Gene expression profiling in multiple myeloma – reporting of entities, risk and targets in clinical routine

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Running title: GEP-reporting in myeloma
Key words: Gene expression profiling, multiple myeloma, molecular entities, risk adapted treatment, targeted therapy

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Statement of Translational Relevance

Multiple myeloma is an example for a cancer entity characterized by a pronounced molecular heterogeneity, transmitting into survival times ranging from months to over 15 years. Gene expression profiling is an ideal tool to assess this heterogeneity in terms of gene expression based biological classifications and prognosis and allows assessment of target genes. However, it is almost never used prospectively in clinical routine, mainly due to the lack of an academic reporting tool giving quality controlled, validated, and clinically digestible information.

We present here a gene expression based report (GEP-R). The GEP-R is a customizable open-source software that can be adapted to novel parameters or other cancer entities. It includes molecular classification, risk stratification, and assessment of target gene expression. Furthermore, the HM-metascore combines validated conventional and expression based risk factors into one superior metascore, thus helping to overcome the confusing and increasing multitude of prognostic factors.


Abstract

Purpose

Multiple myeloma is an incurable malignant plasma cell disease characterized by survival ranging from several months to over 15 years. Assessment of risk and underlying molecular heterogeneity can be excellently performed by gene expression profiling (GEP), but its way into clinical routine is hampered by the lack of an appropriate reporting tool and the integration with other prognostic factors into a single “meta” risk stratification.

Experimental Design

The GEP-report (GEP-R) was built as an open-source software developed in R for gene expression reporting in clinical practise using Affymetrix microarrays. GEP-R processes new samples by applying a documentation-by-value strategy to the raw-data to be able to assign thresholds and grouping-algorithms defined on a reference cohort of 262 multiple myeloma patients. Furthermore, we integrated expression-based and conventional prognostic factors within one risk-stratification (HM-metascore).

Results

The GEP-R comprises i) quality control, ii) sample identity control, iii) biological classification, iv) risk stratification, and v) assessment of target genes. The resulting HM-metascore is defined as the sum over the weighted factors gene-expression based risk-assessment (UAMS-, IFM-score), proliferation, ISS-stage, t(4;14), and expression of prognostic target-genes (AURKA, IGF1R) for which clinical grade inhibitors exist. The HM-score delineates three significantly different groups of 13.1, 72.1 and 14.7% of patients with a 6-year survival of 89.3, 60.6 and 18.6%, respectively.

Conclusion

GEP reporting allows prospective assessment of risk and target gene expression and integration of current prognostic factors in clinical routine, being customizable regarding novel parameters or other cancer entities.
Introduction

Multiple myeloma is an incurable malignant disease of clonal plasma cells which accumulate in the bone marrow causing clinical signs and symptoms related to the displacement of normal hematopoiesis, bone marrow neovascularization, formation of osteolytic bone lesions, and production of monoclonal protein (1, 2). On a molecular level, multiple myeloma is characterized by a pronounced heterogeneity in terms of chromosomal aberrations and altered gene expression (2-5). This molecular heterogeneity is thought to transmit into survival ranging from months to 15 years or more (6-8). Within this time, almost all myeloma patients relapse, necessitating a careful planning of subsequent treatment regimen, and an appropriate selection of (further) compounds to be used, e.g. as an individualized or add-on-treatment.

