The Role of Fluorescence in Situ Hybridization and Gene Expression Profiling in Myeloma Risk Stratification

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SUMMARY

Multiple myeloma patients' survival under treatment varies from a few months to more than 15 years. Clinical prognostic factors, especially beta2-microglobulin (B2M) and the international staging system (ISS), allow risk assessment to a certain extent, but do not identify patients at very high risk. As malignant plasma cells are characterized by a variety of chromosomal aberrations and changes in gene expression, a molecular characterization of CD138-purified myeloma cells by interphase fluorescence in situ hybridization (iFISH) and gene expression profiling (GEP) can be used for improved risk assessment. iFISH allows a risk stratification with presence of a translocation t(4;14) and/or deletion of 17p13 being the best documented adverse prognostic factors. A deletion of 13q14 is no longer considered to define adverse risk. Patients harbouring a t(4;14) seems to benefit from a bortezomib- or lenalidomide containing regimen, whereas patients with deletion 17p13 seem only to benefit from a high dose therapy approach using long term bortezomib (in induction and maintenance) and autologous tandem-transplantation as used in the GMMG-HD4 trial, or the total therapy 3 concept. Gene expression profiling allows the assessment of high risk scores (IFM, UAMS), remaining prognostic despite treatment with novel agents, and prognostic surrogates of biological factors (e.g. proliferation) and (prognostic) target gene expression (e.g. Aurora-kinase A). Thus, assessment of B2M and ISS-stage, iFISH, and GEP is considered extended routine diagnostics in therapy requiring multiple myeloma patients for risk assessment and, even now, to a certain extent selection of treatment.

Keywords: multiple myeloma; prognostic factors; FISH; GEP

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, formation of osteolytic bone lesions, and production of monoclonal protein [1]. Multiple myeloma cells harbour a high median number of chromosomal aberrations [2, 3] and multiple changes in gene expression compared to normal bone marrow plasma cells [4-9]. This molecular heterogeneity is thought to transmit into very different survival times ranging from a few month to 15 or more years [10], with a median survival after conventional treatments of 3–4 years and 5–9 years after high-dose treatment (HDT) followed by autologous stem-cell transplantation (ASCT) [11]. Within this time, almost all myeloma patients relapse, necessitating a careful planning of subsequent treatment regimen, and an appropriate selection of compounds to be used.

Why should risk stratification be performed in multiple myeloma? The main reason for risk stratification is the high variability in survival of therapy requiring myeloma patients as stated above. It is of very high importance for patients and treating physicians to have an appropriated estimation of the expected survival time, with regards to the patients life planning and willingness to undergo risk-prone treatments, e.g. allogeneic stem cell transplantation. Two main approaches are currently used for risk stratification: i) easily assessable clinical prognostic factors (e.g. B2M, ISS-stage) [12, 13, 14] and ii) molecular diagnostics, using a) chromosomal aberrations as assessed by interphase fluorescence in situ hybridization (iFISH) [15-19], and b) changes in gene expression investigated by DNA-microarrays [7, 20-23] (Table 1).

Why should molecular diagnostics be performed in multiple myeloma? The two main clinical reasons are to obtain a better risk assessment (as detailed above) and an eventual help in choosing appropriate compounds or treatment regimen for a given patient. Whereas clinical prognostic factors like B2M or ISS allow a certain amount of risk stratification, they do not, i) cover the variation in survival, ii) identify (very) high risk patients, or iii) allow identification of patients that might benefit from a given treatment (personalisation of treatment) [12]. The latter is even more important, as not all patients harbour targets for current treatment, but cannot be treated with all available compounds due to side effects but also the expensiveness of novel treatments.

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Table 1. Risk stratification by conventional prognostic factors (beta2-microglobulin (B2M), the international staging system (ISS), interphase fluorescence in situ hybridisation (iFISH) and gene expression profiling (GEP)

Feature	Clinical parameters	iFISH	GEP
Power of risk assessment	++	+++	++++/+++++
Clinical consequences – risk adapted treatment	+	++	+++
Clinical consequences – personalization of treatment	No	Limited	Theoretically very high, in practise currently limited (lack of compounds)
Can the initial data be easily interpreted in clinical routine?	Yes	Yes	Yes*
Can the metadata data be easily interpreted in clinical routine?	Yes	Yes	Yes
Scope of information obtained	Very limited	Limited number of probes	Genome wide data
Information independent of necessity of pre-selection of request?	n.a.	No	Yes
Assessment of clonal heterogeneity (e.g. subclones) possible?	n.a.	Yes	No
Number of cells needed for analysis	n.a.	10000**	100000**
Cost	Minimal	Moderate****	Moderate****
Covered by insurance	Yes	Yes	Not yet

