From cell sorting to plasma cell identification and detection chromosomal aberrations in multiple myeloma

Immunomagnetic cell separation for clinical use

Abstracts and Application Manual

Cytogenetic and Immunogenetic Workshop

10 - 11/10/2007

Brno, Czech Republic

ILBIT MU Brno, Kamenice 5

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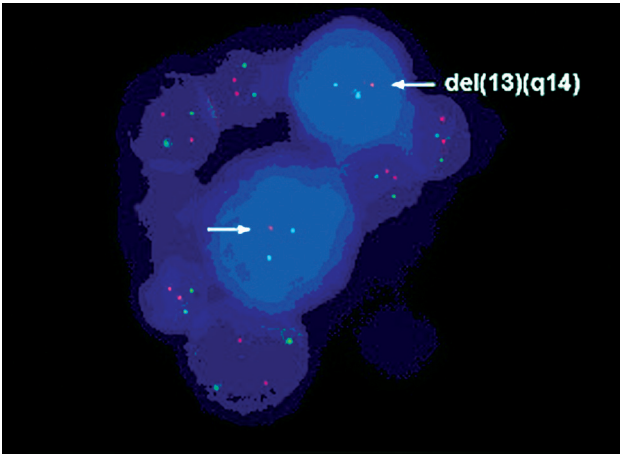

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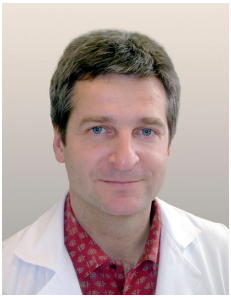

Dear Ladies and Gentlemen,

I am pleased to welcome you to our Second Workshop MACS&FISH focused on FISH methods used in multiple myeloma and on separation technologies aimed at pure cell population obtaining which represents a necessary entrance point of a wide range of research applications. I really appreciate the practical part of the workshop allowing to perform and practice respective methodologies directly in laboratory.

I would like to express my thanks to my colleagues for an excellent preparation of worshop.
I believe that you will enjoy the stay in the areas of our new Masaryk University campus. Our research team has been working here already for three years. Additionally, I am pleased to have the unique occasion to announce official launch of the research ensemble titled „Czech Myeloma Group – University Research Center". One of its main goals is, besides the research in the first instance, the transfer of techniques and support of research in the field of multiple myeloma in the Central and Eastern Europe. This part of our activities is supported by the Czech Myeloma Group and the International Myeloma Foundation.
Your ideas for cooperation and coordination of research activities in monoclonal gammopathies are very welcome!
I wish you a pleasant stay and many new scientific inspirations.

Roman Hajek
Chairman of Czech Myeloma Group
and chairman of CMG-University Research Center

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Scientific programme:

MACS & FISH
From cell sorting to plasma cell identification and detection chromosomal aberrations in multiple myeloma

10.10.2007

9:00 am – 9:15 am	**Chairman's introduction** **Roman Hajek, Brno, Czech Republic**
9:15 am – 10:30 am	**Theoretical part of the workshop**
9:15 am	Poor prognosis associated with gain of chromosome 1q21 in Multiple Myeloma may be overcome by treatment with a Bortezomib combination **Johannes Drach, Vienna, Austria**
9:35 am	Nuclear organization of PML bodies in Multiple Myeloma and leukemic cells **Eva Bartova, Brno, Czech Republic**
9:55 am	Results of multicentric molecular cytogenetic study of 233 patients with multiple myeloma. **Zuzana Zemanova, Prague, Czech Republic**
10:10 am	Prognostic impact of 1q21 amplification for newly diagnosed and relapsed/refractory multiple myeloma patients enrolled in Brno, Czech Republic **Pavel Nemec, Brno, Czech Repuplic**
10:20 am	Clinical implications of chromosomal aberrations (13q14 and 17p13 deletion, translocation t(4;14) and 1q21 amplification) in patients with relapsed multiple myeloma treated by thalidomide or bortezomib (Velcade) **Romana Zaoralova, Brno, Czech Republic**
10:40 am– 11:00 am	**Coffee break**
11:00 am – 12:00 am	**Practical part of workshop (part I)** Detection of chromosomal aberrations in multiple myeloma using simultaneous immunofluorescent labelling of malignant plasma cells and fluorescent *in situ* hybridization (clg FISH) **Romana Zaoralova, Henrieta Greslikova, Petra Rudolecka, Renata Kupska, Hana Filkova, Pavel Nemec, Petr Kuglik, Brno, Czech Republic**
12:00 am – 13:00 pm	**Sample transport demonstration for CMG 2008 clinical study**
13:00 am – 14:00 pm	**Lunch break**
14:00 am – 17:00 pm	**Practical part of workshop (part II)** clg FISH and image analysis in multiple myeloma **Dusan Kadlec, Petr Kuglik, Brno, Czech Republic**
17:00 pm – 17:30 pm	**Chairman's conclusion** **Roman Hajek, Brno, Czech Republic**

Immunomagnetic cell separation for clinical use

11.10.2007

9:00 am – 9:05 am	**Chairman's introduction** **Roman Hajek, Brno, Czech Republic**
9:05 am – 9:25 am	**Identification and immunomagnetic separation of tumor - specific T cells** **Jaroslav Michalek, Brno, Czech Republic**
9:25 am – 9:50 am	**Immunomagnetic isolation of myeloma cells** **Jana Zelena, Darina Ocadlikova, Brno, Czech Republic**
9:50 am – 10:15 am	**Coffee break**
10:15 am – 10:35 am	**Monocyte enrichment for dendritic cell generation by using of CliniMACS System** **Miltenyi Biotec GmbH, Germany**
10:35 am – 11:05 am	**Practical part of workshop III.** AutoMACS versus MidiMACS - Imunomagnetic separation of myeloma cells **Andreas Nicodemou, Anje Bauwens, Biohem s.r.o., Miltenyi Biotec GmbH, Germany**
11:05 am – 11:25 am	**MACS® Technology: The gold standard in cell separation** **Anje Bauwens, PhD., Miltenyi Biotec GmbH, Germany**
11:25 am – 11:45 am	**Discussion**
11:45 am – 12:00 am	**Chairman's conclusion** **Roman Hajek, Brno, Czech Republic**
12:00 pm – 12:45 pm	**Lunch break**

