Validation of a 40-Gene Expression Profile Test to Predict Metastatic Risk in Localized High-Risk Cutaneous Squamous Cell Carcinoma

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Abstract

Background: Current staging systems for cutaneous squamous cell carcinoma (cSCC) have limited positive predictive value (PPV) 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 was developed using a discovery cohort (n=202) and validated in a separate, non-overlaping, independent cohort (n=324).

Results: A prognostic, 40-gene expression profile (40-GEP) test was developed and validated, stratifying high-risk cSCC patients 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 (MFS) rates were 91.4%, 80.6%, and 44.0%, respectively. A PPV of 60% was achieved for the highest-risk group (Class 2B), an improvement over staging systems; while 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.

Keywords: cutaneous squamous cell carcinoma; gene expression profile; prognostication; metastasis; risk

Capsule Summary:

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

Introduction

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 US,[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 50-83% and <40% for patients with regional and distant metastasis, respectively.[16, 20–22] Since 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 manage patients. 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) Staging Manual uses clinicopathologic features of the primary tumor with four 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] as many patients categorized as high risk do not develop advanced disease.[28, 29] The Brigham and Women's Hospital (BWH) staging system includes four T-stages (T1, T2a, T2b, and T3) categorizing tumors by number of high-risk features observed. For BWH, T2b-T3 tumors are generally combined to

identify “high-risk” disease. Sensitivity is comparable between BWH and AJCC, while 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. Gene expression profiling (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 three classes (Class 1, 2A, and 2B) of cSCC patients with different likelihood of developing metastasis within 3 years of diagnosis. The 40-GEP test is an independent predictor of outcomes and improves upon 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 using archival primary cSCC formalin-fixed paraffin-embedded tissue. The primary 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 cases included in the study were primary cSCC tumors (Figure 1). Cases with local recurrence only were not considered as having a metastatic event.

Expression of 140 candidate genes, identified by discovery efforts or literature review[30–36], was 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 Data Supplement 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 prior to validation. Power calculations indicated that the validation cohort (cohort 2, samples passing QC, 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 de-identified clinical data were obtained from 23 independent centers following Institutional Review Board approval. Clinicopathological and outcomes data were entered into a secure case report form. All reported patient data were monitored on-site, 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 (Figure 1). Complete protocol inclusion/exclusion criteria are summarized in the Data Supplement. The protocol targeted enrollment of cases with at least one high-risk feature as defined by NCCN guidelines or by AJCC or BWH staging >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 (5μm) were freshly cut at contributing institutions and collected at a central CAP-accredited laboratory. Tumor tissue, including tumor stroma, was macrodissected from slides and processed to generate RNA and cDNA as previously described.[37] cDNA underwent a 14-cycle preamplification step prior to dilution, and then was mixed 1:1 with 2x TaqMan Gene Expression Master Mix. Quantitative PCR was then performed using high-throughput microfluidics gene cards containing primers specific to the genes of interest and the QuantStudio 12K Flex Real-Time PCR System (Life Technologies). 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, accuaracy metrics, and sensitivity analysis (see Data Supplement).

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 1). The algorithm selected for validation was comprised of two 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.

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 comprised of 321 primary cSCC cases (52 with documented metastasis, and 269 cases without an event) (Table 1). The algorithm demonstrated 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 following Kaplan-Meier survival analysis (Figure 2, log-rank test, $p<0.0001$). 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 ($p<0.01$, $p<0.0001$), and for disease-specific death were 5.4 and 8.8 ($p<0.05$, $p<0.01$), 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=0.021; Class 2B HR=9.55, p<0.0001) or BWH (Class 2A HR=2.27, p=0.016; Class 2B HR=8.72, p<0.0001) T-stage (Table 2 and Supplemental Table 2). Multivariable analysis with individual clinicopathological features also demonstrated independent prognostic value of the 40-GEP signature (Supplemental Table 3). Supplemental Table 4 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. Since 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, posthoc sensitivity analyses were performed. These analyses yielded similar effect sizes and significance, demonstrating the robustness of the primary analysis despite the assumption of null values for missing data (Supplemental Table 5).