* provided appropriated GEP-reporting tools are used (as GEP-R); ** theoretical number lower, typical number for clinical routine;
*** therapy requiring myeloma; **** cost of iFISH and GEP are comparable and depending on number of probes tested for iFISH

What comprises molecular profiling in multiple myeloma? Molecular profiling is performed on CD138-purified plasma (myeloma) cells and routinely comprises iFISH and gene expression profiling (GEP; Table 1). It allows excellent risk stratification with at least some clinical consequences to be drawn (see below). Essential iFISH-probes comprise t(4;14) and 17p13, an extended prognostic set, also 1q21 and, as a matter of discussion, t(14;16)(q32;q23) [15, 16, 17] (Table 2). GEP of CD138-purified myeloma cells is most frequently performed with Affymetrix U133 2.0 DNA-microarrays, and can now be routinely performed in clinical practice [4, 5, 23] (Table 1).

What can molecular diagnostics be used for in current treatment paradigms and concepts? As of now, two main treatment paradigms and three main treatment concepts exist in multiple myeloma. The two main paradigms are either to i) combine all or most available compounds in an attempt to achieve a molecular complete remission and thus eventually cure the patient, or ii) subsequently apply a new compound after each relapse, and thus try to treat myeloma as a chronic disease. The treatment concepts comprise: i) risk adapted strategies based on the assumption that aggressive protocols are more effective (but also more toxic and lethal), and concomitantly the willingness to take a higher risk is increased if the prognosis is bad; ii)

The concept of tailored treatment is based on the assumption that not malignant plasma cells of all patients harbour the target for current compounds, exemplified by a remission [24] induced by single agent lenalidomide or bortezomib in about one third of patients [25, 26, 27]. Using a respective compound only if the "target" or a respective surrogate marker is expressed would spare the patient the unnecessary toxicity (and expenses). A third concept combines a highly effective treatment backbone (e.g. lenalidomide, bortezomib, adriamycin, and dexamethasone (RPAD) with compounds targeting only a subfraction of myeloma cells, e.g. inhibitors of Aurora-kinase A or insulin-like growth factor 1 receptor [4, 28, 29].

PROGNOSTIC FACTORS

Conventional prognostic factors

Several clinical adverse prognostic factors have been identified in multiple myeloma at diagnosis and before initiation of treatment [30,31]. The most significant include the international staging system (ISS) based on serum albumin [12] and serum b2-microglobulin (B2M) alone [32, 33, 34]. B2M with different cut-off values remains a prog-

Table 2. Fluorescence in situ hybridisation, recommended probes. In our institution, the initial investigation comprises an IgH-split probe for the detection of any IgH-translocation. If a split is detected, subsequent testing includes t(4;14) and t(14;16).

Necessity of testing	Test	Event	Grade of evidence (prognosis)	Test frequency
Minimal required set	t(4;14)(p16;q32)	Primary	Validated in several trials (multivariate analysis)	Once
	17p13 deletion	Progression	Validated in several trials (multivariate analysis)	May be repeated
Extended set (prognosis)	t(14;16)(q32;q23)	Primary	Controversial*	Once
	1q21+	Progression	Validated in several trials**	May be repeated
Further markers (prognosis)	Hyperdiploidy***	Primary	Weak prognostic impact in several trials, almost always favourable	Once
	t(11;14)(q13;32)	Primary	Weak prognostic impact in several trials, almost always favourable	Once
	13q14 deletion	--	Prognostic in univariate analysis	May be repeated
		--	Absence of prognostic relevance in multivariate analyis	
		--	Validated in several trials	

* published data show prognosis for t(14;16) by iFISH for patients with conventional therapy but no prognostic significance in a large series of 1030 patients treated in part by aggressive treatment; for HDT only GEP-based assessment of t(14;16) shows prognostic sigificance;
** conflicting results for multivariate analysis depending on parameters investigated; *** e.g. using probes for chromosomes 5, 9, 15

nostic factor independent of addition of bortezomib (e.g. in comparison of TT2 vs. TT3) or lenalidomide to respective treatment schedules e.g. (RAD-trial, see below) [35, 36]. B2M and ISS have likewise been shown to be independent of prognostic chromosomal aberrations as detected by iFISH (especially t(4;14)(p16;q32) and deletion 17p13) and changes of gene expression [16, 18].