Gene expression profiling (GEP) is an ideal tool to assess this molecular heterogeneity in terms of gene expression based classifications of distinct biological entities (9-13). Importantly, prognostic groups with highly different survival have been defined, namely using the gene expression based risk scores of the University of Arkansas for Medical Sciences (UAMS) and the Intergroup Francophone du Myélome (IFM) as well as a gene expression based proliferation index (GPI) (14-16). Moreover, gene expression profiling allows the assessment of target gene expression, e.g. aurora kinase A (AURKA), fibroblast growth factor receptor 3 (FGFR3), insulin like growth factor 1 receptor (IGF1R) (3, 5, 9), for which clinical inhibitors are available (e.g. VX-680, CHIR-258, NVP-AEW541) (3, 5, 17) and potential targets of vaccination strategies (e.g. MAGE3) (18, 19). GEP can currently be performed in about 80% of therapy requiring patients in terms of available material, i.e. purified myeloma cells. It has been performed on large cohorts of patients within clinical trials, but except in a very limited number of cancer centers, only retrospectively. This is surprising in that the prerequisites, i.e. purification of malignant plasma cells, can be performed at most academic institutions (20). The same holds true for gene expression profiling using core facilities or commercial service providers. Thus, the main difficulty
remaining for prospective use of GEP in clinical routine is an affordable (i.e. academic) reporting tool giving quality controlled, validated, and clinically digestible information that can be used by clinicians without extensive bioinformatics training. We present here such a gene expression based report (GEP-R) including molecular classification, risk stratification and assessment of target gene expression. Within the GEP-R, an implemented meta-score integrates current expression based and conventional prognostic factors into one superior prognostic classification. The GEP-R is a non-commercial software framework adaptable to other cancer entities that can be downloaded at http://code.google.com/p/gep-r. A “LiveDVD” for testing without the need to install the program is available at https://gliserv.montp.inserm.fr (see Results for details).
Materials and Methods

Software design. The GEP-R software is developed within the open source software environments R (21) and Bioconductor (22) using a graphical user interface (GUI; see Supplementary Fig. S1) based on the gWidgets package (23). It processes a single Affymetrix U133 Plus 2.0 .CEL-file using a “documentation by value (docval)-strategy” (24) modified for GC-RMA preprocessing (available at http://code.google.com/p/gep-r). This allows using preprocessing information of our reference cohort of 262 quality controlled myeloma samples (E-MTAB-372) and thus generated thresholds to be applied to a subsequent microarray, a prerequisite for prospective use of a gene expression report based on GC-RMA preprocessed data. A detailed description of the GUI and how to use it is provided in the Supplementary Materials and Methods.

Quality control. QC implemented is based on the Bioconductor packages affyQCReport (25), affyPLM (26-28), simpleaffy and yaqcaffy (29) adapted for GC-RMA preprocessing and PANP-based (30) assessment of presence/absence of gene expression. To limit computation time, six samples from the reference cohort have been chosen randomly as QC-reference.

Sample validation. Using prediction analysis for microarrays (PAM; (31)) predictors for sex, the IgL- (lambda and kappa) and IgH-type (IgA, IgG, IgD) have been implemented using one part of the reference cohort as training set and the other one as test set. For further validation, the UAMS total therapy 2 (TT2-) cohort (GSE24080) and the Multiple Myeloma Research Consortium data (MMRC; www.broadinstitute.org/mmgp) were used.

Risk stratification. Two gene expression based risk scores and a risk delineating GPI are implemented: i) The IFM 15-gene model (15) developed on one-channel DNA Unité Mixte de Génomique du Cancer (UMGC) microarrays transferred to respective genes represented on Affymetrix U133 Plus 2.0 arrays. Cutoffs for low/high risk have been generated on the reference cohort defining the 25% of patients with the highest risk score as high risk, showing comparable results to the original publication (15). ii) The UAMS 70-gene-risk-score was
calculated as published on MAS5 preprocessed data using published cutoffs (14) iii) The GPI(16) with cutoff-values for low/medium/high risk was re-generated on the reference cohort. The attribution of the TT2-cohort to the respective risk stratification can be found in Supplementary Table S1.

**Assessment of gene expression.** Expression height and presence/absence of expression by PANP (30) is assessed for i) potential “target genes” for whose products clinical grade inhibitors exist (currently AURKA, FGFR3 and IGF1R), ii) potential vaccination targets, and iii) genes frequently aberrantly (i.e. not expressed in normal plasma cells) or differentially expressed in myeloma (i.e. expression value greater/lower than the respective value from normal plasma cells ± three standard deviations). In the report’s appendix, values are put in comparison to normal plasma cells (n = 10) and myeloma cell samples (n = 262; Supplementary Fig. S2).