10/10/2007 - ABSTRACT OF LECTURES

POOR PROGNOSIS ASSOCIATED WITH GAIN OF CHROMOSOME 1q21 IN MULTIPLE MYELOMA MAY BE OVERCOME BY TREATMENT WITH A BORTEZOMIB COMBINATION

V. Sagaster,[1]V. Odelga,[1]H. Kaufmann,[1]J. Ackermann,[1]M. Galhuber, [2]N. Zojer, [3]H. Ludwig, [3]R. Wieser, [2]C.Zielinski,[1]J. Drach1

Medical University of Vienna, [1] Dept. of Medicine I/Clinical Division of Oncology, and [2] Dept. of Human Genetics/Clinical Institute of Medical and Chemical Laboratory Diagnostics; [3] Wilhelminenspital, Dept. of Medicine I/Center for Oncology and Hematology, Vienna, Austria

Background:

Bortezomib is an active agent for treatment of multiple myeloma (MM) and may even be effective in patients (pts) with adverse prognostic factors including unfavorable cytogenetic abnormalities. However, it is unknown whether or not bortezomib may overcome the negative prognostic impact of a chromosome 1q21 (CKS1B) gain, which has recently been reported as a negative prognostic factor even in the setting of a total therapy approach.

Aim:

We therefore evaluated chromosome 1q21 among other abnormalities in 46 pts with relapsed/refractory MM who were treated with single-agent bortezomib (1.3 mg/m2 on days 1, 4, 8, and 11 every 3 weeks) and in 28 pts treated with a bortezomib combination (bortezomib/dexamethasone in 43%, bortezomib/chemotherapy in 46%, bortezomib/thalidomide/dexamethasone in 11%).

Patients and Methods:

Median age of pts was 63 years (range, 40 – 82) and median time to bortezomib therapy was 40 months (median number of prior therapies: 3; 96% of pts had high-dose pulsed dexamethasone, 61% thalidomide, 85% alkylating agents, and 41% high-dose melphalan). Chromosome 1q21 was evaluated by interphase FISH with a CKS1B-specific probe. Results were correlated with clinical outcome.

Results:

Among patients treated with single-agent bortezomib, gain of 1q21 was observed in 20 of the 46 pts (43.5%). Treatment outcome after bortezomib was negatively affected by presence of a 1q21 gain: The overall response rate was 30% (versus 58% in pts with normal 1q21; $P = .06$) and the CR/near-CR rate was 10% (versus 23%). Moreover, gain of 1q21 was associated with shortened time to treatment failure (TTF) (median, 2.4 versus 6.6 months; $P = .043$) and overall survival (OS) (median, 4.4 versus 19.8 months; $P = .003$) compared to pts with normal 1q21. Beta-2-microglobulin and 14q32 translocations were unrelated to treatment outcome after single-agent bortezomib, but median OS was short in the presence of low serum albumin (4.8 versus 17.8 months; $P = .036$). In the group of pts treated with a bortezomib-combination, 11 of the 28 pts had a 1q21 gain (39%). There were no significant differences between pts with 1q21 gain and normal 1q21 regarding overall response (54.5% versus 64.7%), CR/near-CR rate (27% versus 29%), median TTF (8.2 versus 6.9 months; $P = .57$) and median OS (not reached versus 17.8 months; $P = .49$).

Conclusion:

FISH-defined gain of 1q21 is associated with poor response, short TTF and short OS after single-agent bortezomib; however, these differences disappeared in the context of a bortezomib-combination therapy. These results provide further evidence for the efficacy of bortezomib-combinations in MM patients with high-risk features.

Nuclear organization of PML bodies in multiple myeloma and leukemic cells

[1] Eva Bartova, [1] Jana Krejci, [1] Andrea Harnicarova, [1] Jana Kurova, [1] Radka Uhlirova, [1] Stanislav Kozubek, [2] Roman Hajek

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[2] Department of Internal Medicine, Hematologic Oncology, Masaryk University Hospital, Jihlavska 20, 625 00 Brno, Czech Republic

The structural and functional compartmentalization of the mammalian interphase nucleus has been studied over the last few decades. Regulation of gene transcriptional activity is also ascribed to the chromatin-associated domains such as transcription factories, nuclear speckles, and other proteinaceous structures such as promyelocytic leukaemia (PML) bodies. These nuclear domains are responsible not only for regulation of transcription, but also for program cell death apoptosis, tumour suppression, and antiviral defence. Therefore, PMLs seem to be important prognostic factors associated with various diseases. Mammalian nuclei contain 10-30 spherical PML structures involving PML protein, encoded by PML gene, which is fused to the retinoic acid receptor (RaRα) gene to form t(15;17) translocation in acute promyelocytic leukemia (APL) (Kiesslich et al., 2002; Ching et al., 2005). PML also mediate interferon alpha induced apoptosis in multiple myeloma cells (Crowder et al., 2005).

In our experiments, we tested following hypothesis: 1) whether the nuclear radial distribution of PML bodies is changed during *in vitro* induced differentiation of leukaemia cells and after cytostatic treatment of CD138-/CD138+ myeloma cell populations 2) Is the nuclear distribution and number of PML bodies influenced by cell cycle changes? 3) In which extent the cell differentiation and cytostatic treatment can influence variants of PML protein determined by western blots. Additionally, we tried to address 4) the relationship of selected oncoproteins such *c-MYC* with PML bodies. Taken together, in multiple myeloma and leukaemia cellular models we have observed remarkable differences in the number and nuclear radial distributions of PML bodies. In various cell types, during differentiation induced and after cytostatic cell treatment, we have found distinct variants (Fig.1) and PML protein levels. In addition, conversely to Smith et al. (2004), we did not detect *c-MYC* in the PML nuclear body compartment in multiple myeloma and leukemic cells (Fig. 1).

1. Crowder C, Dahle O, Davis R E, Gabrielsen O S, Rudikoff S: PML mediates IFN-alpha-induced apoptosis in myeloma by regulating TRAIL induction. Blood, 2005, 105: 1280-1287.
2. Ching R W, Dellaire G, Eskiw C H, Bazett-Jones D P: PML bodies: a meeting place for genomic loci? J. Cell Sci., 2005, 118: 847-854.
3. Kiesslich A, vonMikecz A, Hemmerich P: Cell cycle-dependent association of PML bodies with sites of active transcription in nuclei of mammalian cells. J. Struct. Biol., 2002, 140: 167-179.
4. Smith K P, Byron M, O'Connell B C, Tam R, Schorl C, Guney I, Hall L L, Agrawal P, Sedivy J M, Lawrence J B: c-Myc localization within the nucleus: Evidence for association with the PML nuclear body. J. Cell. Biochem., 2004, 93: 1282-1296.