Chromosomal aberrations and prognosis

Metaphase Cyctogenetics

The presence of an abnormal karyotype in metaphase cytogenetics or the detection of abnormal metaphases are already associated with a shorter overall-survival [19, 37], but this method requires metaphases (i.e. proliferating cells), only applicable in about one third of patients [19]. Thus, a large part of the prognostic values seems to be carried out by the proliferation state of the myeloma cells and can thus be covered by other measures of myeloma cell proliferation (see below).

iFISH can be used for almost all myeloma patients as well as patients with monoclonal gammopathies. It allows to group myeloma according to chromosomal aberrations into different molecular groups and subsequently investigate whether different groups or the appearance of markers of disease progression correlate with different survival [16, 18, 19, 38]. A further advantage is that iFISH allows the assessment of the presence of clonal or subclonal aberrations. The main disadvantage of iFISH is the ability only to allow investigation of a limited set of pre-selected chromosomal regions (as opposed to genome-wide assessment by array-CGH or GEP).

Several chromosomal aberrations determined by iFISH show a prognostic relevance. Among aberrations thought to be associated with pathogenesis of myeloma, (here: IgH-translocations) especially the translocation t(4;14) has shown to be of adverse prognostic value independent of conventional or HDT with conventional agents, either in univariate or multivariate analyses [15, 18, 19, 39]. Several aberrations associated with disease progression, i.e., deletion of 17p13, gain of 1q21 or deletion of 13q14 are associated with adverse prognosis in univariate analyses [18, 19, 37]. Different data are published regarding independence of these latter aberrations within multivariate analyses especially the prognostic relevance of deletions of 13q14 seem to be dependent on the simultaneous correlated presence of t(4;14) or deletion 17p13. Thus, a deletion of 13q14 as a single aberration can no longer be considered to be of prognostic significance [16, 18].

Increasing evidence suggests that the impact of prognostic factors, notably t(4;14) and deletion 17p13, are dependent on the treatment schedule, especially in terms of addition of bortezomib or lenalidomide. Barlogie et al. showed in a comparison of prognostic factors between total therapy 2 and 3, with one main difference between the treatment schedules being the long-term addition of bortezomib in TT3, t(4;14) and deletion 17p13 to be of adverse

prognostic impact in TT2 only [10]. Additional support for an independence of bortezomib activity is given by data obtained from the Vista-trial (bortezomib (Velcade), melphalan, prednisone), in which the time-to-progression and the overall survival did not show a significant difference in patients with "high risk cytogenetics" defined as presence or absence of either t(4;14), t(14;16) or deletion 17p13p [39]. In this trial, however, only 26 patients showed any of these "high risk aberrations", and no analysis for the individual aberrations was presented. Recent data from Avet-Loiseau et al. however show an independence of prognosis from t(4;14), but a remaining adverse impact of deletion 17p13 when using brotezomib containing induction treatment [40]. Recent unpublished data from our GMMG-HD4-trial testing VAD vs. PAD induction treatment followed by tandem-HDT and ASCT and either thalidomide or bortezomib maintenance showed that in the bortezomib containing treatment arm the adverse impact of deletion 17p13 was significantly reduced. Patients carrying a t(4;14) showed only a trend to better survival. The difference in the latter results compared to the French study is likely due to a worse outcome in the control (VAD) arm of the French study.

In terms of lenalidomide-treatment, deletion 17p13 remains of adverse prognostic value within the MM-016 trial (130 pts. investigated by iFISH) [41] or RAD (lenalidomide, adriamycin, dexamethasone)-treatment (in relapsed or refractory patients, 69 patients investigated) in terms of progression-free and overall survival [36,41] as well as a recent report from our group [42], whereas t(4;14) does not remain a prognostic factor. Using lenalidomide and dexamethasone in the E4A03 trial, patients with "high risk cytogenetics" (n=21/126 investigated) defined as presence or absence of either t(4;14), t(14;16) or deletion 17p13 showed an adverse outcome compared to those lacking any of these aberrations.

