Dissecting the clonal architecture of multiple myeloma

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A B S T R A C T

Plasma cell dyscrasias are characterized by accumulation of malignant plasma cells in the bone marrow with differentiation between stages by tumor mass and presence or absence of clinical signs and symptoms. The two main pathways leading to plasma cell dyscrasias are hyperdiploidy and IgH-translocations. The unifying feature is an aberrant- or overexpression of D-type cyclins. A plethora of further aberrations on DNA- and RNA-level exists without a characteristic mutation or other genetic alteration being present: clonal heterogeneity between different patients (inter-patient heterogeneity). It is also present within a single patient (intra-patient heterogeneity). In the latter case, differences can be observed *in loco aspiratio* (subclones, e.g. at presentation), as well as at different times (temporal heterogeneity) and sites (spatial heterogeneity). Understanding clonal dynamics can help in clinical decision-making, e.g. regarding re-exposure of patients *versus* a previous treatment line, and define treatment strategies, e.g. biological rationale for combination or early treatment. Sequential molecular characterization is thus indicated in plasma cell dyscrasias.

Learning goals

At the conclusion of this activity, participants should be able to:
- describe the basis for the delineation of different plasma cell dyscrasias;
- explain the basic mechanisms leading to multiple myeloma;
- understand and explain different levels of inter- and intra-patient heterogeneity;
- formulate clinical conclusions from intra- and inter-patient heterogeneity.

Plasma cell dyscrasias

Definitions

Plasma cell dyscrasias are characterized by accumulation of malignant plasma cells in the bone marrow. The earliest discernable stage is a condition termed monoclonal gammopathy of unknown significance (MGUS) that evolves to asymptomatic myeloma (AMM).[1-3] The two disease stages are delineated by surrogates of tumor mass, i.e. the diagnosis "myeloma" prerequisites a plasma cell infiltration in the bone marrow of 10% or more and/or a monoclonal (M)-protein of 30 or more g/L.[4] [The M-protein is a relative measure as it depends on the number of myeloma cells (tumor mass) times the ability to produce and deliver immunoglobulins or parts thereof, which can vary, to the greatest extreme in oligo-/asecretory myeloma.] If the plasma cell accumulation leads to clinical signs and symptoms, the disease is termed "symptomatic myeloma" and treatment is initiated. Signs and symptoms can be grouped in those related to the interaction with the bone marrow microenvironment, first Bone disease (including hyperCalcemia), and secondly those related to displacement of normal hematopoiesis (Anemia, proneness to infection), and thirdly those related to the production of monoclonal protein (Renal impairment). These signs and symptoms are "menomiced" as "CRAB"-criteria.[5,6] For the last decade, this second delineation has thus been made not directly by myeloma cell accumulation (tumor mass) but consequences thereof. In a recent revision of the IMWG-criteria, also high tumor mass (i.e. clonal plasma cell infiltration ≥60%, involved:uninvolved free light chain ratio ≥100, or >1 focal lesion in magnetic resonance imaging) are now seen as (symptomatic) multiple myeloma.[6] Asymptomatic myeloma evolves from MGUS with 5% probability and progresses to symptomatic myeloma with 50% probability in five years.[4,7] In any case, malignant plasma cells are located at the same place as their normal counterparts, i.e. in the bone marrow. Other than in solid cancers, e.g. in carcinoma the ability of transiting the basal membrane, they need not acquire additional lesions to locate in their site of accumulation.

Inter-patient heterogeneity

Basic genetic alterations leading to myeloma: hyperdiploidy and IgH translocations

Two principal pathways target plasma cell precursors during their maturation and are seen as primary events in multiple myeloma pathogenesis: hyperdiploidy and translocations involving the IgH-locus.[8]

Hyperdiploidy represents the gain of odd-numbered chromosomes, most frequently 3, 5,

7, 9, 11, 15, 19, and 21.[9] In hyperdiploid patients, frequently low aberrant expression of cyclin-D1 (CCND1), especially in case of a gain of 11q13,[10] and overexpression of cyclin-D2 (CCND2) can be found.[8]

