrence in the NCCN HR study cohort (n=414).

Risk factor	Without 40-GEP			With 40-GEP	
	Hazard ratio (95% CI)	*P*-value		Hazard ratio (95% CI)	*P*-value
Immunocompetent	Reference	--		Reference	--
Immunosuppressed	2.0 (1.1-3.9)	0.035		2.3 (1.2-4.4)	0.013*
PNI not present or not reported	Reference	--		Reference	--
PNI[a]	5.6 (1.9-15.9)	0.001		3.7 (1.2-10.8)	0.019*
40-GEP Class 1	--	--		Reference	--
40-GEP Class 2A	--	--		2.6 (1.3-5.1)	0.005*
40-GEP Class 2B	--	--		5.3 (1.1-24.4)	0.034*

[a]PNI <0.1mm or unspecified nerve diameter. *Statistically significant (P-value <0.05).*40-GEP*: 40 gene expression profile; *NCCN HR*: National Comprehensive Cancer Network high-risk group; *PNI*: perineural invasion; *CI*: confidence interval.

predict LR. This process also resulted in identifying immunosuppressed patient status and small caliber PNI as significant risk factors, in addition to tumor location (head and neck or special site) and tumor diameter (≥2cm; **Table 7**). In multivariable analysis including 40-GEP with the 'best' clinicopathologic factors, Class 2A, Class 2B, immunosuppression, and small caliber PNI, again, were found to be significant with hazard ratios of 2.3, 5.1, 2.5, and 3.3, respectively (all *P*<0.05). Tumor location and tumor diameter were not significant predictors in the model (**Table 8**). Model likelihood ratios were compared for the clinicopathologic-only (20.14) and

clinicopathologic plus 40-GEP (27.85) models. The results again showed that 40-GEP significantly improved accuracy in predicting LR even when significant clinicopathological factors were considered (ANOVA, *P*=0.021). As BWH and AJCC staging were not significant predictors of LR, similar analyses with these staging systems were not performed.

DISCUSSION

Treatment decisions for patients with NCCN HR cSCC pose a clinical challenge. While most NCCN HR patients are treated successfully with definitive surgery, over 30% of LR or metastases occur in this heterogeneous group. It is therefore important to identify the subset of NCCN HR patients with a higher likelihood of developing poor outcomes to improve management decisions within established NCCN guideline treatment pathways. As demonstrated here and discussed elsewhere, BWH and AJCC staging within the NCCN HR patient group does not identify increased risks of LR or metastasis. As a result, BWH or AJCC staging of NCCN HR patients is not effective in guiding treatment decisions nor does it improve clinical decision-making.[24-26]

Studies have shown that the majority of clinicians agree that a >20% metastatic risk, also the level of estimated risk associated with a BWH T2b tumor, is sufficient to recommend pursuing ART, while a risk of <10% is appropriate to recommend deferral of ART.[37-39] Similarly, the majority of clinicians believe that a 5-15% metastatic risk is sufficient to consider surveillance imaging for cSCC patients, with most recommending imaging when the risk is >10%.[4,26,40] Our results demonstrated that the 40-GEP test can identify NCCN HR patients who are at an elevated risk for LR as well as metastasis. Patients with a Class 2A result would exceed the 10% threshold noted above, such that a risk-aligned recommendation for surveillance imaging and consideration of ART would be appropriate. Similarly, a Class 2B result identifies a group of patients who would cross the 20% threshold and results in a risk aligned appropriate recommendation that would then be made to pursue ART. In contrast, patients with a Class 1 result have a risk for LR and metastasis that is well below the 10% threshold, which would then enable risk-aligned de-escalation. De-escalated patients would still receive a more frequent follow-up schedule, a safeguard which has already been established within the guidelines for management of NCCN HR patients.

Clinicopathologic risk factor based staging systems are used to obtain more granular risk stratification as part of the clinician's personal algorithm used for treatment or management decisions. Our results show that while the 40-GEP Class 2A and Class 2B results successfully identified patients at higher risk of LR and metastasis (**Figure 3**), the staging systems based solely on clinicopathologic factors failed to identify those same at-risk patients within the NCCN HR cohort. Poor LR stratification in BWH T1 and T2a tumors has been reported by Zakhem et al., which is concordant with our study results.[23,25]

The 40-GEP was previously shown to be a significant independent predictor of metastatic risk within the context of individual clinicopathologic risk factors. The study therefore assessed which risk factors may be predictive of LR in NCCN HR patients, and two multivariable modeling approaches demonstrated that Class 2A, Class 2B, small caliber PNI, and immunosuppression were significant predictors of LR, while other NCCN HR factors were not. Moreover, our

Table 7. Initial clinicopathologic Multivariable Cox regression model of the starting set of NCCN HR clinicopathologic features used to generate the forward/backward elimination-derived clinicopathologic multivariable model predicting risk of local recurrence (n=414).