The t(14;16)(q32;q23) as assessed by iFISH up to now has shown to be of adverse prognosis if conventional chemotherapy is applied, but failed to show prognostic impact in a recent large retrospective analysis in 1030 patients with aggressively and non-aggressively treated patients. For HDT alone, a prognostic value has been demonstrated using GEP-based assessment (spiked maf-expression) [7, 15, 17]. Our group recently reported a worse outcome of 6 patients treated with lenalidomide compared to those not carrying the aberration [42]. Basically, due to the rarity of the translocation, the prognostic role as a single aberration is still a matter of debate [16, 17].

The gain of 1q21 (1q21+) has also been described as adverse prognostic factor in multivariate analyses, being associated with disease progression. In our own studies, it initially appeared as prognostically significant in univariate but not multivariate analysis including several other chromosomal aberrations by iFISH [16, 37]. A recent analysis of patients treated within the GMMG-HD4 trial, however, showed a prognostic relevance of greater than three copies of 1q21 maintained in a multivariate analysis.

Other aberrations including hyperdiploidy, defined by additional copies of two of the three chromosome regions

5p/5q, 9q34, and 15q22, or the t(11;14) seem to be of only weak prognostic impact [16, 18, 19].

iFISH, risk and personalized treatment

For VAD-based HDT regimen, t(4;14), deletion of 17p13 are the best documented cytogenetic risk factors, being independent of conventional factors like B2M and ISS-stage and thus carry orthogonal information. Gains of 1q21 are probably associated with high risk, whereas the impact of t(14;16) remains controversial.

Taken all together, patients harbouring a t(4;14) seem to benefit from a bortezomib- or lenalidomide containing regimen, whereas patients with deletion 17p13 seem only to benefit from a HDT approach using long-term bortezomib (in induction and maintenance) and autologous tandem-transplantation as used in the GMMG-HD4 trial, or the TT3 concept.

Array Comparative Genomic Hybridization

Array based comparative genomic hybridization (aCGH) is a complementary analysis to iFISH as it allows the assessment of copy number changes at millions of chromosomal sites (e.g. Affymetrix 2.7M arrays – 2.7 million sites). aCGH allows delineation of different pathogenetic and prognostic groups [43,44]. aCGH, however, does not allow the determination of subclonal aberrations or balanced translocations.

Gene expression profiling and prognosis

GEP is performed on CD138+ purified myeloma cells. It has the advantage that expression of (almost) all genes can be assessed simultaneously without the need of a pre-selection of interesting genes or regions, if iFISH is applied. GEP (e.g. using Affymetrix U133 2.0 arrays) can now be used in clinical routine in approximately 80% of therapy requiring myeloma patients. GEP, however, does not allow the assessment of tumour clone heterogeneity.

Risk stratification by gene expression profiling is applied using four different strategies: i) grouping multiple myeloma into "molecular groups" (entities) subsequently investigating differences in survival between these groups; ii) assessing (high) risk based on association of gene expression with survival; iii) assessing expression of a gene representing a potential target and to investigate its prognostic relevance; and iv) assessing surrogates of biological variables and their respective prognostic relevance.

Of the first, four proposals have been made to delineate etiologic groups in myeloma: A delineation of myeloma in "MGUS" or "myeloma cell line like" groups, in which groups do not show a prognostic significance [6], a classification based on differential gene expression (molecular classification), in which only some groups (e.g. "proliferation", maf-expression, MMSET overexpression) show dif-

ferent survival [7], a classification based on translocations and D-type cyclin expression (TC-classification) without prognostic relevance [20, 21], or chromosomal aberrations and resulting changes in gene expression (EC-classification) with only one group (t(4;14) and FGFR3-expression) showing adverse prognosis [23].

The second strategy comprises the high risk-scores of the UAMS (17/70 genes) and the IFM (15 genes) [22, 23] by building a score over a set of genes associated with survival. Both scores allow delineating a group of patients (13% and 25%, respectively) with very adverse prognosis in the IFM- and TT2-dataset (both not including bortezomib), whereas in the TT3-cohort only the UAMS-score remains significant in univariate analysis. Thus, the UAMS-score remains its prognostic relevance if bortezomib is added to the treatment regimen (TT2 vs. TT3) [22, 23]. In relapsed patients treated with bortezomib within the APEX, SUMMIT and CREST trial (n=188), both scores significantly delineate different outcome, whereas in patients treated with dexamethasone within these trials (n=76), only the UAMS score significantly delineates a high risk group. For initial treatment of patients using lenalidomide and dexamethasone within the E4A03-trail (n=45), both scores significantly delineate groups of high risk patients [45].

