Research article

Risk prediction in cutaneous melanoma patients from their clinico-pathological features: superiority of clinical data over gene expression data

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ABSTRACT

Risk assessment in cutaneous melanoma (CM) patients is one of the major challenges in the effective treatment of CM patients. Traditionally, clinico-pathological features such as Breslow thickness, American Joint Committee on Cancer (AJCC) tumor staging, etc. are utilized for this purpose. However, due to advancements in technology, most of the upcoming risk prediction methods are gene-expression profile (GEP) based. In this study, we have tried to develop new GEP and clinico-pathological features-based biomarkers and assessed their prognostic strength in contrast to existing prognostic methods. We developed risk prediction models using the expression of the genes associated with different cancer-related pathways and got a maximum hazard ratio (HR) of 2.52 with p-value $\sim 10^{-8}$ for the apoptotic pathway. Another model, based on combination of apoptotic and notch pathway genes boosted the HR to 2.57. Next, we developed models based on individual clinical features and got a maximum HR of 2.45 with p-value $\sim 10^{-6}$ for Breslow thickness. We also developed models using the best features of clinical as well as gene-expression data and obtained a maximum HR of 3.19 with p-value $\sim 10^{-9}$. Finally, we developed a new ensemble method using clinical variables only and got a maximum HR of 6.40 with p-value $\sim 10^{-15}$. Based on this method, a web-based service and an android application named 'CMcrpred' is available at (https://webs.iiitd.edu.in/raghava/cmcrpred/) and Google Play Store respectively to facilitate scientific community. This study reveals that our new ensemble method based on only clinico-pathological features overperforms methods based on GEP based profiles as well as currently used AJCC staging. It also highlights the need to explore the full potential of clinical variables for prognostication of cancer patients.

1. Introduction

Cutaneous melanoma (CM), which accounts for less than 5% of all skin cancers, has been reported to be the most aggressive and fatal form of skin cancer [1]. Melanoma also referred to as malignant melanoma or cutaneous melanoma, arises due to genetic mutations in the melanocytes. Melanocytes are the cells that produce the pigment melanin (the pigment responsible for skin colour) and are located in the skin, inner ear, eye, vaginal epithelium, leptomeninges, heart and bones [2, 3, 4, 5]. In the last 30 years, the number of deaths has increased drastically due to melanoma. According to the estimation by The American Cancer Society, in 2019, about 96480 new melanomas would have been diagnosed (57220 in men and 39260 in women) in the US alone, out of which around 7230 people were expected to die (about 4740 men and 2490 women) [6].

Cutaneous melanomas are curable when diagnosed in the earlier stage (~98% survival rate) but once metastasised, they are tough to cure (~25% survival rate); thus, early identification of melanoma has been deemed crucial for the patient's survival [7, 8]. This uncertainty between patient's death or survival is directly affected by the choice of the therapy given to the patients, which in turn depends on the features that were used for prognostication. According to the present AJCC cancer staging criterion for melanoma, features derived from biopsies and/or blood samples, are primarily used for this purpose in CM patients [9]. There are also demographic factors like ethnicity of a patient, which has been associated with Melanoma occurrence [10]. While features like Breslow thickness, Ulceration status, Metastatic staging etc. can be clinically observed and monitored, underlying molecular factors such as alteration of protein expression and genetic mutations are difficult to incorporate during clinical observation of a patient, due to a huge plethora of

genes/proteins to account for. Thus, the problem for risk prediction with the help of minimal but relevant features in addition to the current clinico-pathological features remains a major challenge. Identification of these features would help in deciding better therapeutic strategies and bridge the uncertainty gap that exists at present.

Over the past decades, protein and gene-based biomarkers have provided valuable prognostic information about patient outcome and aided in the design of therapy. Several of these gene/protein based biomarkers have been utilized in the case of prognosis and risk prediction for various diseases such as breast cancer, colon cancer, prostate cancer, lung cancer, leukemia, and melanoma [11, 12, 13, 14]. Along with these biomarkers and due to the modern techniques used for purifying RNA from formalin-fixed paraffin-embedded (FFPE) tumour samples, there have also been efforts to identify/develop multiple protein and multiple genes-based biomarkers associated with prognostication of SNL (sentinel lymph node status), locoregional recurrences, distant metastases, and survival [15]. In the case of melanoma, several protein candidates have been significantly associated with prognostication, including lactate dehydrogenase (LDH), C-reactive protein and S100B [16, 17, 18, 19]. Out of these, only LDH has been included in the AJCC staging system for categorizing metastasis. However, it is shown to function well only in Stage IV patients. NCOA3, SPP1, and RGS1 is another known example of multiple protein-based biomarker [20] that has been shown to be a significant predictor of SLN status and disease-specific survival as compared to other clinical features. Though validated [21], this 3-protein marker was also not included in the AJCC staging criteria. Amongst single and multiple-gene expression profile (GEP) based markers, TRPM1 expression [22], NRAS mutation status [23], BRAF mutation status [24], circulating miRNA biomarkers [25], DecisionDx-Melanoma (31 GEP) [26, 27, 28, 29], Melagenix (9 GEP), ITLP group [30] and 53-gene immune GEP [31] are a few mainstream examples in the scientific community. The prognostic marker Melagenix (9 GEP) was able to differentiate high and low risk patients on the basis of overall survival, DecisionDx-Melanoma segregated patients on the basis of RFS (relapse free survival), DMFS (distant metastasis-free survival) and MSS (microsatellite instability) while models such as 53-gene immune GEP and ITLP group are prediction models for metastasis progression and SLN positivity respectively. While almost all of these models compare their results with the clinical factors (mainly by employing multivariate Cox-PH analysis), only a few of these models incorporate the combined effect of clinical factors with gene expression. However, unlike other cancers, none of the independent gene based or GEP based prognostic biomarkers for melanoma have been included in the AJCC staging system so far.