**Gene expression based classification of myeloma.** Three gene expression based classifications of multiple myeloma are assessed: i) The TC-classification by Bergsagel et al. (10, 11) derived on Hu95Av2 DNA-microarrays (Affymetrix) using MAS5-preprocesion was implemented on U133 2.0 (Affymetrix) DNA-microarrays as described by Chng et al. (32). ii) The molecular classification of multiple myeloma (9). We first performed an unsupervised clustering of the reference cohort based on the 700 genes published (being de facto 687 genes). The resulting 7 clusters could be attributed to the 7 groups of the molecular classification. A “myeloid group” could not be delineated (see Results). A PAM-based predictor using these 687 genes was calculated on 162 patients of the reference cohort and validated on 100 patients, respectively. iii) The EC-classification (12) was re-implemented on the reference cohort and assessed by a PAM predictor consisting of 188 genes. The attribution of the TT2-patients to the respective gene expression based classifications can be found in Supplementary Table S1.
Chromosomal aberrations - presence of t(4;14). The presence of a translocation t(4;14) has been assessed by iFISH as previously published (16) and a PAM-based predictor using parts of the reference cohort as training set and the other part as test set has been generated (36 genes).

Calculation of the HM-metascore. The calculation of the HM-metascore is described in the Results section (see below).
Results

**GEP-R.** After input of patient information (e.g. sex, IgH/L-type), the GEP-R software performs MAS5 and GC-RMA preprocessing as detailed above and subsequently applies i) quality control of gene expression data, ii) sample validation, iii) gene expression based classification, iv) risk stratification, and v) target assessment. The physician preparing the report comments respective findings within the report in an editable text field. A PDF-file containing the final report is generated by the software using the Sweave package (33). The GEP-R consists of two parts: i) the report given to the treating physician (Fig. 1), and ii) an appendix containing detailed quality control and validation information, as well as details of the assessment of gene expression (Supplementary Fig. S2). Analyzing a .CEL-file with GEP-R takes about 20 minutes on a standard computer (2+ GB RAM required). The GEP-R runs on Linux, MacOS and Windows systems. Optionally, results and gene expression data can be written to a PostgreSQL database using PostgreSQL through pgUtils (34) and maDB (35) packages, provided a running postgresQL database. For metascore-generation (HM-score, see below), the patient’s ISS-stage needs to be entered.

**Description of the graphical user interface.** The GEP-R graphical user interface (GUI) is divided into two parts (Supplementary Fig. S1). **Left Part.** Within the left part of the GUI, the user 1) loads an Affymetrix U133 Plus 2.0 raw-file into the software, starts the analysis, can save or load previous analyses, and creates the GEP-R as a PDF-document. The user enters 2) patient and 3) sample specific data (e.g. patient name, IgH-/IgL-type) necessary for identification and validation. Afterwards, the .CEL file is analyzed by the GUI calling a R-script performing MAS5 and GC-RMA preprocessing using preprocessed information of the reference cohort. The user here 4) comments the parameters within the report and 5) includes a concluding statement. **Right part.** Here, the results of the analysis are presented in three tabs for 1) parameters analyzed (e.g. risk scores), 2) quality control parameters and plots, and 3) identity control parameters that appear if entered patient data do not match predicted values.
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(e.g. IgH-type), or a data field misses an entry (Supplementary Fig. S2-3). A detailed description of the GUI and how to use it is included in the Supplementary Materials and Methods.

**Exemplary report.** An example of a GEP-R (final PDF-based patient report given to clinicians and patients) is depicted in Fig. 1, the full report including the appendix in Supplementary Fig. S2.

**Quality control.** Quality control (QC)-metrics assessed are average background, percent present calls as detected by the PANP-algorithm, 3’, mid and 5’ expression of β-actin and GAPDH. Six plots allow the assessment of reproducibility, QC-statistics, spike-in performance, normalized unscaled standard error (NUSE) and relative log expression (RLE), pseudo images, e.g. allowing the detection of artifacts, and RNA degradation (Supplementary Fig. S3). Supplementary Table S2 depicts samples from the initial cohort of 280 patients failing these criteria. In all, 18 samples (6.4%) failed in at least one criterion and were excluded. One criterion was failed by 10 (3.5%), two criteria by additional 4 (1.4%) and three or more by four (1.4%) further samples, respectively.

**Validation.** The GEP-R software compares predicted sex, IgH- and IgL-type with user entered patient information and gives a warning in case of inconsistency. For prediction errors see Supplementary Table S3. Predictors were validated on the UAMS TT2 cohort and the MMRC data set (Supplementary Table S3).