Translocations: the five main translocations seen in multiple myeloma comprise the translocations t(11;14) in 15%-20%, t(6;14) in 2%, t(4;14) in 10%-15%, t(14;16) in 2%, and t(14;20) in 2% of myeloma patients, leading to a direct aberrant- or overexpression of *CCND1*, *CCND3*, *FGFR3* and *MMSET*, as well as *MAF*, and *MAFB*, respectively.[11-14] [Direct, i.e. targeting the respective gene by the translocation, e.g. *CCND1* on 11q13 by the t(11;14). Indirect e.g. by impacting on the signaling chain, e.g. *CCND2*-expression related to the t(4;14)]. The latter patients likewise show a CCND2-overexpression.

Unifying event: expression of D-type cyclins

From the studies mentioned above and previous work, especially by Bergsagel *et al*., a dysregulation of D-type cyclins, is seen as a unifying property and event in multiple myeloma.[15] Normal bone marrow plasma cells express *CCND2* and (arguably) *CCND3* at a low level. Malignant plasma cells aberrantly (i.e. not present in normal plasma cells) express *CCND1*, or over-express either *CCND2* or *CCND3*. Rarely, both *CCND1* and CCND2, or *CCND3* are expressed.[8,15] Patterns of D-type cyclin expression are explained by the underlying genetic alteration. The t(11;14) and t(6;14) places *CCND1* at 11q13 and *CCND3* at 6p21 under the control of the IgH-enhancer, directly leading to a high expression of either *CCND1* or *CCND3*, respectively. In contrast, *CCND2* is almost never overexpressed due to translocations involving the *CCND2* locus at 12p13, or copy number alterations; *CCND2* expression is indirect.

When do hyperdiploidy or translocations happen?

Malignant plasma cells resemble terminally differentiated bone marrow plasma cells and, in an individual patient, have undergone the same V(D)J rearrangement and somatic hypermutation,[16,17] producing the same monoclonal protein or parts thereof; in this regard they are clonal. Thus, either myeloma cells originate from a bone marrow plasma cell that (re-)acquired the ability to proliferate, or the transformation occurred earlier, when precursors still proliferated, and did not interfere with the maturation to terminally differentiated plasma cells.

For hyperdiploid myeloma, it is not known when (and how) the immortalization takes place. Translocations originate from the aberrant rejoining of DNA double strand breaks occurring at distinct sites in the genome.[18] Translocations can arise during different time points of the generation of terminally differentiated plasma cells by five mechanisms: 1) aberrant class switch recombination (CSR); 2) aberrant V(D)J rearrangement; 3) homologous recombination; 4) somatic hypermutation; or 5) receptor-revision rearrangement.[16] Investigating 61 samples with IgH-translocation, Walker *et al*. found two-thirds showing a breakpoint within the switch regions upstream of the IgH constant genes being generated via CSR in mature B cells. While in 14 samples with a t(4;14) and 5 samples with a t(6;14), all were generated through a CRS-mediated mechanism, the frequency of CRS-generated translocations in samples with a t(14;16), or t(11;14), respectively, was only 50%. In 6 of 29 samples with a t(11;14), and 1 of 4 with a t(14;20), the generation of the translocations was mediated via a D_H-J_H rearrangement. The latter occur as early as at the pro-B-cell stage in the bone marrow indicating that at least a subset of translocations in myeloma seems to appear in pre-germinal center cells.[16]

Further chromosomal aberrations

As discussed above, IgH-translocations and hyperdiploidy are seen as primary events. A high number of other recurrent aberrations are present,[12,19-26] here exemplified by the most well described deletions of 13q14 (46%), 17p13 (10%), and gains of 1q21+ (36%).[20] All three aberrations have been associated with adverse survival in symptomatic myeloma patients.[17,20,27-29] One potential explanation is that gains of 1q21 and deletion of 13q14 are associated with a higher proliferation rate.[23] It has, however, proved difficult to conclusively identify genes targeted by these aberrations. Despite there being several candidates, e.g. on 1q (*CKS1B*, *ANP32E*, *BCL9*, *PDZK1*),[30] 13q14 (*RB*, *DIS3*),[31-34] and 17p13 (*TP53*),[35,36] regularly the whole chromosomal region is affected. At the same time the indicated chromosomal aberrations very rarely appear as single aberrations,[11,12,34,37,38] and the number of adverse aberrations simultaneously present, rather than the single aberration, has been described as transmitting into adverse progression-free and overall survival.[37] Therefore, as for hyperdiploidy, the question is whether it is not a more subtle impact of copy number change with consecutive deregulation of a number of genes inside and outside the region of focus.