In the present study, we make a systematic attempt to identify/develop new prognostic markers based on the overall survival (OS) of CM patients to predict risk. For this purpose, we utilized the TCGA-SKCM RNAseq expression dataset to build machine learning-based regression (MLR) and prognostic index (PI) models based on GEP and clinical factors. GEP based models were constructed based on genes involved in different cancer related pathways, most of which have been related to melanoma carcinogenesis in the past while others have been attributed a role in other cancers. Models were also developed using best genes associated with survival as obtained via rfSRC feature selection method. Combinations of GEP models and clinical features were also analyzed. Briefly, we present here a comparative assessment between these various models and show that a new model based on clinico-pathological features alone outperforms GEP based models.

2. Materials and Methods

2.1. Dataset and pre-processing

The original dataset consisted of RNAseq expression values for 458 Skin Cutaneous Melanoma (SKCM) patients that was obtained from The Cancer Genome Atlas (TCGA) using TCGA Assembler 2 [32]. Out of which, information about OS time and censoring was available for 449

patients. Thus, the final dataset was reduced to 449 samples constituting RNAseq values for 20530 genes. Following the approach similar to [33], genes with 'NA' or zero expression data for more than 50% of the samples were removed. Table 1 presents a summary of clinicopathological features in the final TCGA-SKCM cohort. Further, the final dataset was normalized by quantile normalization method, which has been extensively used in the past for similar studies [34, 35, 36].

2.2. Survival analysis

Hazard ratios were computed to predict the risks of death associated with high-risk and low-risk groups based on overall survival time of patients. These were stratified on the basis of mean and median values of various factors, using the univariate unadjusted Cox-Proportional Hazard (Cox-PH) regression models. Kaplan-Meier (KM) plots were used to compare survival curves of high risk and low risk groups. Survival analyses on these datasets were performed using 'survival' and 'survminer' packages (V.2.42–6) in R (V.3.4.4, The R Foundation). Statistical significance between the survival curves was estimated using log-rank tests. Wald tests were performed to estimate the significance of the explanatory variables used for HR calculations. Concordance index (C) provided the strength of predictive ability of the model [37, 38, 39]. p-values less than 0.05 were considered as significant.

2.3. Prognostic genes

Cox-Proportional Hazard models were used to find the genes that are related to CM patient survival. A cutoff of HR > 1.2 and p-value<0.05 was used. Univariate Cox-PH analysis, at median expression cutoffs, revealed a total of 1343 good prognostic marker (GPM) genes and 1294 bad prognostic marker (BPM) genes. GPM genes are defined as genes whose expression is positively correlated with patient OS time and viceversa for BPM. For a GPM gene, patients with GPM gene expression < median (GPM gene expression) are at high risk and for a BPM gene, patients with BPM gene expression > median (GPM gene expression) are at high risk. Supplementary S1 TableA shows the survival associated parameters such as HR, p-value, C, and Cox regression coefficient-Beta (β) corresponding to each gene. The distribution of the GPM and BPM genes based on Hazard Ratio (HR > 1.2 and p < 0.05) is represented in Supplementary S2 Figure S1.

2.4. Machine learning based regression (MLR) models

Regression models from 'caret' package (V.3.4.4, The R Foundation) were implemented to fit the gene expression values against the OS time. Various regressors such as Support vector machine (SVR), Decision-tree (DT), Random-forest (RF), K-nearest neighbors (KNN), Ridge, Lasso and Elastic-Net were used. The fitting and test evaluations were carried using a five-fold cross-validation scheme. Combination of all five evaluated test datasets (predicted OS) was then used to classify the actual patient survival time (OS) at mean and median cutoffs to estimate HR, CI and p-values. Hyperparameter optimization and regularization was achieved using the in-built function 'expand.grid'.

2.5. Five-fold cross-validation

The dataset is shuffled randomly and divided into 5 subsets. After these groups are prepared, an iterative process begins. During each iteration, a unique group is taken as a test dataset and combination of remaining groups as a training dataset. Model is fitted on the training dataset and evaluated on the test dataset. Model's performance is evaluated using standard parameters viz. RMSE (root mean squared error) and MAE (mean absolute error). The process is repeated five times and each sample is processed once as a testing data point and four times as training data point. Five-fold cross-validation has been successfully implemented in previous studies [40, 41, 42, 43, 44, 45, 46].

Table 1. Summary of TCGA-SKCM cohort used in this study.

Factor	Value	Percentage
Age (at diagnosis), in years		
Mean	58.006	
Median	58	
Range	15–90	
Gender		
Male	280	62.36
Female	169	37.63
Ethnicity		
Non-Hispanic/Non-Latino	426	94.88
Hispanic/Latino	10	2.23
Unknown	13	2.90
Race		
White	426	94.88
Black/African American	1	0.22
Asian	12	2.67
Unknown	10	2.23
Known primary melanoma tumor		
Yes	402	89.53
No	47	10.47
Location of primary melanoma		
Head and Neck	35	7.80
Trunk	155	34.52
Extremity	194	43.21
Other	13	2.90
Unknown	52	11.58
Breslow thickness (mm)		
Mean	5.49	
Median	3	
Unknown	105	23.39
Clark level		
I–III	98	21.83
IV–V	211	46.99
Unknown	140	31.18
Ulceration status		
Yes	159	35.41
No	141	31.40
Unknown	149	33.18
Mitotic rate		
<1	19	4.23
≥ 1	151	33.63
Unknown	279	62.14
T stage		
Tis	7	1.56
T0	23	5.12
T1	10	2.23
T1a	22	4.90
T1b	10	2.23
T2	30	6.68
T2a	31	6.90
T2b	15	3.34
T3	14	3.12
T3a	37	8.24
T3b	37	8.24
T4	15	3.34
T4a	23	5.12
T4b	104	23.16
TX	44	9.80
Unknown	27	6.01