**Risk stratification.** Using the GEP-R based assessment, the gene expression based risk scores of the UAMS and IFM significantly delineate a high-risk population of 29.2% and 22.9% of myeloma patients, respectively. The same holds true for the GPI significantly delineating 6.3%, 47.9% and 45.8% of patients as high, intermediate and low risk (Supplementary Fig. S4). The risk stratification according to the UMAS 70-gene score for the UAMS patients is available as Supplementary Table S1.
Assessment of target gene expression. On our reference cohort, the assessed target genes AURKA, IGF1R and FGFR3 are expressed in 29.3, 28.2 and 13.4% of myeloma samples, respectively. Expression of each gene is significantly associated with adverse survival on our cohort (Supplementary Fig. S4). For the reporting, see Supplementary Fig. S1-2.

Gene expression based classification of myeloma. The TC-classification is implemented as suggested by the authors (32) (see above). Grouping is validated by published data of the TT2-dataset (Supplementary Table S1). The EC-classification implemented as stated above can be predicted with an overall error rate of 3% (Supplementary Table S3B). In contrast, grouping according to the “molecular classification” proposes a problem as there is no clear delineation algorithm of the so-called “myeloid group” published. Nevertheless, the molecular classification (7 groups, without the “myeloid group”) can be predicted with an overall error rate of 11%. For details, see Supplementary Table S3B. The overlap of our prediction with the grouping according to Zhan et al. (9) is visualized in Supplementary Table S1.

Chromosomal aberrations - presence of t(4;14). The presence of a translocation t(4;14) can be assessed using a PAM-based predictor without error (Supplementary Table S3C). Its expression is significantly associated with adverse prognosis (Supplementary Fig. S4).

Building a meta-score including current prognostic information. To integrate depicted expression-based and clinical prognostic factors within one prognostic (meta-) score (HM-score), a weight of 0, 0.5 or 1 is given for each of the named prognostic factors. The HM-score is calculated as the sum over these weights for gene-expression based assessment of risk (0, 0.5, 1 for none, one, or both of the UAMS- and IFM-scores depicting high risk, respectively), proliferation (0, 0.5, 1 for GPI low, median, or high, respectively), ISS (0, 0.5, 1 for ISS-stage 1, 2, and 3, respectively), t(4;14) (0 for absence, 1 for presence of the aberration), and expression of prognostically relevant target genes (0 or 1 if none or at least one of the genes AURKA or IGF1R is expressed, respectively). The model delineates three significantly different groups of 13.1, 72.1 and 14.7% of patients with a HM-score of 0
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(none of the risk-factors), 0.5 - 3, and >3, and a 6-year survival of 89.3, 60.6 and 18.6% of our patients (HM-cohort), respectively. It is likewise prognostic for event-free survival (Fig. 2). The HM-score is validated using published information on the respective parameters on the TT2-cohort (Fig. 2) (3, 5, 16).

**Downloadable version and files.** The GEP-R software is open-source and can be downloaded at [http://code.google.com/p/gep-r](http://code.google.com/p/gep-r). The source code can be freely modified and customized to user requirements. The GEP-R is currently validated for use of Affymetrix gene expression data obtained by double amplification protocol. A “LiveDVD” for direct testing of the report containing three exemplary *.CEL-files of myeloma patients is available at [https://gliserv.montp.inserm.fr](https://gliserv.montp.inserm.fr) (user: gepr, password: review).
Discussion

In this article, we introduce a reporting tool (GEP-R) allowing automated interpretation of Affymetrix U133 Plus 2.0 gene expression profiles. It enables the prospective use of GEP within clinical routine in terms of molecular classification, risk stratification, and assessment of target genes. This represents the critical first step in a process that will eventually allow for reproducible approaches and a standardization of the analyses across different centers as well as for personalized treatment.

In 2009, the International Myeloma Working group stated: “Our group also recognizes that the greatest prognostic ability for multiple myeloma resides in the comprehensive analysis of GEP. At a minimum, all clinical trials should consider incorporation of GEP into the correlative science studies to identify subgroups of high-risk disease. We also propose that methodology to include GEP into the clinical testing is urgently needed and methods for implementation should be identified.” (16). In the following, we discuss use, interest in, and clinical applicability of GEP and the GEP-R, respectively.