Same and different prognostic impact of chromosomal aberrations in asymptomatic and symptomatic myeloma

Only recently, we and others have shown that the chromosomal aberrations gain of 1q21, deletion of 17p13, translocation t(4;14), and hyperdiploidy are significantly associated with shorter time to progression from asymptomatic towards therapy-requiring myeloma.[39,40] The first three aberrations are likewise adverse prognostic factors in symptomatic patients regarding progression-free and overall survival.[17,20,27] Hyperdiploidy is different: an adverse prognostic factor in AMM-patients,[39] a positive predictive factor in symptomatic (treated) patients.[12,41] This implies first that part of the predictive impact of chromosomal aberrations is due to innate and treatment independent properties of myeloma cells (a prognostic factor in the strict sense), and a part only plays out in interaction with a specific treatment (as, for example, the case of hyperdiploid patients in whom the prognostic impact is changed by treatment, i.e. a predictive factor), and secondly, that different aberration patterns can lead to the same phenotype, in this case, faster progression.

Different molecular entities defined by gene expression profiling

Several attempts have been made to define different myeloma subentities by gene expression profiling.[15,42-45] The group of John Shaughnessy first classified myeloma samples according to their similarity to either plasma cells from individuals with MGUS or human myeloma cell lines.[46] The "molecular classification" of Shaughnessy *et*

al. is based on unsupervised clustering and prediction of clustered groups,[47] whereas the TC-classification by Bergsagel *et al*. is centered on IgH-translocations and CCND-expression as an early unifying event in multiple myeloma.[15] Gene expression profiling also allows delineating groups of 10%-25% of high-risk patients with very adverse overall survival.[23,42,48-52]

No unifying mutation in myeloma

Whole exome and whole genome sequencing approaches revealed 20-40 non-synonymous variants per myeloma cell,[31-33] i.e. higher than other hematologic malignancies such as hairy cell leukemia (n=5)[53] or acute myeloid leukemia (n=8),[54] but considerably lower than solid tumors (e.g. n=540 in non-small-cell lung cancer).[55] There is no unifying mutation in myeloma in contrast to some other hematologic malignancies where a common mutation is thought to be the primary driver, e.g. in hairy cell leukemia, (the BRAF V600E in all samples[53]) and in Waldenströms macroglobulinemia (the MYD88 L265P in 91% of samples[56]). In myeloma, the most frequent mutations are *NRAS* (23%), *KRAS* (26%), *BRAF* mutations (4%), the first 3 in ERK pathway, *FAM46C* (13%), and *TP53* (8%). The ERK pathway plays a role in 53% of multiple myeloma. Other mutations include those on chromosome 13, *DIS3* (10% mutated), being deleted in approximately half of the patients (del13q14).[31-33]

Without explicit use of the term, we have described above the presence of molecular inter-patient heterogeneity. Different genetic alterations lead to the same phenotypes of plasma cells dyscrasias; only those that do so are denominated "multiple myeloma". Further alterations are present on DNA (chromosomal aberrations, SNV), and RNA-level (changes in gene expression, different gene expression profiling-based molecular entities). Multiple myeloma is, therefore, multiple myelomas with the unifying genetic feature of leading to an accumulation of terminally differentiated plasma cells.

Intra-patient heterogeneity

Besides different individual genetic background related to the two general patterns hyperdiploidy and IgH-translocations and additional aberrations, i.e. inter-patient heterogeneity, heterogeneity also exists within an individual patient: intra-patient heterogeneity. This can be present in terms of: i) a heterogeneity *in loco aspiratio* at presentation, i.e. in the site of clinical bone marrow aspiration; ii) in terms of a *spatial* heterogeneity, e.g. a different pattern of genetic alterations in focal lesions *versus* random aspirates; and iii) temporal heterogeneity, i.e. change of the clonal composition / emergence of new subclones over time, especially evidenced under treatment.