Table 1 (*continued*)

Factor	Value	Percentage
N stage		
N0	223	49.67
N1	16	3.56
N1a	18	4.01
N1b	38	8.46
N2	6	1.34
N2a	13	2.90
N2b	20	4.45
N2c	9	2.00
N3	53	11.80
NX	34	7.57
Unknown	19	4.23
M stage		
M0	400	89.09
M1	5	1.11
M1a	4	0.89
M1b	5	1.11
M1c	9	2.00
Unknown	26	5.79
Pathological Stage		
Stage 0	6	1.34
Stage I	30	6.68
Stage IA	18	4.01
Stage IB	29	6.46
Stage II	30	6.68
Stage IIA	17	3.79
Stage IIB	27	6.01
Stage IIC	58	12.92
Stage III	40	8.91
Stage III A	15	3.34
Stage III B	45	10.02
Stage III C	66	14.70
Stage IV	22	4.90
I/II NOS	10	2.23
Unknown	36	8.02
Anatomic site		
Primary Tumor	91	20.27
Regional Lymph Node	218	48.55
Regional Cutaneous or Subcutaneous tissue	73	16.26
Distant Metastasis	64	14.25
Unknown	3	0.67
Overall Survival (months)		
Mean	52.96	
Median	29.5	
Range	0–362.33	
Vital Status		
Alive	295	65.70
Dead	154	34.30

2.6. Prognostic index (PI)

As implemented in [33, 47, 48], PI for a set of *n* genes was evaluated as:

$$PI = \beta_1 g_1 + \beta_2 g_2 + \dots + \beta_n g_n$$

where β represents regression coefficient obtained for a gene *g* from a univariate Cox-PH model. PI for different set of genes was used for stratifying risk groups and standard metrics such as HR, p-value etc. were estimated.

3. Results

3.1. Multiple gene expression profile (GEP) based risk prediction

3.1.1. Models based on genes associated with cancer pathways

Several signaling pathways have been attributed a role in cancer progression and development in the past. We collected the list of 11 cancer-related pathways and the genes associated with those pathways from a recent study [49]. These are namely Apoptosis, MYC, NRF2, NOTCH, P53, WNT, HIPPO, CELL CYCLE, PI3K-AKT, RAS and TGF-BETA pathways. Amongst these pathways, many have been associated with

melanoma tumorigenesis. Table 2 shows the PMIDs of the studies which have explored the role of these pathways in CM progression and/or development. Table 2 also shows the original gene-count in the pathway, number of GPM and number of BPM genes as a result of univariate analysis. Combined gene count is the sum of GPM and BPM genes. Supplementary S2 Figure S2 shows the upset plot representing gene overlaps between different pathways in the original gene set. MLR and PI based models were built using the GPM, BPM and combined (i.e. GPM + BPM) genes in these pathways, as explained in the following sections. The information regarding genes involved in original gene set and the three filtered gene sets is provided in Supplementary S1 TableB.

Machine learning based regression (MLR) models: MLR models were built using GPM, BPM and combined gene sets for each pathway. Various regressors were used to predict OS time by employing the expression data for the genes in the corresponding gene set (Materials and Methods). Similar models have also been used in the past with protein concentrations as independent variables [45].

Table 3 provides detailed results for each gene-set and the corresponding best model. The Lasso regression model that predicts the survival time on the basis of the combination of GPM and BPM genes (7 genes) of the NOTCH pathway shows the best results, where patients with pred OS $\leq$median (pred OS) are at 2.34 times higher risk than patients with pred OS $>$ median (pred OS) with a p-value of the order of 10^{-7} and C = 0.62. Second best results are seen in the case of the RF model built using GPM genes of the apoptotic pathway (29 genes), where HR is 2.24 with a significant p-value ($\sim 10^{-6}$) and C = 0.59, at median cutoff. However, it should be noted that the combination of the apoptotic GPM geneset and NOTCH combined geneset (36 genes) improves the risk stratification further with HR = 2.54 and p$\sim 10^{-8}$. For mean cutoff, see Supplementary S1 TableC.

Prognostic Index (PI) based models: PI was evaluated for the three types of gene-sets corresponding to each pathway. Based on PI, the patients were stratified into high and low risk groups such that patients with PI $\geq$median (PI) were at higher risk than patients with PI $<$ median (PI) (similarly with mean in Supplementary S1 TableD). Results for each pathway are shown in Table 4. As evident from the results, PI for apoptotic GPM genes improved the risk prediction over the models developed previously. High and low risk patients show a 2.52 fold difference in survival when stratified using the PI for apoptotic GPM genes at median cutoff, with a p-value $\sim 10^{-8}$ and C value of 0.62. 10-year survival rate for high risk patients in this case is around 30% whereas low risk patients have around 60% survival probability. Figure 1a shows the Kaplan Meier plot for median cutoff. The PI model built using the combination of apoptotic GPM genes and NOTCH GPM and BPM genes, is again seen to enhance the risk stratification with HR = 2.57 though p-value and C remain of the same order as apoptotic GPM geneset alone. KM plot for this model is shown in Figure 1b.

3.2. Models based on complete set of genes

In addition to developing models for pathway-specific gene sets, we also developed similar models for the total GPM (1343), BPM (1294) and combined gene set (2637). Feature selection was performed to obtain the most important genes using random survival forests-variable hunting (rfSRC) for 100 iterations, on each of these three gene sets. rfSRC resulted in 58 GPM genes, 52 BPM genes and 129 combined genes (Supplementary S1 TableE). This feature selection method has been extensively used in similar survival-based studies in the past [47, 50, 51, 52]. First, MLR models were built and predicted OS was used to stratify high and low risk patients, similar to the pathway-specific analysis before. Subsequently, prognostic index based stratification was performed using the selected GPM, BPM and combined genes. Results are provided in Supplementary S2 Table S1. The SVR regression model based on 52 BPM genes performs the best out of all these models with highest HR value of 2.77, lowest p-value $\sim 10^{-9}$ and concordance index of 0.63. A comparison of apoptotic genes based PI models, NOTCH genes based regression models, apoptosis and NOTCH genes combined models with the current 52 total BPM based SVR model shows that HR, p-value and concordance have improved. KM plot corresponding to this model is shown in Figure 2.