Risk factor	Hazard ratio (95% CI)	*P*-value
Age, continuous	1.0 (1.0-1.0)	0.668
Biological sex, female	Reference	--
Biological sex, male	1.8 (0.7-4.9)	0.227
Immunocompetent	Reference	--
Immunosuppressed	2.5 (1.2-5.3)	0.014*
Location: trunk and extremities	Reference	--
Location: special site[a]	1.8 (0.3-9.8)	0.513
Location: head and neck	3.4 (0.8-15.3)	0.103
PNI not present or not reported	Reference	--
PNI[b]	3.4 (1.1-10.5)	0.035*
Tumor diameter, <2cm or unknown	Reference	--
Tumor diameter, ≥2cm	1.8 (0.9-3.7)	0.102
Tumor thickness, <2mm or unknown	Reference	--
Tumor thickness, ≥2mm	1.7 (0.4-7.7)	0.481

[a]Special site includes acral, anogenital, and pretibial. [b]PNI <0.1mm or unspecified nerve diameter. *Statistically significant (P-value <0.05). *40-GEP*: 40-gene expression profile; *NCCN HR*: National Comprehensive Cancer Network high-risk group; *PNI*: perineural invasion; *CI*: confidence interval.

Table 8. Multivariable Cox analyses of the NCCN HR study cohort using a stepwise-derived clinicopathologic model predicting local recurrence with and without 40-GEP class result (n=414).

Risk factor	Hazard ratio (95% CI)	*P*-value	Hazard ratio (95% CI)	*P*-value
Immunocompetent	Reference	--	Reference	--
Immunosuppressed	2.2 (1.1-4.4)	0.020	2.5 (1.3-5.0)	0.007*
Location: trunk and extremities	Reference	--	Reference	--
Location: special site[a]	1.9 (0.3-10.3)	0.474	2.1 (0.4-11.5)	0.401
Location: head and neck	4.2 (1.0-18.0)	0.056	3.8 (0.9-16.3)	0.075
PNI not present or not reported	Reference	--	Reference	--
PNI[b]	4.2 (1.4-12.4)	0.009	3.3 (1.1-9.6)	0.031*
Tumor diameter, <2cm or unknown	Reference	--	Reference	--
Tumor diameter, ≥2cm	1.9 (0.9-3.8)	0.081	1.8 (0.9-3.7)	0.096
40-GEP Class 1	--	--	Reference	--
40-GEP Class 2A	--	--	2.3 (1.2-4.6)	0.015*

40-GEP Class 2B	--	--	5.1 (1.1-23.4)	0.037*

[a]Special site includes acral, anogenital, and pretibial. [b]PNI <0.1mm or unspecified nerve diameter. *Statistically significant (P-value <0.05). *40-GEP*: 40-gene expression profile; *NCCN HR*: National Comprehensive Cancer Network high-risk group; *PNI*: perineural invasion; *CI*: confidence intervals.

results demonstrated that the tumor biology-based 40-GEP test has extended discriminatory capability beyond clinicopathologic factors and that it provides prognostic value alongside two highly recognized risk factors associated with LR and metastasis; thus, the Class 2A and Class 2B result may be considered as risk factors for upstaging. Moreover, the augmented prognostic information provided by the 40-GEP test further improves risk-aligned decision-making within the established NCCN treatment pathways.

A strength of the current study was in the specific assessment of patients treated with Mohs surgery, which is the standard of care treatment modality for NCCN HR tumors, as it enables 100% margin assessment. This allowed for stringent margin control, the best means of assessing LR risk, and limited the confounding factor of residual disease and its impact on poor outcomes in the Mohs-treated NCCN HR subset. The risk of LR in patients undergoing standard excision is greater than that of Mohs surgery, because less than 1% of the surgical margin is evaluated in breadloafed sections and residual disease may not be reliably identified in these specimens. We reported analysis of a broader NCCN HR cohort that included patients treated by Mohs or WLE and we observed similar significant stratification of risk for LRFS and MFS. The results presented can be considered agnostic to surgical methods in this broadened NCCN HR cohort. A limitation of this study is the retrospective study design with reliance on existing data that may have missing information. Consistent with practices in this disease state, some clinical and pathologic factors were not recorded on pathology reports that were accessed for data capture. To address this limitation, (i) histologic review was performed on 100% of cases for specific factors by an independent, board-certified dermatopathologist (blinded to outcomes and 40-GEP results) and (ii) one-hundred percent monitoring was performed. It should be noted that validation of NCCN, BWH and AJCC systems have also been performed using retrospective study designs.

CONCLUSION

Neither BWH nor AJCC staging systems provide clinically relevant risk stratification within the NCCN HR group of cSCC patients. However, 40-GEP testing did significantly stratify these patients in terms of their local recurrence-free and metastasis-free survival and adding prognostic value beyond that of individual clinicopathologic factors. The results of this study therefore validate the extended utility of the 40-GEP test within the NCCN HR patient cohort by providing information that not only predicts metastasis and response to ART but also extends to prediction of LR. Furthermore, 40-GEP Class 2A results increased the likelihood of LR and metastasis above the 10% threshold at which most clinicians would recommend surveillance imaging and consider ART. A Class 2B result had an increased risk of LR and metastasis above the 20% threshold, at which most clinicians would recommend proceeding with ART. In contrast, a Class 1 result had a decreased likelihood of LR and metastasis significantly below the 10% threshold, such that a recommendation to defer ART could be provided. The addition of 40-GEP testing in NCCN HR patients therefore improves risk-aligned treatment

planning within established NCCN pathways, further enhancing our ability to individualize and optimize the care of a patient subgroup whom we were previously unable to reliably identify. This is not only a practice-changing development for physicians who treat cSCC but also a life-changing development for NCCN HR cSCC patients, who can now be stratified according to their risk of developing LR and metastasis, resulting in appropriate escalation or de-escalation of their management.

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