This work was supported by grant LC06027.

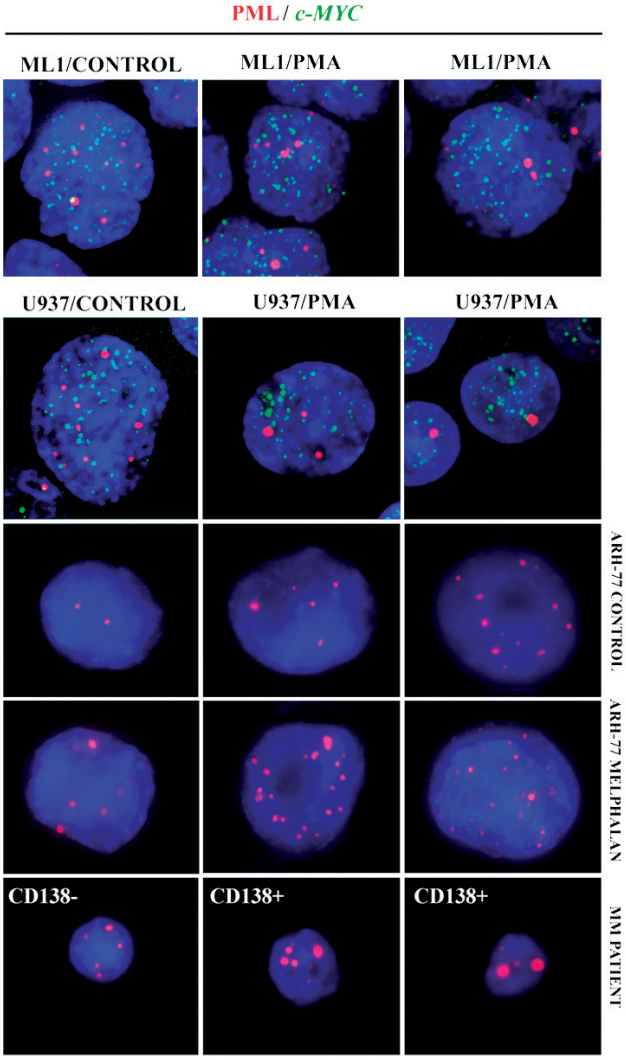

Figure 1 No association of the c-MYC protein (green) with PML bodies (red) in leukeamia ML1 and U937 cell lines. These cells were differentiated by phorbol esters (PMA), which was characterized by a reduced number of PML. Conversely, cytostatic treatment by Melphalan induced remarkable increase of PML bodies. CD138+ fraction from the bone marrow of patient with diagnosed multiple myeloma was characterized by increased volume and nuclear repositioning of PML nuclear bodies.

Results of multicentric molecular cytogenetic study of 233 patients with multiple myeloma in the trial CMG 2002.

Zemanova Zuzana[1], Michalova Kyra[1,2], Tajtlova Jana[1], Pavlistova Lenka[1], Oltova Alexandra[3], Filkova Hana[3], Kuglik Petr[3], Jarosova Marie[4], Holzerova Milena[4], Rabasova Jana[5], Hruba Martina[6], Hajek Roman[7] and The Czech Myeloma Group (CMG).

[1]*Center of Oncocytogenetics, Institute of Clinical Biochemistry and Laboratory Diagnostics, General Faculty Hospital and 1st Medical Faculty of Charles University, Prague;* [2]*Institute of Hematology and Blood Transfusion, Prague;* [3]*Department of Medical Genetics, Faculty Hospital Brno;* [4]*Department of Hemato-Oncology, Faculty Hospital Olomouc;* [5]*Department of Medical Genetics, Faculty Hospital Hradec Kralove;* [6]*Institute of Medical Genetics, Faculty Hospital Plzen;* [7]*Department of Internal Medicine – Haematooncology, University Hospital, Brno; Czech Republic.*

Finding of clonal genomic changes in plasma cells is considered as one of the most important and independent prognostic factor in patients with multiple myeloma (MM). We analyzed frequency and prognostic value of the most common chromosomal aberrations in a homogenous series of patients with newly diagnosed multiple myeloma enrolled to the phase III trial CMG 2002. All patients underwent single autologous bone marrow transplantation according to the protocol of the trial after 4xVAD induction chemotherapy. Patients were randomized after transplantation into two maintanance/consolidation treatment arms.

All the 233 patients enrolled until December 31, 2005 were systematically examined by conventional G-banding technique and by I-FISH for the deletion/monosomy of chromosome 13 and *IgH* gene rearrangements. I-FISH analyses were done on plasma cells labeled by the Amca Anti-Human kappa-chain, Amca Anti-Human lambda-chain and Amca Anti-goat IgG monoclonal antibodies (Vector Laboratories). I-FISH was done by locus-specific DNA probes (Abbott-Vysis, Des Plaines, Illinois, USA and Kreatech Biotechnology B.V, Amsterdam, The Netherlands).

In patients with previously proved rearrangement of *IgH*, I-FISH with LSI *IgH*/CCND1 probe for detection of the translocation t(11;14)(q13;q32) was performed. *IgH*/CCND1 negative patients were further examined and *IgH* translocation partner was searched using the LSI *IgH*/FGFR3 probe for the t(4;14)(p16;q32) at first and if negative the LSI *IgH*/MAF probe to recover the t(14;16)(q32;q23) was used. In addition, 132 patients were also screened for the deletion of 17p13 and 92 cases were examined for amplification of 1q21 chromosomal region. Chromosomal changes were observed in 81.1% of the patients. Abberations of chromosome 13 and *IgH* rearrangement were present in 53.3% and 54.6% of the patients, respectively. t(11;14)(q13;q32) was ascertained in 26.0% of cases and other translocations affecting 14q32 region in 24.4% of patients. Besides translocations, different variants of total and/or partial deletions of *IgH* gene were detected in 18.8% of patients. Molecular cytogenetic findings were correlated with clinical and laboratory parameters. Results of detailed statistical analysis will be presented at the workshop.

Method of immunofluorescent labeling of plasma cells improves sensitivity of detection of chromosomal aberrations by I-FISH technique and therefore contribute to diagnosis and prognosis of the disease. Comprehensive molecular cytogenetic analyses of genomic changes in plasma cells of patients with MM will improve our understanding of the origin and progress of the disease.

