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Characterization of the Karyotype in Patients with Multiple Myeloma by the Combination of Karyotype Analysis, FISH and CGH

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ABSTRACT Cytogenetic and molecular cytogenetic investigations from bone marrow samples were performed in 83 patients with multiple myeloma. Karyotype analyses were made after cultivation with and without growth factors. The polyploidy level ranged from 3n to 6n. For each patient the composite karyotype was delineated. The number of abnormalities per aberrant cell was in a range from 1 to 17 ($\bar{x}$ =3,7). The comparison of CGH and FISH results showed an accordance of 92%. In total, 57% of cells showed chromosomal aberrations.

INTRODUCTION

Changes in chromosomal karyotype are a major predictor of prognosis in multiple myeloma (Schmidt-Wolf et al. 2006). Diverging genetic findings in tumor studies made it obvious that methodical improvement is necessary to gain relevant results. This is only possible by combining various techniques. Therefore, we tested five significant parameters in a collective of patients with multiple myeloma. Cytogenetic, and molecular cytogenetic investigations were performed in 83 patients with multiple myeloma to gain a better characterization of the aberrant karyotype.

MATERIAL AND METHODS

In total, bone marrow of 83 patients with multiple myeloma was analysed. In 5 cases second bone marrow samples were received after a time interval.

Sex distribution of the patients was 52 males and 31 females. Mean age at the time of investigation was 60 years (39 to 84 years). The majority of patients (n=55) showed a multiple myeloma stage III (66 %), 14 % (n=12) were in stage II and 6 % (n=4) in stage I (13 % (n=12) without exact staging). In 48 out of 83 patients, a therapy with cytostatic substances was documented, 25 were analysed before chemotherapy and in 10 no information to a pre-treatment could be achieved.

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METHODS

MACS (Magnetic Cell Separation): The mononuclear bone marrow cells were treated with RPMI and Percoll to isolate the leucocytes and they were counted in a Neubauer-counting chamber. The plasmacells were magnetic labelled with CD 138 MicroBeads (Miltenyi Biotech) according to the instruction of the manufactures. The positive selection of the CD 138^{+}-cells was performed in a VarioMACS-magnet (Miltenyi Biotech).

***Vitality Testing*:** Cell activity after magnetic labelling was controlled by vital fluorescence staining with Acridineorange (3,6-Bis (dimethylamino)-acridine-dihydrochlorid).

Cell Cultivation: As the cultivation of the bone marrow cells is difficult, various cultivation methods were tested, to achieve a high number of analysable metaphases. Test series were made, including lymphocyte cultures of a control group of 100 persons. Various parameters were variegated to optimize the results. Cell cultures with untreated and separated plasmacells were prepared parallel. The culture medium contained RPMI-1640 (Biochrom) (with Penicillin-Streptomycin und Glutamin), fetal serum albumine and

phytohemagglutinin. In a second series of cultures granulocyte macrophage-colony stimulation factor (GM-CSF) und interleukine-6 (IL-6) were added. The time of incubation varied from 24 to 96 h. For the preparation of the cells standard methods were used. Metaphases were harvested according to standard protocols.

Mitotic Index (MI): Cell proliferation was determined per 1000 cells.

Chromosome Analyses**:** The chromosomes were analysed after GTG-banding. The C-metaphases were karyotyped according the ISCN (2009). A normal karyotype was defined when at least 30 cells did not show recurrent aberrations. A clonal origin of a numerical chromosome aberration was obvious when at least 2 cells had the same chromosome hyperploidy. In the case of a hypoploidy, the aberration had to be analysed in at least 3 cells.

The composite karyotype was documented. Other parameters documented were the level and frequency of polyploid cells.

The *comparative genomic hybridization* (CGH) was realized to register gains and losses of whole chromosomes, deletions and amplifications. In the samples with a low level of plasma cells the myeloma cells were first separated magnetically and results could only be achieved when the frequency of plasma cells in the sample was at least 50 %. The DNA of the plasma cells was isolated with QIAmp DNA-Blood-Mini-Kit (Quiagen). DNA was amplified by *Degenerate Oligonucleotide Primer* P*olymerase Chain Reaction* (DOP-PCR). Tumor DNA and reference DNA for CGH were labelled by nick-translation with biotin und digoxigenine respectively. After the following hybridization on metaphase preparations, the detection was done by FITC and TRITC.