Heterogeneity in loco *at presentation*

Several lines of evidence confirm the presence of heterogeneity *in loco aspiratio* (at the site of bone marrow aspiration). By interphase fluorescence *in situ* hybridization (iFISH), chromosomal aberrations can be present in different percentages within the malignant plasma cell population of a given patient. Whereas IgH-translocations as t(4;14) or ploidy state (hyperdiploidy) usually appear in the majority of myeloma cells, the frequency of malignant plasma cells in which a deletion 13q14 can be detected varies between 20% and 100%;[57,58] the same holds true for deletion of 17p13 or gains of 1q21.[11] If one chromosomal aberration appears in 60% or more of myeloma cells whereas another only appears in a smaller percentage of this population, a "subclonal aberration" is present.[22,26] Their appearance is a sign for an evolution of the malignant plasma cell clone, in which the subclonal aberration appeared after the clonal aberration (temporal heterogeneity; see below).[11] Using next generation sequencing and analyzing 7 serial myeloma cell samples from an individual patient at diagnosis, remission, four relapses, and ultimately plasma cell leukemia, Keats *et al*. were able to show different (sub)clones being present at myeloma diagnosis.[59] In this patient with a t(4;14), longitudinal array comparative genomic hybridization (aCGH) and iFISH analyses revealed the presence of two major clones with a dominant one being present in 72% of cells, as well as two major subclones that emerged from the first. While one major subclone was already present at diagnosis in 11% of cells, the second one seemed to have emerged at a later time point, i.e. after treatment was initiated, or was below the threshold at diagnosis. One subclone differed by only six copy number alterations (2 lost and 4 gained) from its parental clone, while the second subclone showed 13 lost and 39 gained copy-number abnormalities.[59] In agreement with this, genetic analysis of sorted single cells in patients with t(11;14) revealed the presence of 2-6 major clones at presentation.[60] Combining SNP-based array data and whole-exome sequencing, Walker *et al*. also found evidence for a clonal heterogeneity in samples of patients with t(4;14) (n=10) *versus* those presenting with t(11;14) myeloma (n=12).[61] Focusing on mutations in the RAS pathway, they found *NRAS* or *KRAS* mutations to be present in 32%-96% and 20%-72% of malignant plasma cells, respectively. Although seen as driver aberration, RAS mutations were thus not always present in the dominant clone, but only in minor fractions of the myeloma cell population, and a subfraction of patients.[31,32,61] In case a RAS mutation was present in a given sample, there was evidence for the simultaneous presence of 1 or more subclones.[61] These findings were confirmed in a larger series of myeloma patients, showing mutations often to be present in subclonal fractions only with numerous clones being predicted to be present at diagnosis.[32] Taken together, there is convincing evidence for the presence of aberrations at a subclonal level.

Spatial hetogeneity

Different bone marrow infiltration patterns of myeloma cells can be simultaneously present in the same patient,[62,63] i.e. focal lesions and/or diffuse spread over the bone marrow, tempting us to assume a connection between the existence of several subclones and different manifestation patterns, i.e. the presence of a spatial heterogeneity. Evidence is given by Zhou *et al*. comparing myeloma cells from random (pelvic) aspirates (RA) with those from computer-assisted tomography-guided fine needle aspiration from MRI-defined focal lesions (FL).[64] Using conventional karyotyping, four patterns of chromosomal abnormalities (CA) can be distinguished: 1) CA detected in both RA and FL, i.e. RA+/FL+ (n=75, 18% of patients); 2) RA+/FL- in 16% of patients (n=67); 3) RA-/FL+ in 18% of patients (n=77); and 4) absence of CA in both, RA-/FL- in 48% of