Three gene expression based classifications delineate molecular groups in myeloma: The “molecular classification” based on differential gene expression in which three of seven groups (“proliferation”, MAF-expression, MMSET-overexpression) show different survival (9); the TC-classification based on translocations and D-type cyclin without prognostic relevance (10, 11) and the EC-classification based on chromosomal aberrations and resulting changes in gene expression with only one of four groups (t(4;14) and FGFR3-expression) showing adverse prognosis (12, 13). Biological classifications likely remain relatively stable in contrast to prognostic factors prone to change with different treatment schedules (see below).

The translocation t(4;14) represents a specific disease entity and is an independent risk factor despite conventional or high-dose treatment (36-41). Treatment with bortezomib or
lenalidomide containing regimen seems to at least lessen the negative prognostic impact of this aberration (6, 42-45) and can thus be selected. t(4;14) is present in 13% of patients in our reference cohort and can be predicted without error by GEP (Supplementary Table S2C).

Gene expression based risk stratification is applied using four different strategies:

i) To investigate differences in survival between molecular groups (see above).

ii) To surrogate biological variables associated with prognosis, e.g. here proliferation as strong adverse prognostic factor in myeloma (46-49) by GPI (16) (Supplementary Fig. S4).

iii) To build a score over a set of genes associated with survival, exemplified by the high-risk scores of the UAMS (70 genes) and the IFM (15 genes) (14, 15). Both scores allowed delineating a group of patients (13% and 25%, respectively) with very adverse prognosis in the IFM and total therapy 2-dataset (not including bortezomib), whereas in the total therapy 3-cohort (including bortezomib) only the UAMS-score remains significant (14, 15). In relapsed patients treated with bortezomib within the APEX, SUMMIT and CREST trial (n = 188), both scores significantly delineate different outcome, in patients treated with dexamethasone (n = 76), only the UAMS score. In our cohort of patients, 32.4% and 25.2% of patients are delineated to be of “high risk”. As implemented in the GEP-R, two groups of patients with significantly different survival can be delineated on our cohort (Supplementary Fig. S4).

iv) To assess the presence of expression of a gene representing a potential target and investigate its prognostic relevance, exemplified by AURKA (3), IGF1R (5) and FGFR3 (12, 13) in the current version of the GEP-R (Supplementary Fig. S4). As these genes are only expressed in 13 - 28% of patients, they are ideal targets for personalized treatment, e.g. add-on for current induction treatment regimen, and respective clinical trials are in preparation. GEP likewise allows the screening for a large number of immunotherapeutic targets, e.g. cancer testis antigens (18, 50), exemplified by MAGEA3 (18, 19). In both cases GER-R easily allows assessment of expression of such target genes thus enabling personalized treatment.
A sufficient number of malignant plasma cells for GEP at a purity of 80%, i.e. $5 \times 10^4$ - $10^5$ cells if a double amplification protocol and Affymetrix U133 2.0 arrays are used, can be obtained in about 80% of therapy requiring myeloma patients at most academic institutions (20), as can be GEP. As global GEP allows simultaneous assessment of (almost) all genes without any need for pre-selection, novel risk stratifications and target genes as well as predictors for response can be applied using existing expression data and easily be included in the GEP-R software framework.

A pressing practical problem for clinical risk assessment is how to integrate the various risk factors into a single clear message given to physicians and patients. The HM-score here combines main current expression-based and conventional prognostic factors and prognostically relevant targetable genes reported in the GEP-R delineating three groups of patients with highly significantly different event-free and overall survival (Fig. 2).

Therefore, the GEP-R here allows overcoming the main difficulty remaining for prospective use of GEP in clinical routine: an affordable reporting tool giving quality controlled, validated, and clinically digestible information that can be used by clinicians without extensive bioinformatics training. It is used by the Universities of Heidelberg and Montpellier in clinical routine as well as within the GMMG-MM5 multicenter trial of the German-Speaking Myeloma Multicenter group (GMMG) and will be validated prospectively in 600 evaluable patients within a multicenter trial funded by the Federal Ministry of Education and Research.

In conclusion, gene expression profiling using GEP-R allows prospective reporting of molecular classifications, risk, and therapeutic targets to clinicians in clinical routine in a digestible manner, and thus represents a major step in translational oncology. It will foster performing clinical trials using risk adapted and personalized treatment strategies.
Disclosure of Potential Conflicts of Interest and Authorship

Conflict-of-interest disclosure: The authors declare no conflict of interest.