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 3); although, the percentages of metastases occurring in low T-stages were higher than previously reported (62% and 75% for AJCC and BWH stages, respectively).[24–27] The 40-GEP Class 2B group demonstrated a PPV of 60% compared to 32.8%, 35.1%, and 16.7% for AJCC, BWH, and NCCN high-risk groups, respectively (Table 3). The Class 1 group was associated with a 91.1% NPV compared with the 87.7%, 86.3%, and 90.5% NPV for AJCC, BWH, and NCCN, respectively. Likelihood ratios, combining sensitivity and specificity to indicate probability that metastasis will (+LR) or will not (-LR) occur

based on Class result, are reported in Table 3. Importantly, 63.0% 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 cSCC patients into prognostic groups; low-risk for metastasis (Class 1, 91.4% 3-year MFS), and high- and highest-risk for metastasis (Class 2A, 80.6%; and Class 2B, 44.0% 3-year MFS). The study was designed to include cases with at least one 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 cSCC patient population.[5, 10, 15]

Clinical decision-making has benefitted from development of multi-analyte 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. NCCN guidelines for cSCC recommend that patients with certain high-risk features consider pre-operative 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 prior to full capture of histopathological features on excisional specimens. In

addition, as the 40-GEP class results demonstrated prognostic value independent from staging, this risk assessment may help guide post-operative decision making.[52]

Contemporary staging systems are limited in accuracy for identifying patients who are at high risk for developing metastatic disease, as only 24%-38% of patients with BWH stage T2b/T3 tumors and 14%-17% of AJCC T3/T4 patients develop metastasis.[24–27] NCCN's expansive definition of high-risk cSCC suffers from a still lower PPV and risks overtreating patients. While 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 intensity of surveillance and treatment for patients with low-risk biology, and improved allocation of healthcare resources to high-risk patients.

The 40-GEP test achieved a PPV of 60% for Class 2B tumors, exceeding the PPV observed for 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 vs. Class 2 tumors, which was comparable to NCCN and 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 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 (e.g., *in situ* to invasive cSCC) have reported differential expression of various genes and miRNAs.[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 6) 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 potential to identify how these genes promote cSCC metastasis.

As with all archival studies, there is possible bias in specimen collection based on 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. Since not all histological 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, under-reporting 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 herein relative to other cohorts (39% and 25%, respectively, versus 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 multi-center cohort studies in target populations for 40-GEP testing should be undertaken to confirm the PPVs and NPVs reported herein, 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.

As cSCC poses a significant burden on the healthcare 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 clinicopathological features alone are limited in their ability to accurately stratify patients, primarily due to low PPV. The 40-GEP demonstrated 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 clinicopathological features with tumor-intrinsic risk, as per the 40-GEP prognostic test developed and validated herein, has potential to improve patient outcomes, quality of life, and appropriate allocation of healthcare resources for cSCC patients.

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Figure Legends

Figure 1. Cutaneous Squamous Cell Carcinoma. Study cohorts: tissue samples and associated data acquisition. CRF, case report form; f/u, follow-up; event, regional or distant metastasis; QC, quality control. Protocol and monitoring are ongoing, assessment performed Oct. 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 discovery and validation assays.

Figure 2. Cutaneous Squamous Cell Carcinoma. Kaplan-Meier analysis of the 40-GEP prognostic test and outcomes from independent validation of cutaneous cSCC cases (n=321).

Tables:

Table 1: Demographics and clinical characteristics of validation cohort (n=321)

Feature	All (n=321)	Non Met (n=269)	Regional/distant met (n=52)	p value
Age: Median years (range)	70 (34-95)	70 (34-95)	72 (44-90)	0.84
Male sex	235 (73.2%)	191 (71.0%)	44 (84.6%)	0.042
Caucasian	320 (99.7%)	269 (100%)	51 (98.1%)	0.16
Non-Hispanic*	312 (97.2%)	262 (97.4%)	50 (96.2%)	0.62
Immune deficient**	76 (23.7%)	59 (21.9%)	17 (32.7%)	0.10
Prior Hx of SCC	135 (42.1%)	109 (40.5%)	26 (50.0%)	0.22
Located on H&N	214 (66.7%)	171 (63.6%)	43 (82.7%)	0.007
Tumor diameter: Mean cm (StDev)***	1.8 (+/-1.9)	1.6 (+/-1.8)	2.8 (+/-2.4)	<0.0001
Tumor thickness: Mean mm (StDev)#	3.9 (+/-6.4)	3.4 (+/-6.6)	7.2 (+/-3.6)	<0.0001
Poorly differentiated	36 (11.2%)	22 (8.2%)	14 (26.9%)	<0.0001
Clark Level IV / V	62 (19.3%)	49 (18.2%)	13 (25.0%)	<0.0001
PNI##				
present (≥0.1mm)	7 (2.2%)	5 (1.9%)	2 (3.9%)	<0.0001
present (<0.1mm or unknown caliper)	29 (9.0%)	16 (6.0%)	13 (25%)	
not present	285 (88.8%)	248 (92.2%)	37 (71.2%)	
Invasion into fat	43 (13.4%)	28 (10.4%)	15 (28.9%)	0.0004
Definitive surgery MMS###	256 (79.8%)	222 (82.5%)	34 (65.4%)	0.032
AJCC8 T Stage				
T1	201 (62.6%)	175 (65.1%)	26 (50%)	0.001
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				
T1	186 (57.9%)	166 (61.7%)	20 (38.5%)	0.0004
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	300 (93.5%)	250 (92.9%)	50 (96.2%)	0.39