patients (n=200). Furthermore, also a discordance in CA, i.e. structural or numerical aberrations being unique to only one of the two sites, was observed in 48 of 75 patients (64%) of the first category. Regarding event-free survival (EFS) and overall survival (OS), the 75 patients showing CA in both sites (RA+/FL+) exhibited the worst outcome with 7-year estimates of 23% and 28%, respectively. The other groups had comparable EFS, while OS was inferior in patients with RS+/FL- (n=67). In agreement with a worse outcome and differences in laboratory parameters (e.g. higher LDH level in the RA+/FL+ group), 52% of patients in the RA+/FL+ group were considered high risk according to the gene expression-based risk score of the same group[42] *versus* 27% in the 49 patients with RA+/FL- *versus* 6% among the 50 patients with RA-/FL+, and 4% of the 130 patients with RA-/FL- for whom gene expression data were available.[64] This study has shown that there can be a difference. It will be interesting to gain further insight into the genetic architecture of samples from random aspirates *versus* those from focal lesions and their association with the infiltration pattern. Corresponding analyses using high-resolution techniques are currently under way.

Temporal heterogeneity

Does the composition of the myeloma cell clone at one site change over time? Treatment-associated changes

Recent data provided evidence for the presence of a temporal clonal heterogeneity. Analyzing 7 serial myeloma cell samples, as described above, Keats *et al*. were able to show different patterns of subclonal composition, i.e. subclones gained or lost dominance during the course of the disease, "clonal tides", with different treatment regimens exerting varying selection pressures on the cells.[59] Egan *et al*. conducted whole genome sequencing of germline DNA and myeloma cells at four time points during tumor evolution of the same patient at diagnosis, first relapse, second relapse, and plasma cell leukemia.[33] Fifteen single nucleotide variants (SNV) were shown to be present at all four time points investigated (*AFF1*, *ATXN1*, *COL2A1*, *CORO1A*, *CNGA3*, *CSMD3*, *LTB*, *MAGI1*, *MSL1*, *KCNIP4*, *KRT9*, *LRRC4C*, *MYPN*, *RNF145*, *TYRP1*), thus sharing a common progenitor. Six SNVs were only detectable at alternating time points, i.e. diagnosis and second relapse (*ACER1*, *C12orf42*, *DOKS*, *PARD3B*, *PPFBP1*, *ZNF557*), seven SNPs could only be observed in first relapse (*ATXN1*, *CACNA1S*, *DSC1*, *PCDH7*, *PTPRD*, *TLR9*, *TUBB6*), and seven uniquely at the last time point, i.e. plasma cell leukemia (*BIRC5*, *RB1*, *ZKSCAN3*, *SUB1*, *TNN*, *TUBB8*, *ZKSCAN3*, *ZNF521*). The first relapse and the plasma cell leukemia sample did not share any unique SNV beyond the ones observed at all four time points (see above). *PDE4DIP* was found uniquely at diagnosis.

In 28 paired samples from symptomatic patients treated with different chemotherapeutic regimens analyzed by aCGH, a median number of 23 copy number abnormalities (CNA) per sample was identified.[59] The number of CNAs increased significantly during the course of the disease with a mean of 19.7 CNAs at baseline to 26.3 CNAs detected at the second time point. A mean of 16.8 CNAs were shared between both time points indicating a certain clonal relation. Three different phenotypic patterns of temporal tumor types can be distinguished according to Keats *et al*.: 1) genetically stable tumors, especially in patients with a more favorable hyperdiploid myeloma, with no detectable CNA changes between paired samples from a given patient (35.7% of patients); 2) a linear evolutionary path in 21.4% of patients characterized by newly acquired CNAs at the second time point; and 3) a changing clonal dominance (42.9% of patients) with gains and losses of CNAs including the reappearance of bi-allelic deleted regions suggesting the existence of different subclones at diagnosis. Interestingly, patients with high-risk myeloma, i.e. presence of a translocation t(4;14), t(14;16), t(16;20), or deletion 17p13, showed significantly more CNAs over time. Presence of deletion 17p13 was also accompanied by a higher number of CNAs at the time of diagnosis.[59]

In conclusion, there is evidence that, with treatment, the subclonal composition in a given patient can shift.[59,65] Is the observed change in turn driven by treatment? The argument for this is that treatment represents a very substantial additional selection pressure, i.e. regularly killing a high proportion of myeloma cells, and thus changing the cellular composition of the bone marrow microenvironment. A different responsiveness of subclones could easily explain the change in subclonal composition. An alternate hypothesis is that there is a continuously ongoing change in the subclonal composition of the bone marrow myeloma cell population, which in turn leads to a different responsiveness. Based on current data, neither possibility can be excluded. Given the above, for us, it would seem very unlikely that the huge additional selection pressure caused by treatment does not impact on the clonal composition, and what we observe is just the image of clonal dynamics, independent of treatment.