3.3. Using clinico-pathological features for prognostication

3.3.1. Univariate analysis shows Breslow thickness as the most significant feature

In order to see whether the models developed earlier in this study perform better than the previously established prognostic markers, patients were stratified using clinical factors such as AJCC pathological staging, age, TNM staging, Breslow thickness, Gender and Ulceration status. These have been reported to be associated with risk in CM patients in the past [53, 54, 55, 56]. Table 5 shows these results.

Though our results agree with the previously reported observations, such as patients with age greater than 63 years [57], males [58], patients with metastasized tumors, stage III/IV patients etc. are at higher risk and thus show a high HR value, some of them are either insignificant (p $>$ 0.05) or have a low HR/high p-value except Breslow thickness. Patients with Breslow thickness more than 3mm (median) were found to be at 2.45 times higher risk (p-value = 3×10^{-6}) than compared to the patients with a lesser Breslow thickness. Nevertheless, both apoptotic GPM based PI model and total BPM based SVR model outperform the individual clinical factors and AJCC staging based risk stratification.

3.3.2. GEP and clinical features based combinatorial models

In one of our previous studies [59], Human Leukocyte Antigen (HLA) alleles-based features were used in combination with clinical information, for the prognostication of melanoma patients. The study used a

Table 2. Genes related to pathways associated with cancer. PMIDs of the studies that relate to role of the pathway in melanoma and gene-count before and after univariate Cox-PH analysis is provided.

S. no.	Pathway	PMID	No. of Genes	No. of GPM	No. of BPM	Combined
1	NRF2	27344172, 18353146	481	27	26	53
2	P53	32377702, 31374895	201	17	16	33
3	Apoptosis	32687246, 32645331	161	29	4	33
4	WNT	32659938, 32073511	151	7	9	16
5	CELL-CYCLE	-	128	4	17	21
6	PI3K-AKT	32626712, 32558531	105	18	11	29
7	TGF-β	31667872, 31599708	86	3	1	4
8	NOTCH	30569717, 30941830	47	3	4	7
9	MYC	32283126	25	2	2	4
10	RAS	32605090, 32568870	23	2	1	3
11	HIPPO	32407182, 32561850	22	1	2	3

Table 3. MLR models for risk stratification. The table shows the best models for each pathway and corresponding gene set used. Patients with predicted OS less than or equal to the median cutoff are at higher risk than patients with predicted OS more than cutoff.

S. no.	Pathway	Gene set	Regressor	HR	p-val	C
1	NRF2	Combined	Ridge	2.17	3×10^{-6}	0.60
2	P53	Combined	RF	2.04	1×10^{-5}	0.59
3*	**Apoptosis**	**GPM**	**RF**	**2.24**	**1.2×10^{-6}**	**0.59**
4	WNT	Combined	RF	2.22	2×10^{-6}	0.59
5	CELL-CYCLE	Combined	RF	1.76	5.7×10^{-3}	0.59
6	PI3K-AKT	Combined	RF	1.90	8×10^{-5}	0.60
7	TGF-β	Combined	KNN	1.73	9×10^{-4}	0.58
8	NOTCH	Combined	Lasso	2.34	3×10^{-7}	0.62
9	MYC	GPM	Lasso	1.68	1.7×10^{-3}	0.57
10	RAS	Combined	Lasso	1.79	3.8×10^{-4}	0.58
11	HIPPO	Combined	KNN	1.11	0.51	0.52
12*	**Apoptosis + NOTCH**	**GPM + Combined**	**RF**	**2.54**	**2.3×10^{-8}**	**0.61**

*Boldface indicates the best results. Hyperparameters for above models are provided in Supplementary S1 TableC.

Table 4. Prognostic index (PI) based risk stratification. The table shows the results for each pathway and corresponding gene set used. Patients with PI less than the median cutoff are at lower risk than patients with PI greater than cutoff.

| S. no.	Pathway	Gene set	HR	p-val	C
1	NRF2	GPM	1.87	1.2×10^{-4}	0.58
2	P53	Combined	2.20	1.5×10^{-6}	0.61
3*	**Apoptosis**	**GPM**	**2.52**	**3.2×10^{-8}**	**0.62**
4	WNT	GPM	1.97	3.6×10^{-5}	0.59
5	CELL-CYCLE	GPM	1.48	1.6×10^{-2}	0.57
6	PI3K-AKT	GPM	1.82	2.4×10^{-4}	0.58
7	TGF-β	BPM	1.48	1.6×10^{-2}	0.53
8	NOTCH	Combined	2.26	9.4×10^{-7}	0.60
9	MYC	BPM	1.67	1.8×10^{-3}	0.57
10	RAS	BPM	1.79	4.5×10^{-4}	0.56
11	HIPPO	Combined	1.67	1.9×10^{-3}	0.55
12*	**Apoptosis + NOTCH**	**GPM + Combined**	**2.57**	**1.5×10^{-8}**	**0.62**