Supported by grant IGA NR/8183-4.

Prognostic impact of chromosome 1q21 amplification for newly diagnosed and relapsed/refractory multiple myeloma patients enrolled in Brno, Czech Republic.

Pavel Nemec[1,3,4], Petr Kuglik[1,2,4], Hana Filkova[2,4], Henrieta Greslikova[1,3,4], Romana Zaoralova[1,3,4], Jana Smejkalova[1,3], Alexandra Oltova[2], Roman Hajek[1,3]

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Introduction

Fluorescence *in situ* hybridisation (FISH) has become a powerful technique for prognostic assessment in multiple myeloma (MM). Amplification of chromosome band 1q21 as well as increased expression of *CKS1B* gene in this area is a frequently mentioned prognostic factor in patients with multiple myeloma. A total of 88 patients enrolled in Faculty Hospital Brno, Czech Republic, have been analysed by FISH for presence of 1q21 amplification (Amp(1q21)). Impact of Amp(1q21) in clinical parameters (treatment responses and selected „end-point" intervals) in 39 patients with newly diagnosed MM enrolled in CMG2002 trial (4 cycles of VAD followed by high-dose therapy - melphalan 200mg/m^2 supported by autologous stem cell transplantation (ASCT)) were statistically analysed. In a similar way, a subset of 49 relapsed patients with at least 1 prior treatment line were analysed separately in groups divided according to treatment based regimen (thalidomide or Velcade). See Table 1 for patients baseline characteristics.

Results valid for newly diagnosed patients:

Amp(1q21) was found in 41% (16/39) patients. Clinical parameters valid for patients with Amp(1q21) versus patients lacking Amp(1q21) were as follows: overall response rate (ORR) achieved 87.5% (14/16) vs. 91.3% (21/23) patients (p=0.404); overall survival (OS) median was 22.4 months vs. not yet reached (NR) (p=0.022); time to progression (TTP) median was 16.1 months vs. NR (p=0.010); progression-free survival (PFS) median was 15.6 vs. 25.2 months (p=0.023); and duration of response (DOR) median was 15.9 months vs. NR (p=0.048).

Results valid for relapsed patients: Amp(1q21) was found in 61.2% (30/49) patients.

Thalidomide based regimen group

Amp(1q21) was found in 40.7% (11/27) patients. Clinical parameters valid for patients with Amp(1q21) versus patients lacking Amp(1q21) were as follows: ORR achieved 83.3% (5/6) vs. 80% (8/10), (p=0.868); OS median was 6.6 months vs. NR (p=0.072); TTP median was 12.1 vs. 8.2 months (p=0.269); and PFS median was 11.7 vs. 8.2 months (p=0.484).

Velcade based regimen group

Amp(1q21) was found in 86.4% (19/22) patients. Clinical parameters valid for patients with Amp(1q21) versus patients lacking Amp(1q21) were as follows: ORR achieved 50% (7/14) vs. 66.7% (2/3), (p=0.316); OS median was NR vs. NR (p=0.937); TTP median was 8 months vs. NR (p=0.442); and PFS median was 6.5 months vs. NR (p=0.637).

Conclusion

In conclusion, patients with Amp(1q21) treated by ASCT have significantly shorter PFS median (15.6 months) when compared with patients lacking Amp(1q21) with PFS median 25.2 months (p=0.023). There was statistically significant difference found in all named „end-point" intervals (OS, TTP, PFS and DOR) between patients with/without Amp(1q21) but not in ORR. This finding is in accordance with previously published work (Chang *et al.*, 2006). Relapsed patients with Amp(1q21) treated with thalidomide based regimen show a trend towards the worst prognosis based on overall survival when compared with patients lacking Amp(1q21) suggesting that thalidomide probably cannot overcome negative prognostic impact of this aberration. None significant differences between clinical parameters of patients with/without Amp(1q21) treated with Velcade based regimen were observed. However, these findings have to be confirmed on a larger cohort of patients. These data are valid for July 2007.

Literature

Hong Chang, Xiaoying Qi, Young Trieu, Wei Xu, Jocelyn C. Reader, Yi Ning, Donna Reece. 2006. Multiple myeloma patients with CKS1B gene amplification have a shorter progression-free survival post-autologous stem cell transplantation. *British Journal of Haematology*, 135, 486–491.

Supported by Monoclonal Gammopathy and Multiple Myeloma Basic Research Centre (LC 06027), Masaryk University, Brno, Czech Republic, and by grant of Ministry of Education (MSM0021622415), Czech Republic, and grant of Ministry of Medicine (IGA NR9317-3), Czech Republic.

Table 1 Patients baseline characteristics

		Thalidomide	Velcade
No. of patients		27	22
Age: range (years)		62 (45-78)	59 (39 - 78)
Ig Isotype	IgA	9 (33%)	10 (45%)
	IgG	14 (52%)	10 (45%)
	B-J	4 (15%)	10 (45%)
Durie - Salmon stage	I.A	-	4 (18%)
	II.A	7 (26%)	9 (41%)
	III.A	17 (63%)	7 (32%)
	IIII.B	3 (11%)	2 (9%)
No. of relaps	First line	2 (7%)	1 (5%)
	1	17 (63%)	8 (36%)
	2	5 (19%)	6 (27%)
	≥ 3	3 (11%)	7 (32%)
Regimen	T	3 (11%)	-
	TD	5 (19%)	-
	CTD	17 (63%)	-
	V	-	4 (18%)
	V-Do	-	4 (18%)
	VD	-	5 (23%)
	VMP	-	5 (23%)
	Other	2 (7%)	4 (18%)

T - thalidomide, D - dexamethason, C - cyclophosphamide, V - Velcade, Do - Doxil, M - Melphalan

Clinical implications of chromosomal aberrations (13q14 and 17p13 deletion, translocation t(4;14) and 1q21 amplification) in patients with relapsed multiple myeloma treated with thalidomide or bortezomib (Velcade)

R. Zaoralova[2] , P. Kuglik[1], H. Filkova[4], H. Greslikova [1,2], P. Nemec[2], A. Oltova[4], L. Pour[2], Z. Adam[3], A. Krivanova[3], M. Krejci[3], R. Hajek[2,3]

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[2] Monoclonal Gammopathy and Multiple Myeloma Basic Research Centre, Masaryk University, Brno, Czech Republic
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Multiple myeloma (MM) is the second most common hematologic cancer, representing 1 % of all cancer diagnoses and 2 % of all cancer deaths. Despite recent progress in the disease management, the survival of myeloma patients is highly variable, ranging from a few months to more than 10 years. The heterogeneity relates mainly to prognostic factors associated with specific characteristics of tumors.