Fluorescence in situ hybridization (FISH) was applied predominantly to confirm the results of the CGH, especially in the detected chromosome sidelines. Centromere- and *single-copy*-probes (Abbott) were used. A co-hybridization of two probes of different fluorescence staining guaranteed, that disomic and trisomic chromosomes could be documented in the same cell (Fig.1). Metaphase and interphase analyses were combined. The probes were chosen in dependence of the CGH results. FISH was done with the following probes (Abbott): centromere specific probes (CEP) of chromosomes 3, 7, 9, 11, 15, 17 and 18 and locus specific probes (LSI) of the loci 4p16.3, 4p11.1-q11.1, 5p15.2, 5q31, 7q31, 7p11.1-q11.1, 11q13, 11p11.1-q11, 11q22.3, 11q23, 11qtel, 13q14, 17p13.1, 22q11.2, 22q13. Some of the loci contain genes relevant in multiple myeloma, as WHSC1, ERG1, Cyclin D1, ATM, MLL, RB-1, p53, N25.

In the combined chromosome and aberration analyses by FISH and CGH the aberrant cells were differentiated according to their frequency into main-lines (> 20 %) and side-lines (< 20 %) of karyotype evolution (table 3). In cases, where the CGH did not allow a differentiation between pathologic single cells and a side-line the aberration in question was further investigated by FISH with the defined DNA-probes.

We also analysed *clinical parameters* as β2-microglobuline, CRP, creatinine, Hb, as well as the amount of plasma cells, tumor stage, and influence of chemotherapy for combination with specifical chromosomal aberrations.

RESULTS

In the present investigation the following different cytologic and cytogenetic methods were combined: mitotic index analysis, conventional chromosome analysis, CGH, and FISH.

As 36 out of 88 bone marrow samples had less than 50 % of plasma cells they were separated by *MACS* to increase the amount of CD138 positive plasma cells. This proved to be a new and very successful method to increase the amount of the target DNA. The average increase leads to 50 % plasma cells in the samples.

The pretreated plasma cells showed a significantly higher amount of chromosomal aberrations than the untreated ones (Table 1).

Cell activity was controlled after magnetic cell separation by Acridine orange staining. The amount of the vital cells was between 74 and 86 %.

Cell cultivation was performed in 211 samples of plasma cells. In 111 of these cases the mitosis-index was determined to check the different cultivation methods. The best results were achieved after incubation for 72 h with medium RPMI-1640 and additional stimulation of the cells by GM-CSF and IL-6. Mitotic indices of the samples were compared to mitotic indices of normal bone marrow cells found in literature. The mitotic index was reduced in comparison to normal bone-marrow-cells in all samples cultivated in vitro even by adding specific growth

Table 1: Comparison of aberration rates in directly prepared bone marrow samples and after increase of plasma cells by MACS (results from one patient MM12)

Region tested and DNA probe applicated	*Aberration rates and type of aberration*			
	In directly prepared bone marrow cells	*Frequency (%)*	*After pretreatment of plasma cells*	*Frequency (%)*
5p15.2 (LSI)	Duplication	22	Duplication	42
5q31 (LSI: ERG1)	Duplication	18	Duplication	38
9 (CEP)	Trisomy	14	Trisomy	3
11 (CEP)	Normal		Normal	
11 q23(LSI:MLL)	Duplication	5	Duplication	19
17 (CEP)	Monosomy	10	Monosomy	17

factors ($\bar{x}$ = 11,32 ‰) . The low mitotic activity of plasma cells in vitro was additionally influenced by the increased age and the chemotherapy of the patients. In 50 out of 211 cell cultures mitotic activity could not be induced at all or was to low for analyses (MI: 0-20 ‰). These insufficient probes were all cultivated after cell separation after MACS.