*Boldface indicates the best results.
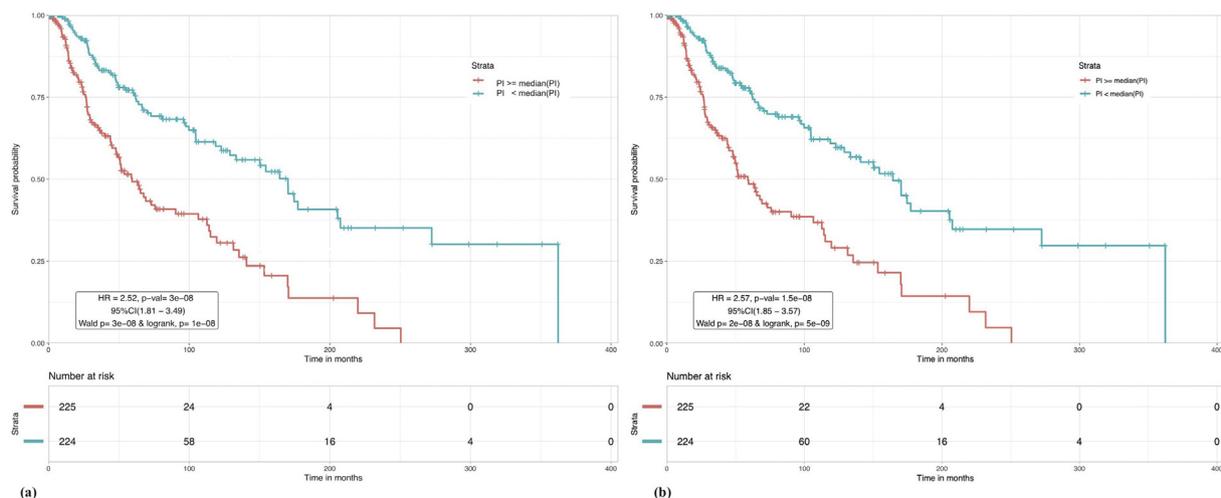
Figure 1. Kaplan Meier plot for risk stratification of CM patients. (a) Based on prognostic index of apoptotic genes. Based on GPM gene set, patients with PI $\geq$ median (PI) are at a greater risk than patients with PI < median (PI) with HR = 2.52 and p-val = 3×10^{-8}. (b) Based on prognostic index of apoptotic GPM and NOTCH combined genes. Patients with PI $\geq$ median (PI) are at a greater risk than patients with PI < median (PI) with HR = 2.57 and p-val = 1.5×10^{-8}.

dataset of 401 patients for developing ML based models. Here, we follow a similar strategy with a bigger dataset (449 patients) and additional features (TNM staging, Ulceration status), to combine clinical-features with the apoptotic GPM set, NOTCH combined set and total BPM set to see whether performance increases. New MLR models and PI based models were built with the combinations and patients were classified based on pred OS and PI in the same way as earlier. Best results based on combination with 'Breslow Thickness' are shown in Supplementary S2
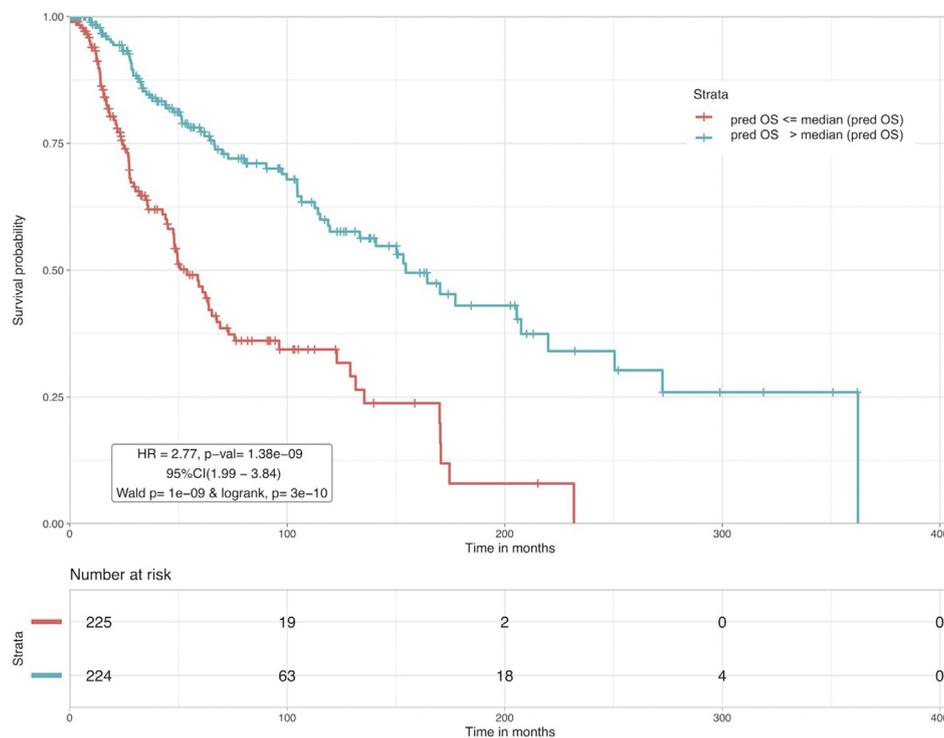
Figure 2. CM patients were stratified based on the predicted survival time (pred OS) by the 52 BPM based SVR model. Patients with pred OS ≤ median (pred OS) are at higher risk than the patients with pred OS > median (pred OS).

Table S2. Clearly, the predicted OS obtained from the SVR model based on a combination of 52 total BPM genes and Breslow thickness boosted the performance even further as compared to using BPM genes alone. An HR value of 3.19 with a p-value $\sim 10^{-10}$ and C value of 0.65 was achieved at median cutoff, which is the best amongst other combinatorial models as well as previous models. At mean cutoff, HR was 3.19, p-value $\sim 9.16 \times 10^{-10}$ and C = 0.65 (not shown in the table). KM plot illustrating the survival rates associated with high/low risk patients corresponding to this model at median cutoff is shown in Figure 3. Gene-enrichment analysis was performed on this set of 52 genes and the results are shown in Supplementary S1 TableF. A pathway enrichment corresponding to the associated proteins showed that the proteins KRT4, KRT13, KRT27 and SPRR3 are involved in the cornification process, which has a direct association with risk of skin cancer [60, 61].