There are well known correlations between prognosis and several chromosomal aberrations detected by cytogenetic analyses including interphase fluorescence *in situ* hybridisation. Deletion of chromosome 13 (del RB1), translocations t(4;14) and deletion of 17p13 (del p53) are known to be associated with poor prognosis. Recently, the amplification of chromosome band 1q21 (amp 1q21) as well as increased expression of *CKS1B* gene were suggested as key marker of poor prognosis. These data are conclusive but valid only for the patients with newly diagnosed MM who are undergoing conventional chemotherapy or autologous transplantation. There is limited knowledge only of prognostic and predictive (resistance vs. sensitivity) features of chromosomal abnormalities when new agents such thalidomide, lenalidomide and bortezomib are used.

The aim of the present work was:

1) To study the incidence of the chromosomal aberrations known as adverse prognostic factors in MM - deletion of 13q14 (RB1), deletion of 17p13 (p53), amplification of 1q21 (CKS1B) and translocation t(4;14) in a group of 40 patients with relapsed MM.
2) To compare the prognostic and predictive significance of these unfavorable chromosomal aberrations in patients with relapsed MM treated with thalidomide or bortezomib (Velcade) based regimens.

Material and methods

Bone marrow (BM) samples from 40 patients with relapsed MM enrolled in Faculty Hospital Brno, Czech Republic, were studied. BM samples were cultured in Panserin 441 medium for 24 hours at 37°C in 5% CO2. The cells were harvested using hypotonic potassium chloride, fixed by methanol/glacial acetic acid (3:1), and then stored at -20°C. The group was divided according to the undergone therapy into 2 subgroups: „T-group" comprises 21 samples from patients treated with thalidomide based regimen; „V-group" comprises 19 samples from patients treated with Velcade based regimen. All subjects provided written informed consent approving use of their samples for research purposes.

Cytoplasmic Immunoglobulin Staining/Interphase Fluorescence *in situ* Hybridization (clg FISH) analysis was done in clonal plasma cells (PCs) detected by immunofluorescence of the cytoplasmic light chain (clg) according to Ahmann *et al*. (1998) with slight modifications:
Bone marrow samples were fixed in 95% ethanol for 15 min, the cells were collected by centrifugations, placed on slides and dried at room temperature. The slides were incubated in 10 mM citrate puffer (pH=6,0) at 95 ^{0}C for 10 min and then briefly rinsed in PBS buffer. Subsequently, the slides were stained with either goat anti-human kappa or lambda light chain conjugated with AMCA (Vector Laboratories) for 1 hour at 37 ^{0}C. After incubation, the slides were washed twice in PBS buffer and stained with anti-goat immunoglobulin conjugated with AMCA ((Vector Laboratories) for 1 hour. The slides were then rinsed in PBS buffer and dehydrated in a series of ethanol (75%, 85%, 100%). The 10 ul of DNA probes was placed on the selected area and the slides were hybridized overnight.
For each patient, 50 AMCA positive plasma cells were scored. We used 30 % as the background level for the probe sets.

DNA Probes used:

del(13q14)/monosomy 13 (Vysis LSI RB1 DNA Probe/ Vysis LSI 13q34 DNA Probe)
del p53 (17p13) (Vysis LSI p53 DNA Probe)
t(4;14) (Vysis LSI *IgH*/FGFR3 DNA Probe)
amp 1q21 LSI 1q21/1p36 DNA Probe (Q-Biogene)

Digital Image Analysis

Fluorescence Microscope Olympus BX-61
CCD Camera Vosskuhler 1300D
software: Lucia 4.80 – KARYO/FISH/CGH, Laboratory Imaging, s.r.o., Prague, Czech Republic

Statistical analysis

Overall survival (OS) and time to progression (TTP) distributions were estimated using the Kaplan-Meier method. The differences among survival curves were analyzed by the log-rank test. P values below 0.05 were considered significant.

Results and conclusions

In our study, we did not find statistically significant difference in OS and TTP between patients with/without del p53, t(4;14), amp 1q21. Therefore, it is possible to estimate that the new drugs overcome the negative prognostic impact. Patients with RB1 deletion have significantly shorter time to progression median (8.9 month) in comparison with patients lacking RB1 deletion. Similarly, patients with any combination of 3 or 4 unfavorable cytogenetic abnormalities have significantly shorter time to progression median (6.0 month) in comparison with patients with 1 or 2 aberrations. These data suggest that cytogenetic abnormalities can define subgroup of patients with relapsed MM not benefiting from thalidomide and Velcade treatment. Data are still preliminary and we need to wait for the results from large randomized trials.

Supported by Monoclonal Gammopathy and Multiple Myeloma Basic Research Centre (LC06027), Masaryk University, Brno, Czech Republic and by grants from Ministry of Educations, (MSM0021622415) and Ministry of Health of Czech Republic (NR9317-3).

METHODOLOGY

Protocol for the identification of malignant plasma cells in bone marrow samples using simultaneous staining of cytoplasmic immunoglobulin with FISH (cIg FISH)

Ahmann, G.J., Syed, M.J. at al.: A Novel Three-Color, Clone-Specific Fluorescence *In situ* Hybridization Procedure for Monoclonal Gammopathies. Cancer Genet. Cytogenet. 101, 7-11, 1998.
Johannes Drach and Jutta Ackermann – personal communication, modification Petr Kuglik

Reagents and equipment:

Solutions:

3 : 1 methanol-acetic acid fix, 96 % ethanol fix, 10 mM citrate buffer - citric acid (1g/500ml H_2O) / natrium citrat (1,45g/500ml H_2O) mix 1:1; pH 6,0, PBS solution, ethanol (70 %, 80%, 96 %), 0,5 x SSC, antifade solution (0,01 % p-phenylendiammine dissolved in glycerol/PBS buffer pH=8,0, 1:1)

Antibodies used (Vector Laboratories)

- Amca Anti-Human Kappa Chain, Catalog No. CI-3060, 1 : 10 (diluted in PBS)
- Amca Anti-Human Lambda Chain, Catalog No. CI-3070, 1 : 10 (diluted in PBS)
- Amca Anti-Goat IgG (H + L), Catalog No. CI-5000, 1 : 20 (diluted in PBS)