NOTE. Data analyzed using Chi-square test or Kruskal-Wallis F test.
Abbreviations: Hx, history; SCC, squamous cell carcinoma; H&N, head and neck; StDev, standard deviation; PNI, perineural invasion; MMS, Mohs micrographic surgery; AJCC8, American Joint Committee on Cancer, Cancer Staging Manual, Eighth Edition; BWH, Brigham and Women's Hospital; NCCN, National Comprehensive Cancer Network. *One patient did not report ethnicity. **67 of 76 immune deficient patients were organ transplant recipients. ***Tumor diameter reported (n=295). [#]Tumor thickness reported (n=115). [##]PNI with nerve caliper ≥0.1mm or in nerve deeper than the dermis are upstaging factors for AJCC. Only nerve caliper ≥0.1mm is an upstaging factor for BWH. 1 of 7 cases met AJCC upstaging but not BWH upstaging. [###]Mohs or wide local excision (n=319) with 2 cases not having additional surgery beyond biopsy.

Table 2. 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)	0.021
Class 2B	9.55 (4.79-19.06)	<0.0001
AJCC8		
T1/T2	1.0	---
T3/T4	2.68 (1.52-4.72)	<0.001
40-GEP		
Class 1	1.0	---
Class 2A	2.27 (1.19-4.35)	0.013
Class 2B	8.72 (4.30-17.71)	<0.0001
BWH		
T1/T2a	1.0	---
T2b/T3	2.03 (1.07-3.88)	0.032

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

593 Table 3. Accuracy of risk prediction of the 40-GEP and risk assessment methods (n=321)

Accuracy Metric	40-GEP (Class 2B v 1/2A)	40-GEP (Class 2 v 1)	AJCC 8* (T3/T4 v T1/T2)	BWH* (T2b/T3 v T1/T2a)	NCCN* (High v 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%

Abbreviations: GEP, gene expression profile; AJCC8, American Joint Committee on Cancer, Cancer Staging Manual, Eighth Edition; BWH, Brigham and Women's Hospital; NCCN, National Comprehensive Cancer Network; LR, likelihood ratio; PPV, positive predictive value; NPV, negative predictive value.
*Missing histopathologic information was treated as negative.