3.3.3. Clinico-pathological information-based ensemble model as a superior prognostic marker

We devised a new ensemble model for integrating the prognostic potential of important clinical features. Here, entries corresponding to each clinical feature were allotted a risk point (r) as r = 1, 0 or -1 based on the risk group (high risk: r = 1, low risk: r = -1, unavailable: r = 0), according to Table 5. For example, in the feature 'Breslow Thickness', entries that were >3mm (high-risk) were given an r = 1, entries that were ≤3mm (low-risk) were given r = -1 and missing/unavailable entries were labelled with r = 0. This method ensured fixed length vectors, each of equal dimensions i.e. 449. Various linear combinations comprising of two or more features were evaluated and the best results were achieved with the combination of Breslow thickness, N staging, M staging and Ulceration status. We termed this combination as Risk Grade (RG) where RG for a patient is defined as:

RG = r (Breslow thickness) +r (N staging) +r (M staging) + r (Ulceration status)

The hazard ratio for RG was 6.40 with a p-value of 2.49×10^{-15}. Patients with RG > 1 were at higher risk than patients that had an RG ≤1, as represented by the KM plot in Figure 4. 10-year survival rate for high risk patients is seen to fall to zero whereas patients in low risk group have a 50% survival probability. Results for other top combinations are shown in Supplementary S2 Table S3. It is important to note the inferior risk stratification achieved using the AJCC 7th edition staging (Table 5) in contrast to RG. It should also be noted that even when the patients with

Table 5. Risk estimation in CM patients using clinical factors. N represents no. of samples for which data was available.

| Factor	Strata	N	HR	p value
Age	>63y vs ≤63y	449	1.83	4×10^{-4}
	continuous	449	1.02	1.9×10^{-6}
AJCC 6th ed.	Stage III, IV vs I,II	138	1.60	0.071
AJCC 7th ed.	Stage III, IV vs I,II	215	2.26	0.025
N staging	N1, N2, N3 vs N0	396	1.82	9×10^{-4}
T staging	T2, T3, T4 vs Tis, T1	378	1.68	4.8×10^{-2}
M staging	M1 vs M0	423	1.90	9.9×10^{-2}
Breslow thickness	>3mm vs ≤3mm	342	2.45	3×10^{-6}
	continuous	342	1.03	10^{-4}
Gender	Male vs Female	449	1.20	0.277
Ulceration status	Yes vs No	300	2.06	5×10^{-4}
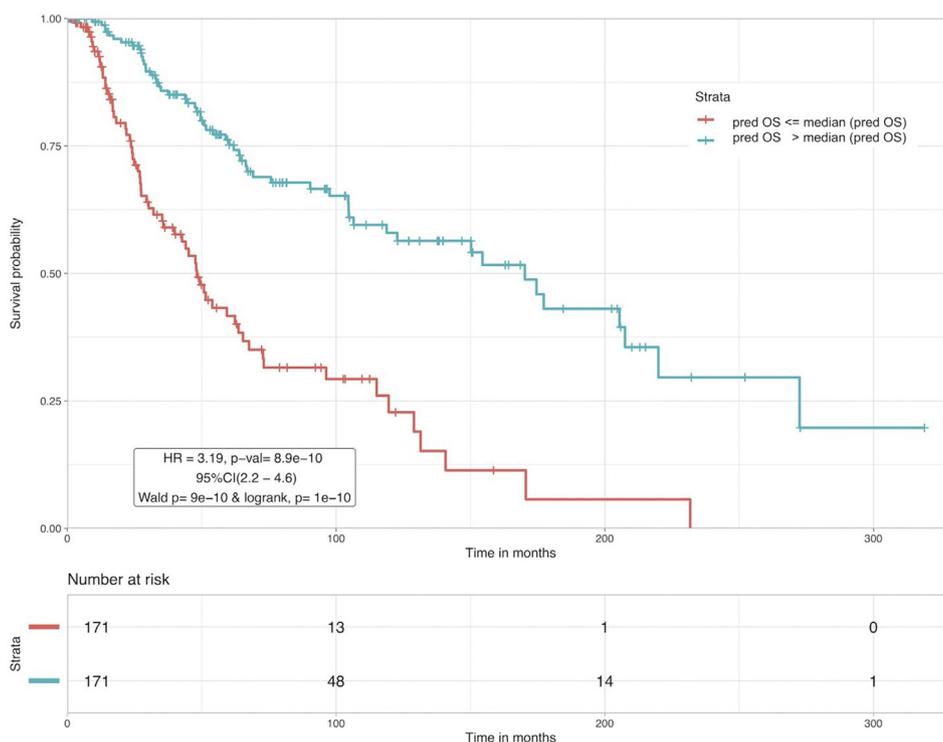
Figure 3. Kaplan Meier plots for risk stratification of CM patients based on SVR model with combination of 52 BPM and Breslow thickness as features. Patients with Pred OS $\leq$ median (Pred OS) are at a greater risk than patients with Pred OS > median (Pred OS) with HR = 3.19 and p-val = 8.9×10^{-10}.

data available for all four clinical features were taken (~259 patients), RG was able to stratify high (RG > 1) and low risk (RG$\leq$1) patients with a significant HR of 4.04 (95%CI 2.09–7.79) with a p-value of 3×10^{-5} (Wald test p-val = 3×10^{-5}, logrank test p-val = 6×10^{-6}), which is still superior than AJCC staging. High risk patients in this case had a 10-year survival probability of zero as compared to a 40% survival chance for low risk patients.