DNA Probe used:

- del(13q14)/monosomy 13 (Vysis-Abbott LSI RB1 DNA Probe/ Vysis – Abbott LSI 13q34 DNA Probe)

Digital Image Analysis

- Fluorescence Microscope Olympus BX-61
- CCD Camera Vosskuhler 1300D
- **Software:** Lucia 4.80 – KARYO/FISH/CGH, Laboratory Imaging, s.r.o., Prague, Czech Republic

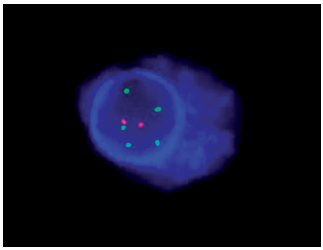
Method:

(this protocol is for use with Abbott Vysis DNA probes)

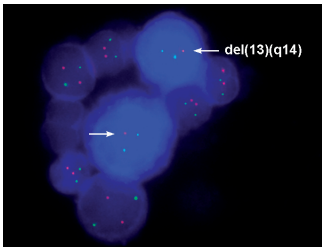
clg FISH is carried out on bone marrow (BM) aspirate. BM samples are cultured in Panserin 441 medium for 24 hours at 37 ^{0}C in 5% CO2. The cells are harvested using hypotonic potassium chloride, fixed by methanol/glacial acetic acid (3:1), and then stored at -20^{0}C.

1. Collects the fixed bone marrow cells by centrifugation at 1500 rpm for 10 min
2. Remove the supernatant, and add 8 ml of 96 % ethanol. Resuspend sediment and incubate 15 min at room temperature
3. Repeat step 1 and 2
4. Resuspend the pellet in 96 % ethanol and place a drop of fixed cells onto a cleaned microscope slide. Let slides air dry, and examine under phase contrast microscope to check cell density
5. Incubate the slides 10 min in glass Coplin jar with 10 mM citrate buffer prewarmed at 95 ^{0} C (water bath)
6. After incubation, remove Coplin jar from water bath and leave cool at room temperature for 30 min
7. Wash slides twice for 2 min in PBS solution (at room temperature)
8. Apply 150 μl of diluted Amca Anti-Human Kappa Antibody or Amca Anti-Human Lambda Antibody onto each slide, and incubate under plastic cover slip in humified chamber for 1 hour (1 – 1,5 hour)
9. Wash slides twice for 2 min in PBS solution (at room temperature)
10. Apply 150 μl of diluted Amca Anti-Goat Antibody and incubate under glass cover slip in wet chamber for 1 hour (1 – 1,5 hour)
11. Wash slides twice for 2 min in PBS solution (at room temperature)
12. View slides under fluorescent microscope to check staining of cytoplasmic immunoglobulin
13. Dehydrate slides by serial ethanol washing (70 %, 80 %, 96 %) for 2 min
14. DNA Probe Preparation: denatured probe - manufacturer ´s protocol is recommended
15. Hybridisation: add 10 μl of DNA Probe per 22 x 22 mm slide area. Apply glass cover slip and seal with rubber cement. Incubate 16 hour in a humified chamber.
16. Post-hybridisation wash: wash slides in 0,5 x SSC for 4 min at 72 ^{0}C, and leave slides in the dark to dry
17. Pipette 10 μl of antifade solution onto each slide, overlay with cover slip and analyze under fluorescent microscope
18. Score 100 Amca positive plasma cells for each patients

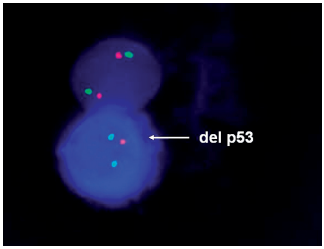
Examples of clg - FISH



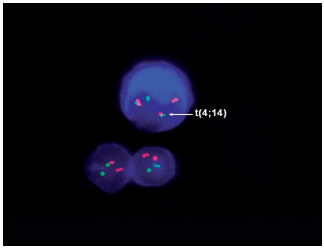
Abnormal malignant plasma cell hybridized with LSI 1q21 (green) / LSI 1p36 (red) probes. The cell shows amplification of 1q21 (Amp(1q21)) as 5 copies of the 1q21 region indicated by five single green signals. Red signals enable to distinguish Amp(1q21) from trisomy or tetrasomy of chromosome 1.



Abnormal malignant plasma cell hybridized with the LSI 13q14 (red)/13q34 (green) probes.The cell shows deletions of one copy of the 13q14 regions of chromosome 13 as indicated by the single red signal.Green signals enable the differentiation of the interstitial deletions from loss of the entire q arms.



Abnormal malignant plasma cell hybridized with LSI 17p13 (red) / CEP17 (green) probes. The cell shows deletion of p53 gene (del 17p13) as one copy of the 17p13 region indicated by one single red signal. Green signals enable to distinguish deletion of 17p13 from monosomy of chromosome 17.



An abnormal interphase cell hybridized with the LSI IgH / FGFR3 Dual Color, Dual Fusion Translocation Probe. The cell shows the one red (FGFR3), one green (IgH), two fusion (der (4) and der (14)) signal pattern indicative of a t(4;14).

Cryoconservation of bone marrow sample for subsequent molecular genetic and cytogenetic analyses

Material

5 ml of bone marrow heparinized

Solutions

- **Hanks' Balanced Salt solution**, SIGMA, No. H-6648, 500 ml, store at laboratory temperature until expiration date
- **Albumin human 20%**, Grifols, No. 59/361/91-C, 100 ml, store at 2 – 8 °C until expiration date
- **Histopaque-1077** (density 1,077 $g.ml^{-1}$), SIGMA, No. H-8899, 500 ml, endotoxin tested, store at 2 – 8 °C until expiration date
- **Ethanol 70%**, 250 ml, store at laboratory temperature
- **Carnoy's solution (fixative)**: Acetic acid 99% : methanol, 1:3 vol.

EQUIPMENT

- Refrigerated centrifuge
- Haematological analyzer or Bürker cell
- Laminar box
- Automatic pipettes
- Syringes 20 ml

EXPENDABLE SUPPLIES

- Sterile syringes, 20 ml and 10 ml
- Pasteur pipette sterile, 3 ml
- Sterile tubes, 50 ml
- Laboratory gloves
- Sterile tips for pipettes – 10-200 μl, 200-1000 μl, 1-5 ml

A. ISOLATION OF MONONUCLEAR CELLS FROM BONE MARROW (MNC)

Work sterile in a laminar box.