The third possibility is exemplified by the expression of Aurora-kinase A [4]. Presence of aurora-kinase A expression delineates significantly inferior event-free and overall survival in two independent cohorts of patients undergoing HDT, independent from conventional prognostic factors. Using GEP, aurora kinase inhibitors as a promising therapeutic option in myeloma can be tailoredly given to patients expressing aurora-kinase A, who in turn have an adverse prognosis.

The fourth possibility is exemplified by a GEP-based proliferation index (GPI). Proliferation of malignant plasma cells, as determined by several methods, has been shown to be a strong adverse prognostic factor [46, 47, 48, 49, 50], independent of clinical prognostic factors, e.g., B2M [50] and can likewise be assessed by gene expression based proliferation indices [7, 22, 25].

Gene expression profiling, risk and personalized treatment: GEP is feasible in extended clinical routine diagnostics. High risk scores, surrogates of proliferation (GPI) and upcoming targets (e.g. Aurora-kinase A) allow advanced risk stratification and begin to influence treatment. Importantly, GEP-based factors remain prognostic in patients treated with novel agents [45].

PRACTICAL CONSIDERATIONS

In our institution, we routinely use bone marrow aspirates of 60-80 ml which are purified by autoMACS (Miltenyi Biotec). Samples indicated for GEP with purity below 80% are subsequently FACSAria-sorted (Becton Dickinson).

iFISH necessitates the identification of plasma cells, which can be either performed by cytoplasmatic light chain staining (cIg-FISH) or CD138-purification, using beads or fluorescence activated cell sorting. In clinical routine and

depending on the number of probes investigated, about 10^3–5×10^4 plasma cells are needed despite the theoretical amount being lower. iFISH analysis is informative in ~95% of cases of therapy requiring myeloma (Table 1).

For GEP, plasma cells need to be purified and about 5×10^4–10^5 plasma cells are needed using e.g. a double amplification protocol and Affymetrix U133 2.0 DNA microarrays. A purity of at least 80% as investigated by flow cytometry (CD138/CD38) is recommended (Table 1). Although under conditions of a multicenter-trial (GMMG-MM5, 237 of 504 patients recruited), about 80% of therapy requiring patients can be assessed by GEP.

CONCLUSION

Besides B2M and ISS-stage, molecular diagnostics including iFISH and GEP massively add prognostic information and begin to influence treatment decisions, and should therefore be considered clinical routine at least in every newly diagnosed patient requiring treatment, and mandatory for patients entering clinical trials.

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Улога флуоресцентне хибридизације *in situ* и одређивања профила експресије гена у прогностичкој стратификацији мултиплог мијелома

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КРАТАК САДРЖАЈ

Преживљавање болесника с мултиплим мијеломом је веома различито: од неколико месеци до више од 15 година. Клинички прогностички фактори, посебно β2 микроглобулин (*B2M*), и међународни систем градирања (енгл. *International Staging System – ISS*) омогућавају дефинисање група различитог степена ризика, али не и препознавање болесника код којих је степен ризика највећи. Како се малигне плазма-ћелије одликују низом хромозомских аберација и различитих генских промена, молекуларна карактеризација пречишћених CD138+ мијеломских ћелија, помоћу интерфазне флуоресцентне хибридизације in situ (енгл. *interphase fluorescence in situ hybridization – iFISH*) и одређивања профила експресије гена (енгл. *gene expression profiling – GEP*) доприноси одређивању прогностичког профила ових болесника. Постојање *t(4;14)*, односно делеције *17p13* утврђено применом *iFISH* до-казан је фактор неповољног прогностичког значаја. Делеција *13q14* се више не сматра лошим прогностичким параметром. Болесници са *t(4;14)* веома повољно реагују на лечење бортезомибом или леналидомидом, док су код болесника са делецијом *17p13* потребни лечење великим дозама лека и дуготрајна примена бортезомиба, попут протокола *GMMG-HD4* или тоталне терапије 3. Одређивање *GEP* омогућава дефинисање високоризичних група (*IFM, UAMS*) и уз прогностички значајне биолошке показатеље пролиферације дефинише генске мете (нпр. Аурора-киназа А) потенцијалног дејства нових терапијских модалитета. Одређивањем *B2M* и *ISS* скора iFISH и *GEP* се сматрају обавезним дијагностичким методама у утврђивању прогностичког профила болесника с мултиплим мијеломом и даљег терапијског приступа.
Кључне речи: мултипли мијелом; прогностички фактори; *FISH*; *GEP*