3.3.4. Comparative validation of 'Risk Grade'

We performed a comparative assessment of the strength of RG as prognostic marker by employing a popular melanoma survival prediction model [62]. To do this, we used our dataset's features as input to the web-server "AJCC individualised melanoma patients outcome prediction tool" for prediction of 5 and 10-year survival probabilities of the patient samples in our dataset. The web-server required a total of seven input features i.e (i) whether patient had localized melanoma or regional melanoma (ii) tumour-thickness (iii) age (iv) tumor burden (v) lesion site (vi) number of nodes and (vii) ulceration status. After the queries were executed, it was able to predict survival rates for 162 patients out of 449 patients in our dataset. A pre-computed RG score based on the ensemble model for these 162 patients was used to classify these patients. Figure 5 shows the web-server predicted survival rates between two risk groups (RG > 1 or High Risk vs RG<=1 or Low Risk) in the form of a boxplot. The high risk group (n = 46) had a ~30% 5-year and ~20% 10-year mean survival rate as compared to the low risk group (n = 116) with a ~65% 5-year and ~55% 10-year mean survival rate. Apart from the risk groups segregation, the strength of RG can also be implied from the minimal number of required input features i.e four features as compared to seven features in the "AJCC individualised melanoma patients outcome prediction tool" for survival prediction.

4. Web-server and mobile application

A web-server and an android application named "CMcrpred" were developed and are freely available at (http://webs.iiitd.edu.in/raghava /cmcrpred/) and Google Play Store. Users can predict the survival outcome and risk of a melanoma patient using these services. The web-server provides a detailed prediction (graphical and tabular output) about the survival outcome of a patient belonging to a specific RG. On the other hand, the android application has been kept less detailed and more user friendly for on-the-go usage by clinicians and/or patients. The web server has been designed by using a responsive HTML template for adjustment to the browsing device. Thus, our web server is compatible with a wide range of devices, including desktops, tablets, and smartphones.

5. Discussion

So far, the primary methods for the prognosis of melanoma patients include patient classification on the basis of various clinico-pathological features — following which the therapy choice is made for the majority of cases. However, alleged inadequate assessment of individual tumor prognosis by clinicopathological variables led to the need for molecular biomarkers that provide sufficient predictive value to allow personal treatment in the case of cancer. Molecular biomarkers such as S100 levels in pembrolizumab treated patients with metastatic melanoma [63], higher levels of serum tryptase levels in deeper melanomas and melanomas with ulceration [64], serum levels of vitamin D in regards to shield site melanoma vs non-shield site melanoma [65] are examples of a few recent studies. Another widely emerging class is GEP based biomarkers and combinations of these biomarkers. These genomic biomarkers are directly related to the intricacies of cancer biology and could adjunct the phenotypic staging system. To that endeavor, many studies in the past have suggested prognostic markers that could enhance the risk management in melanoma patients notably [22, 23, 24, 25, 26, 27, 28, 29]. While there has been a major interest in this area, none of the GEP based prognostic markers have been included in the AJCC staging system as of yet.

In this study, we were able to present new models for risk assessment in melanoma patients, based on OS time. We first analyzed various cancer
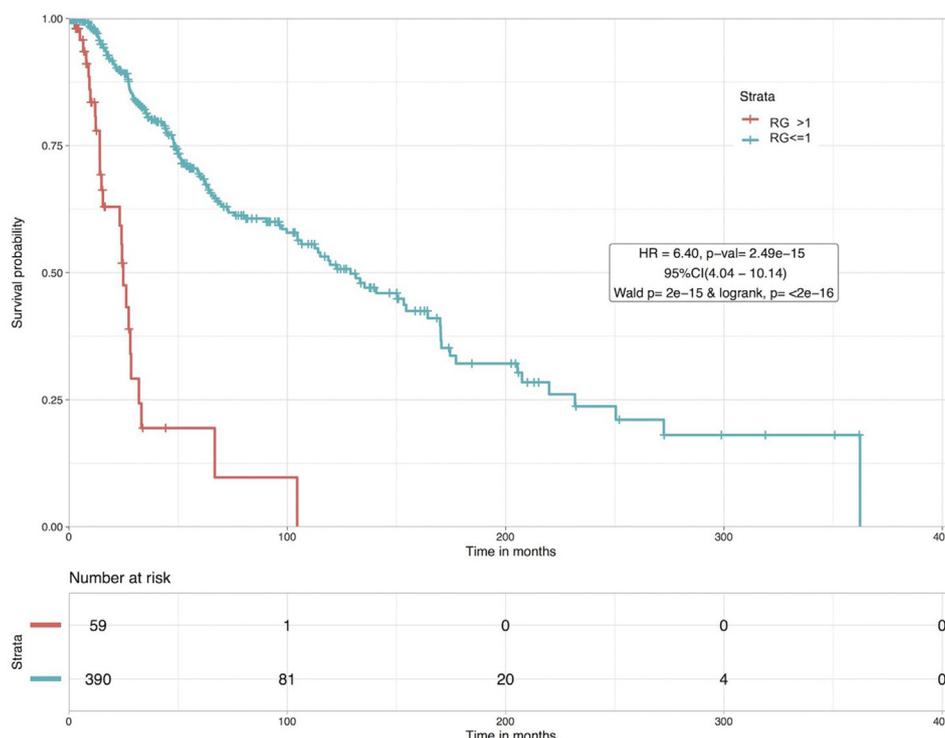
Figure 4. Kaplan Meier plot for risk stratification of CM patients based on Risk Grade (RG). Patients with RG > 1 are at a greater risk than patients with RG ≤ 1 with HR = 6.40 and p-val = 2.49×10^{-15}.