1) Put the bone marrow into sterile tube and add the same amount of Hanks' solution. Mix thoroughly.
2) Gradient density centrifugation:
   - Layer using 20 ml syringe with needle:

1. Histopaque
2. Histopaque: sample, proportion 2 :1 vol.
   - Max. volume in one 50 ml tube is 30 ml of sample and 15 ml of Histopaque.
   - Centrifugation 400G / 35 min/ 4 °C or 200 G / 45 min / laboratory temperature

3) After centrifugation aspire the layer containing the MNC (above the Histopaque medium) by the Pasteur pipette and put it into a new sterile tube. Note: The layer could not be visible.
4) Add a redundant amount of the Hanks' solution to the tube.
5) Centrifugation 300 G / 10 min / 4 °C or 400 G / 8 min / laboratory temperature.
6) After centrifugation waste the supernatant and resuspend the sediment in Hanks' solution. Final volume should be about 5 ml. Measure the cellularity.

B. THE TREATMENT OF MNC FOR FISH

1. Aliquote the isolated mononuclear cells:

• 1×10^6 MNC for DNA analysis	1 tube	- 80 °C
• 1×10^6 MNC for RNA analysis	1 tube	- 80 °C
• 2×10^6 MNC in Carnoy's fixative for FISH	1 tube	- 20 °C

2. Process the aliquots:

- **MNC for DNA analysis:**
  Centrifuge 500 G / 10 min / laboratory temperature
  Waste supernatant
  Label the cryotube by the code due to the evidence paper
  Freeze the sediment in cryotube to – 80 °C
  Store in an appropriate box

- **MNC for RNA analysis:**
  Centrifuge 500 G / 10 min / laboratory temperature
  Waste supernatant
  Label the cryotube by the code due to the evidence paper
  Freeze the sediment in cryotube to – 80 °C
  Store in an appropriate box

- **MNC in Carnoy's fixative for FISH:**
  Centrifuge 500 G / 10 min / laboratory temperature
  Waste supernatant
  Add a redundant amount of the Carnoy's solution
  Resuspend thoroughly
  Incubate for 1 hour
  Centrifuge 500 G / 10 min / laboratory temperature
  Waste supernatant
  Add the Carnoy's solution 1,5 – 2 ml
  Resuspend thoroughly
  Label the cryotube by the code due to the evidence paper
  Freeze to – 20 °C
  Store in an appropriate box

EUROPEAN MYELOMA NETWORK RECOMMENDATIONS FOR FISH IN MYELOMA 2007

Ross FM (1), Avet-Loiseau H (2), Drach J (3), Hernandez Rivas JM (4), and Liebisch P (5) on behalf of the European Myeloma Network FISH Working Party

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A workshop to discuss the problems particular to FISH in myeloma was held at the Royal Marsden Hospital, London on 11 March 2005 and attended by representatives of 31 European laboratories. The following recommendations are the result of agreement at the time combined with updates resulting from e-mail discussion between the participants in May 2007. The recommendations apply only to newly diagnosed or relapsed myeloma; monitoring of disease or testing of plasma cell dyscrasias with very low levels of marrow involvement may require different criteria. **Results found by applying the recommendations in this document should not yet be used to make treatment decisions except in the context of a clinical trial.**

1. It is not acceptable to report FISH results in myeloma without either concentrating the plasma cells or employing some means of plasma cell identification so that only these cells are scored. Haemodilution is a universally reported serious problem in myeloma. Clinicians should be encouraged to send part of the first draw of the aspirate for FISH, and further aspiration should involve repositioning of the needle.

2. It is strongly recommended that bone marrow aspirates are not performed on Friday.
Processing for FISH is time-consuming and results depend on good viability plasma cells.

3. Purification and simultaneous immunostaining and FISH (cIgFISH) are equally valid methods.
Choice of method depends on individual laboratory requirements. In general, the expense of purification is best justified in the context of a cell bank. Differences in purification methods should not affect FISH results.

4. Purified plasma cells must be checked for the proportion of plasma cells (by morphology or immunostaining)

5. Purified plasma cells should be fixed in 3:1 methanol:acetic acid. The choice of doing this to the cell suspension (with or without prior hypotonic treatment) or to cytospin slides is up to the individual laboratory. Both slides and suspensions can be successfully stored at -20°C for prolonged periods.

6. Immunostaining for light chains is recommended for cIgFISH. This gives a stronger result that CD138.

7. Cells for cIgFISH should be subjected to red cell lysis or density gradient centrifugation and fixed in 3:1 methanol:acetic acid. Slides can be made directly or the suspension stored at -20°C. Bone marrow smears can be used for cIgFISH but only when they are very fresh.

8. Cut off levels should be 10% for dual fusion and break-apart probes, and 20% for numerical abnormalities and single fusion results with dual fusion probes. These are conservative figures based on mean+3SD results of 5-10 controls in several laboratories but it is recognised that suitable control material is difficult to obtain and myeloma cells are prone to artefacts, thought to be due to the paraprotein levels. Any laboratory setting up myeloma FISH should ensure that their results are compatible with these levels. Laboratories with low mean+3SD for deletions may wish to consider results in the 10 – 20% range to be borderline for their own records but they should not be reported to clinicians as positive.

9. The 2005 recommendation that control probes should be used for all deletion probes is no longer considered essential. This recognises that each probe behaves in a unique way and that experience of the probes helps interpretation. In practice many laboratories prefer to use a control probe. In 2005 it was recommended that 13q and p53 should not be used together because of the frequency of double deletions. However, this objection has also been withdrawn.

10. A single experienced analyst is considered enough for reporting all abnormalities in the majority of cells from good preparations. However, results should always be checked where there is an equivocal signal pattern, where there are few plasma cells for clg FISH or where purified plasma cells make up <30% of the total. Smaller labs are recommended to use 2 analysts with a third to check any results with a discrepancy of <5%.

11. Wherever possible 100 cells should be scored. Results are only acceptable from <50 cells where all cells are identical or >75% have the abnormality.

12. p53, t(4;14) and 13q should be tested in all cases. The t(14;16) also has h*IgH* priority and many still consider t(11;14) worth doing. Where all these tests are performed there will be extremely few 'normal' 13 results reported incorrectly due to unsuspected near-tetraploidy. However, if an *IgH* break-apart strategy is used to decide which cases to test for fusion genes then some *IgH* negative near-tetraploid cases will be missed. The Vysis 5/9/15 probe is recommended to avoid this problem. Alternatively reports should be qualified with regard to the 13q and p53 results.