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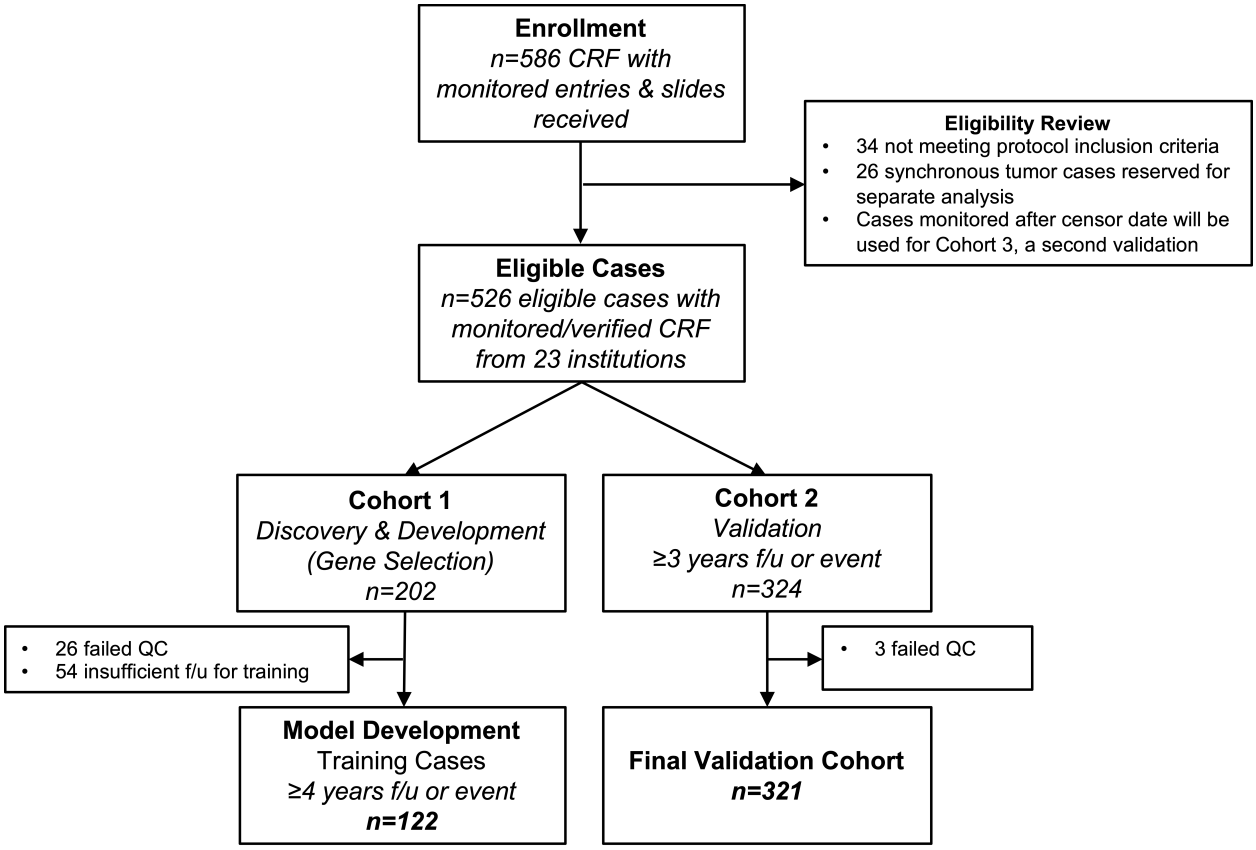
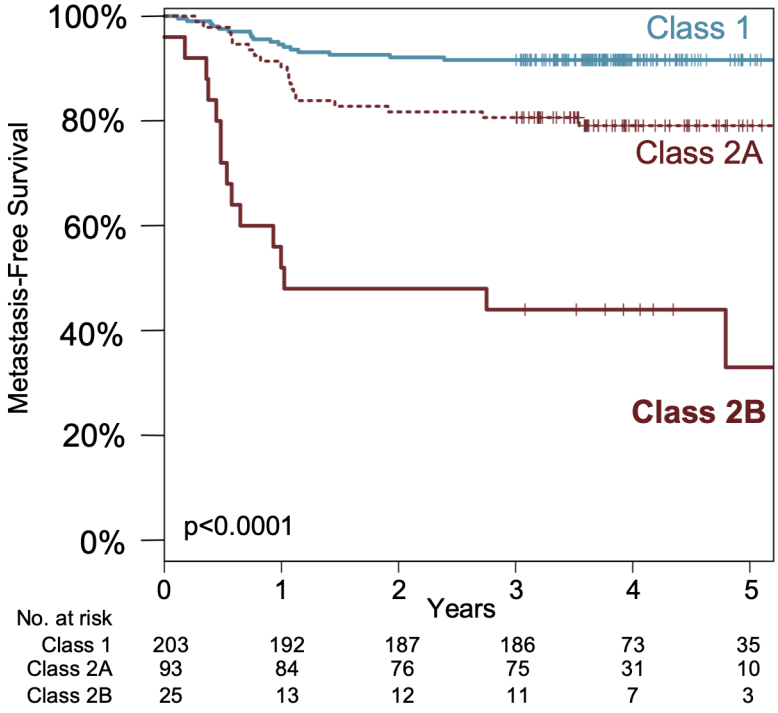

Enrollment
n=586 CRF with monitored entries & slides received
Eligibility Review
• 34 not meeting protocol inclusion criteria
• 26 synchronous tumor cases reserved for separate analysis
• Cases monitored after censor date will be used for Cohort 3, a second validation
Eligible Cases
n=526 eligible cases with monitored/verified CRF from 23 institutions
Cohort 1
Discovery & Development
(Gene Selection)
n=202
Cohort 2
Validation
≥3 years f/u or event
n=324
• 26 failed QC
• 54 insufficient f/u for training
• 3 failed QC
Model Development
Training Cases
≥4 years f/u or event
n=122
Final Validation Cohort
n=321



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%