pathway associated genes and showed that a PI constructed with the GEP of 29 apoptotic pathway linked genes could be used for risk prediction in melanoma. These genes mainly encode for proteins that are directly related to promoting cell death and are under-expressed in tumor cells. Examples include MCL1, XIAP, FAS, FASLG, Caspases [3, 7, 8], which are key players in the intrinsic and extrinsic apoptotic pathway [66, 67]. The PI for these 29 genes was able to significantly discriminate between high and low-risk patients by a 2.5 hazard ratio. Another model employed the genes involved in both Apoptosis and NOTCH pathways and increased

the HR, though only by a marginal amount. The strong biological roles of these pathways in the general case of cancer are well established [68, 69, 70, 71]. Further, a popular feature selection method, rfSRC was used to filter out important survival associated genes, which were then used to make MLR and PI-based models. We show that OS time predicted by using GEP of a set of novel 52 genes was able to stratify high and low-risk patients with a higher HR (2.77) than 29-apoptotic genes based PI. The PI-based on these 52 genes also performed better than the 29-apoptotic genes based PI. By integration of this 52 GEP and Breslow thickness in
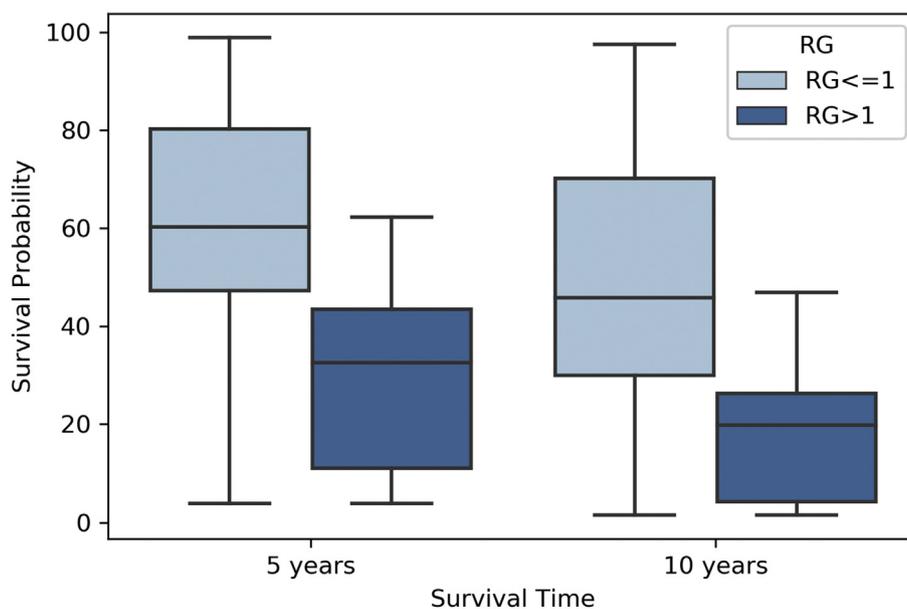

Figure 5. Boxplot representing the distinct segregation of risk groups by RG on the basis of 5- and 10- year survival outcomes predicted by "AJCC individualised melanoma patients outcome prediction tool". A total of 162 predictions were made using the tool out of which 116 were low-risk patients (RG<=1) and 46 were high-risk (RG > 1).

an SVR based regression model, we were able to enhance the risk classification further (HR = 3.19).

Further, to assess the comparative strength of the clinico-pathological features against the GEP based prognostication, we developed models with combinations of two or more clinical features. To achieve this, we employed a simple categorization method, which helped to avoid data loss and consequently guaranteed a bigger dataset. "Risk Grade (RG)" built on the information from N stage, M stage, Breslow thickness, and Ulceration status of a patient was shown to stratify high and low-risk patients with an HR value almost two-fold (HR = 6.40) of the 52 GEP and Breslow thickness-based model. Though, the efficacy of RG was also shown for a subset of 259 patients where data for every clinical feature was present. The strength of this risk segregation scheme can be compared with the current AJCC staging. While in the conventional AJCC staging criteria, T stage encompasses the information about Breslow thickness and Ulceration, here we constructed RG with the explicit use of both of these features. It is also necessary to point out that even though we have shown the performance of RG in comparison to AJCC 7th and 6th editions, it is bound to perform equally well when compared with 8th edition, since the major change in AJCC 8th edition was within the substages (primarily in stage III sub staging) [16]. Briefly, as compared to using any of the GEP based methods, the number of required prognostic features was reduced to a minimum, with a huge improvement in risk stratification. Although these models are based on features that are already involved in the AJCC staging system, an integration of these features, such as the one done in this study, has been missing in the previous studies. We exhibit the strength of using the clinico-pathological features over the molecular biomarkers by means of combinatorial models. We also show by making a comparative assessment with an existing survival prediction tool that our model which employs information about only four clinical features displays both risk segregation and survival prediction ability. Clearly, there needs to be more development and integration of prognostic biomarkers, such as the one presented here, for therapeutic decision-making in melanoma. To promote this concept further and facilitate the scientific as well as clinical community, we have also developed a web-server (http://webs.iiitd.edu.in/raghava/cmcrpred) and an android application "CMcrpred", based on this model.

6. Limitations of the study

The *in-silico* analysis presented here was performed by employing a recent RNAseq dataset obtained from TCGA which contained clinical as well as bio-specimen information. To the best of our knowledge, only publicly accessible free dataset which contains all the clinical features (i.e Breslow Thickness, N staging, M staging and Ulceration status) that are required as input to our model, is the TCGA-SKCM cohort. Though, we have used cross-validation techniques and comparative analysis with another prediction tool to assess the risk prediction models, an independent validation on external dataset is crucial for the application of the model in a clinical setting. Absence of such an external validation dataset and constraints within TCGA dataset are major limitations of this study and thus demand future efforts.

Declarations

Author contribution statement

Chakit Arora: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Dilraj Kaur: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Anjali Lathwal: Contributed reagents, materials, analysis tools or data.

Gajendra P.S. Raghava: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Competing interest statement

The authors declare no conflict of interest.

Additional information

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