A *composite karyotype* was established for the samples with analysable metaphases. The average amount of polyploid mitoses in the investigation group was 1.6 % compared to 0.02 % in a control group and comprised triploid, tetraploid, pentaploid, and hexaploid mitoses. Aneuploid and pseudodiploid cells were analysed by karyotyping and had an average frequency of 41 % of the mitoses. In mitoses with structural chromosomal aberrations 5 different types of abnormalities were diagnosed: translocations, inversions, duplications, deletions, and derivates. The majority of aberrant cells (48 %) showed numerical aberrations, 24 % structural, and 28 % a combination of both (Table 1).

One patient had an additional heterochromatic marker chromosome (constitutional karyotype: 47,XY,ish+der(22)(p10. is q10) analysed by GTG,QFQ, CBG,DA/DAPI,D22Z1, WCP22, D22S75).

CGH was performed in 37 samples, 28 of them after MACS treatment. These investigations could only be performed because the MACS resulted in a sufficient selection of plasma cells. This made it possible to analyse the side-lines of karyotype evolution besides the main-lines (Table 2). 90 % of the investigated patients showed aberrations. The number of abnormalities in the aberrant cells ranged from 1 to 17 (x =3,7). In CGH-analyses the majority of aberrant cells (52 %) showed a combination of numerical and structural abnormalities, 33 % only structūral, and 15 % only numerical (gains, losses, amplifications).

FISH analyses were performed on interphase cells of 30 patients with 16 different DNA probes as showed in the chapter before. The results of 117 FISH investigations showed 44 % of the cells with hypo- and/or hyperploidy and 53 % with structural aberrations (Table 2, Fig. 1).

We analysed the different results of the methods. Thus, a concordance of FISH and CGH results could be delineated in 92 % of the cases. In the remaining 8 % the differences between

Table 2: Main- and side-lines of karyotype aberrations (selection of CGH and FISH results in 4 tumors)

Tumor	*Main-lines*		*Side-lines*	
	Type of aberration (detected by CGH)	*Frequency (%) (detected by FISH)*	*Type of aberration (detected by CGH)*	*Frequency (%) (detected by FISH)*
1	+3	41	del (17(p13.1)	14
	dup(11)(q21-qter)	31	del(22)	11
2	-13	77	t(3;18)	14
			+5	16
3	+3	20	dup(8)(q22-24)	< 10
			dup(10q)	< 10
			dup(11)(q22-23)1	< 10
4	+9	43	dup(11(q23)	19
	dup(5)(p15.2)	42	del(17)(p13.1)	19
	dup(5)(q31)	38	del(22q)	13

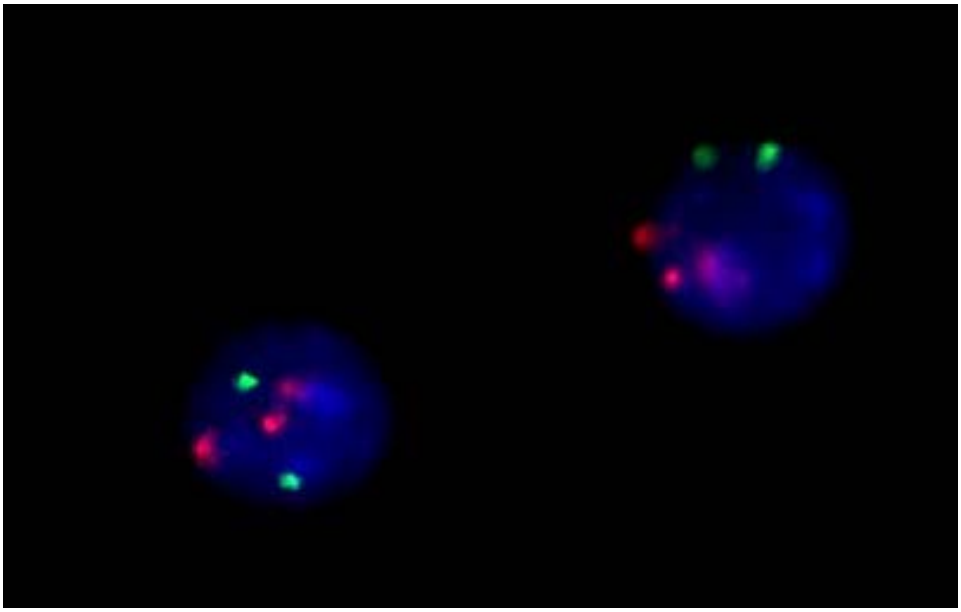

Fig. 1. In situ hybridization with 2 DNA-probes. 3 signals for chromosome 3 (orange) and 2 signals for chromosome 9 (green) (trisomy 3, disomy 9)

FISH and CGH were assumed to be caused by the different size of detectable structural aberrations by FISH and CGH.