Changes associated with disease progression: without treatment, only "natural" selection pressures, e.g. competition for myeloma survival niches or faster accumulation, are present; this is the situation in MGUS-AMM-MM transition. What evidence for changes is there here? First, in cohort studies (transversal samples), an increased frequency of aberrations later associated with disease progression, e.g. 1q21,[39] has been found in none of 14 individuals with MGUS, 43% (206 of 479) of newly diagnosed, 72% (32 of 45) of relapsing myeloma patients, and 93% (21 of 23) of myeloma cell lines.[66] A further example is t(4;14).[38] These finding are traditionally interpreted as chromosomal aberrations appearing in higher frequencies at later stages to be associated with an evolution of the malignant plasma cell clone during different stages of plasma cell dyscrasias, "multi-step-model".[65] However, all chromosomal aberrations detected in symptomatic myeloma can be detected already in MGUS or asymptomatic myeloma.[39,40,67-72] In longitudinal samples, gains and losses of aberrations have been found.[65,73,74] Examples comprise whole exome sequencing data of paired samples as asymptomatic- and symptomatic myeloma (n=4 patients) by Zhao *et al*. showing no newly acquired SNVs in 3 patients, while in the fourth patient only one new SNV of unclear significance was detected in a single gene (*BBOX1*).[75] Although the degree of loss of heterozygosity (LOH) was greater at baseline in samples from progressing patients (n=4) *versus* those who did not progress (n=6), there was no shared pattern. In addition, the LOH pattern was largely maintained with only few changes in the samples at the time of progression identified, but for 2 patients with newly acquired regions with LOH.[75] A com-

parable result has been found by Walker *et al*. who, but for inactivating mutations in *RUNX2* and acquired translocations into *BRCA2* and *UNCSD*, "could not identify truly acquired genetic abnormalities" between paired AMM and MM samples "despite thoroughly checking for coding SNVs, indels and copy number abnormalities".[65] Whereas there is convincing evidence that, also without treatment, changes in the clonal composition at least at the level of SNV can appear, these have as of yet not convincingly been associated with disease progression.

In the light of what we have discussed so far, there is evidence for possible clonal change, and clonal evolution if selection pressure is applied by treatment, but not for clonal evolution driving progression.

Clinical considerations

The findings described above have several clinical implications. First, the potentially changing malignant plasma cell clone should be sequentially assessed by molecular characterization at presentation and during subsequent relapses. Second, given the evidence of "clonal tidings" during treatment, re-treatment with a regimen A to which a patient had responded but finally progressed seems worthy of consideration if the patient responded in-between to a different treatment regimen B. A potential biological background is the re-emergence of a clone sensitive to treatment A if, without the selective pressure leading to its emergence, a different selection pressure is put in place by compound B. Third, given the clonal heterogeneity, selection pressure by treatment with single agents, especially targeted treatment options like BRAF-inhibitors, will likely be prone to emerging resistance due to a higher chance of a subclone harboring a protective mutation, instead of a combination treatment with several non-cross resistant drugs; a situation potentially analog to the treatment of tuberculosis with a compound combination.[76,77] With this, clonal heterogeneity favors combination or even "total" therapy.[78] Fourth, regarding the treatment of "asymptomatic myeloma". On the one hand, if reports about a lower genetic heterogeneity in early stage disease[65,73,74] hold true in larger patient populations, this would advocate early treatment,[79] alongside the presence of lower tumor-mass (and thus lower probability of these mutations), and less changes in the bone marrow environment, eventually making normalization under treatment easier to achieve.[78,80,81] On the other hand, early treatment could remove more "indolent" clones leaving room for more aggressive ones to expand. In the end, only clinical trials can give the answer to this question, preferably with sequential characterization of the malignant clone.

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