Contribution: T. Meißner performed programming and creation of the GEP-R and participated in writing of the paper, A.S. participated in designing the research and writing of the paper; T.R. participated in the creation of the GEP-R; T.H. participated in statistical analysis and the modification of the docval-package; T. Möhler, N.K., B.K. and H.G. participated in the analyzing of the data and in the writing of the paper, D.H. designed research, wrote the paper, and participated in creating the GEP-R.

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Legends

Figure 1. Gene expression report (GEP-R) as given to referring physicians. For the complete report including validation-, and quality control-metrics as well as detailed depiction of gene expression, see Supplementary Fig. S2.

Figure 2. Prognostic value of the HM-metascore. HM-metascore combining gene expression based high risk scores, assessment of proliferation, ISS-stage, t(4;14), and expression of prognostically relevant target genes (AURKA and IGF1R). (A) Significant delineation of event-free survival within our (HM-cohort) and the TT2-cohort. (HM-cohort, P<0.001, low versus medium P=0.02, medium versus high P<0.001, TT2-cohort, P<0.001, low versus medium P<0.001, medium versus high P<0.001, respectively). (B) Significant delineation of overall survival (HM-cohort, P<0.001, low versus medium P=0.03, medium versus high P<0.001, TT2-cohort, P<0.001, low versus medium P=0.006, medium versus high P<0.001, respectively).
Gene expression profiling - report (GEP-B)

Name: Peppermint, Patty

Address: INF 350, Heidelberg, 69120, Germany

Date of birth: 02.10.1950

Clinical Diagnosis: Multiple Myeloma

Date of first Diagnosis: 09.01.2006

Clinical Data:

<table>
<thead>
<tr>
<th>IGH-type</th>
<th>IgL-type</th>
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<td>A</td>
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Report

1. Quality control passed.
2. Identity control passed.
3. Genes frequently over- or aberrantly expressed in myeloma: Cyclin D2, MMSET
4. Gene expression based classification of myeloma:
   - Molecular Classification: MS-group
   - TC Classification: 4p16-group
   - EC Classification: E22-group
5. Gene expression based risk stratification:
   - IFM high risk
   - UAMS high risk
   - GPI medium risk
6. Targets for Immunotherapy: Expression of HM1.24
7. Targets for individualized treatment: Expression of AURKA, FGFR3 and IGFR1

Conclusion

Gene expression based risk assessment. The 70 gene score of the UAMS delineates 10-15% of patients with high risk, i.e. adverse prognosis (Shaughnessy et al. 2007); the IFM-score 25% of patients (Decaux et al. 2007), and the gene expression based proliferation index 42% of patients as medium and 8% as high risk, respectively (Hoe et al. 2008). Taken together, gene expression based risk scores delineate Mrs. Peppermint as having high risk myeloma.


The patient shows the presence of a translocation t(4;14) frequently associated with adverse prognosis. Current clinical data suggest the negative prognostic impact of this translocation seems to be at least in part overcome by lenalidomide or Bortezomib containing treatment regimen (Reese et al. 2005, Aver-Loiseau et al. 2010).

Integrating prognostic information from the ISS-score and GEP-based risk assessment (HM-meta-score), Mrs. Peppermint shows high-risk myeloma.

Targets for Immunotherapy. Cancer testis antigens are frequently expressed or overexpressed in malignant plasma cells (Condamines et al. 2007, 2009). Of the tested antigens CTAG1, HML124, MAGEA1, MAGEA3, MUC1 and SSX2, malignant plasma cells of Mrs. Peppermint only express HML1.24. This antigen could act as vaccination target depending on ow cytometric validation.

Targets for individualized treatment. Depending on clinical situation and treatment response, additional treatment with inhibitors of Aurora-Kinase, IGFR1, and FGFR3 could be considered.

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69120 Heidelberg

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Figure 2.

A

EFS HM-group

- Low risk
- Medium risk
- High risk

P < 0.001

B

OS HM-group

P < 0.001

EFS SH-group

- Low risk
- Medium risk
- High risk

P < 0.001

OS SH-group

P < 0.001
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