Finally, different *clinical parameters* were tested for combination with specific chromosomal aberrations. In 7 of 10 patients with a deletion 13q14 β2-microglobuline was increased as well as CRP in 6 of 9 patients with deletion 17p13.1 and Kreatin in 3 of 9 patients with trisomy 11. Seven of these patients were in tumor stage III, 2 in stage IIA. Trisomy 3 and 9 and deletion 22q11.2 showed no correlation to the amount of β2-microglobuline, CRP, creatinine, Hb, amount of plasma cells, tumor stage, or chemotherapy.

DISCUSSION

The present study combined various methods because the treatment of plasma cells is difficult and we tried to find better results in karyotype-analyses, CGH, and FISH.

The increase of plasma cells after MACS lead to significantly better results in FISH and CGH but it was unsuccessful for chromosome analyses as it reduced the mitotic index too much. This shows that even in samples with tested cell activity, the cell cycle was interfered.

The differences in the aberration spectrum between karyotype analyses and CGH are

Table 3: Frequency of selected chromosome aberrations in the present investigation with a comparison to findings from the literature (see Weh et al. 1993; Sawyer et al. 1995; Hagen et al. 1998; Gutiérrez et al. 2000; Zojer et al. 2000; Dalton et al. 2001; Avet-Loiseau et al. 2002; Kuehl and Bergsagel 2002; Fonseca et al. 2003; Gonzalez et al. 2004; Chen et al. 2005; Schmidt-Wolf et al. 2006.

Type of chromosome aberration		*Frequencies in the studies from literature (%)*	*Frequencies in the present investigation (%)*	
			Tumor stage I-II	*Tumor stage III*
Numeric	+3	17-41	12	19
	+9	17-42	10	13
Structural	dup(5)(p15.2)	8-24	35	31
	dup(5)(q31)	4-55	25	25
	dup(11)(q23)	30-44	25	35
	del(13)(q14)	30-55	10	13
	del(17)(p13.1)	4-40	25	25

thought to be caused by two main factors: A cell selection in favour of normal and less aberrant cells during the cultivation before karyotyping and the possibility to analyse types of structural aberrations by CGH which are not detectable in chromosome investigations. The first factor could be verified by the different maxima of abnormalities per aberrant cell which was about twice in CGH in comparison to chromosome investigation, and the second after reinvestigation of chromosomes with pathologic CGH profiles by FISH (Table 2).

The differences of the FISH results compared to karyotyping and CGH were caused by the selection of DNA probes in this investigation. In cases of non-significant changes in the CGH profiles additional FISH analyses were performed. Side-lines of karyotype evolution could be demonstrated and quantified in 12 out of 17 cases by combination of FISH and CGH. The chromosomes most frequently aberrant in the investigation group presented here where trisomy 3 and 9, duplications 5p15 and q31, 11q23, and deletions 13q14 and 17p13, which were also found in other studies (Table 3). These aberrations were mainly observed in tumor stage III, less frequent in stage II and not in stage I.

Concerning the analysed clinical parameters in relation to specific chromosomal aberrations we found that they are in general agreement with observations from the literature (Hallek et al. 1998; Liebisch et al. 2003). Differences to the results of other authors are partially caused by the choice of different cut-off-values, which can lead to false positive or negative results.

In conclusion, the investigation of plasma cells in patients with multiple myeloma enabled us to gain qualitative and quantitative results in karyotype evolution by the combination of cytogenetic and molecular cytogenetic techniques. Parallel changes of specific clinical parameters combined with the development of characteristic chromosome changes were delineable. A combination of specific numerical and structural chromosomal aberrations could be shown. The newly established combinations of MACS for an increase of plasma cells in the sample with DOP-PCR lead to a significant improvement of the results from CGH and FISH.

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