13. Probes to use: **All commonly used probes in 13q14 are acceptable** for 13q deletion which is usually of the whole chromosome.
Fusion strategies must usual dual fusion probes that cover a large enough area on the donor chromosome to encompass all breakpoints and allow detection of unbalanced translocations (eg loss of der(14) in t(4;14)). For the t(11;14), the difference between the Vysis standard and Tx probes was not thought to be significant
17p probes should be specifically for p53.

14. Reporting results.

The method of plasma cell identification should be reported.

The proportion of affected plasma cells should be reported. It is not clear what is the cut-off level for clinical significance. It is suggested that abnormalities in <40% cells should be emphasised in the text as being at a low level (although a few laboratories prefer not to report these).

All abnormalities should be expressed as clearly as possible. Thus the workshop did not endorse the use of ISCN.

Fusion probe results (especially for the t(4;14)) should record whether there was a dual or single fusion.

These recommendations should be reviewed yearly by email, with further workshops being held if there is significant controversy.

Anyone wishing to be included in the discussions should contact fiona.ross@salisbury.nhs.uk

A quality assurance scheme has been set up by Hervé Avet-Loiseau. The first round is underway but anyone wishing to be included in future rounds should contact herve.avetloiseau@chu-nantes.fr

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11/10/2007 - ABSTRACT OF LECTURES

Identification and immunomagnetic separation of tumor- specific T cells

J.Michalek, D.Ocadlikova, ,J.Zelena ,E. Matejkova, P. Vidlakova, D.Kyjovska ,I.Buresova, R.Hajek

Background:

Autologous hematopoietic stem cell transplantation has been considered recently as part of a standard treatment strategy in patients with multiple myeloma (MM). Here we atempted to enhance the immunotherapeutic potential of autologous T cells based on selection of myeloma-reactive lymphocytes in vitro.

Aims:

The aim of this study was to identify and characterize autologous myeloma-reactive T cells in vitro and to evaluate their cytotoxic effect.

Methods:

Irradiated myeloma cell line ARH 77 or patient's myeloma cells were used as tumor antigen for dendritic cells loading. Peripheral blood mononuclear cells of 8 healthy volunteers and 10 MM patients were used for repeated stimulation of T lymphocytes. Activated T cells producing interferon gamma were isolated using immunomagnetic separation (MACS) (Miltenyi Biotech) and expanded in vitro by phytohemaglutinin and high concentrations of interleukin 2. A specific cytotoxicity against myeloma cells was tested after the expansion with propidium iodide or 7-amino actinomycin D. Activated T cells were labeled with CFSE. Allogeneic T cells and interferon gamma negative fraction of T cells served as controls.

Results:

In an allogeneic setting with ARH 77 cells the enrichment of interferon gamma positive T cells by magnetic beads in healthy donors started from a median of 2.83% (1.97-4.58%) to 48.57% (15.14- 82.98%) after MACS and from 1.91% (1.14-3.4%) to 73.14% (3.9-88.75%) after MACS in CD3+CD4+ and CD3+CD8+ T cells, respectively. Interferon gamma positive T cells were further expanded in vitro from $0.5x10^6$ to a median of $160x10^6$ ($150x10^6$-$420x10^6$) T cells within 4 weeks and the test of cytotoxicity has demonstrated a high degree of specific killing of ARH 77 myeloma cells 69.17% (38.04-78.23%). Cytotoxicity of expanded interferon gamma negative T cells was negligible.

In an autologous setting with autologous myeloma cells used as an antigen, the enrichment of interferon gamma positive T cells from MM patients started from 1.12% (0.27-6.2%) to 7.85% (0.42-12.6%) after MACS and from 1.9% (0.37-14.4%) to 14.7% (1.28-71.4%) after MACS in CD3+CD4+ and CD3+CD8+ T cells, respectively. Interferon gamma positive T cells were expanded *in vitro* from $0.12x10^6$ ($0.05x10^6$-$0.4x10^6$) to 88.5x106 ($35x10^6$-226 $x10^6$) within 8-12 weeks and the test of cytotoxicty has demonstrated only a modest specific killing of autologous multiple myeloma cells (18.88%) and allogeneic ARH 77 cells (18,21%).

Conclusion:

These data demonstrate a promising tumor-specific effect of allogeneic myeloma-reactive T cells but only a modest effect in an autologous setting in patients with MM. Whether that is due to a low MACS enrichment or low immunogenicity of autologous myeloma cell needs to be further clarified.

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Immunomagnetic isolation of myeloma cells by autoMACS

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The standard method used for cell separation is Magnetic Antibody Cell Separation, MACS. In our laboratories, we use autoMACS separator (Miltenyi Biotec, Bergisch Gladbach, Germany) designed for positive selection as well as depletion of magnetically labeled cells. The separator operates with separation programs according to cell abundance and the intensity of marker expression. The separated cells are immediately ready for experiments, cell analysis or further subset sorting.
Within minutes can be isolated up to 2x108 pure target cells per sample. AutoMACS allows positive selection and depletion of cells even if the antigen used for selection is expressed at low levels.

CD138+ myeloma cells can be isolated from whole blood or bone marrow (BM) or from mononuclear cells prepared using gradient centrifugation from BM of the patients with multiple myeloma. The CD138 antigen, also known as syndecan-1, is found on malignant plasma cells in the BM. The CD138+ myeloma cells are isolated via positive selection of human plasma cells using CD138 micro beads. The sample containing labeled cells is loaded onto an autoMACS column which is placed in a magnetic field. The magnetically labeled CD138+ cells are retained within the column. The unlabeled cells run through as negative fraction.

Isolated plasma cells can be used for molecular biology studies such as PCR analysis, microarrays, and protein analysis, and for analysis of cell surface markers of intracellular antigens, for example, immunoglobulin light chains.

Using possel d program, we obtained 77,6% cells (8,9-112,4%), average (min-max), with purity of positive fraction 83,8% (63,7-98,9%). In this report, we would like to inform about our achieved results in immunomagnetic separation of myeloma cells from patients using several separation programs.

Supported by MSMT LC0602

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Notes:

Notes:

From cell sorting to plasma cell identification and detection chromosomal aberrations in multiple myeloma

Immunomagnetic cell separation for clinical use

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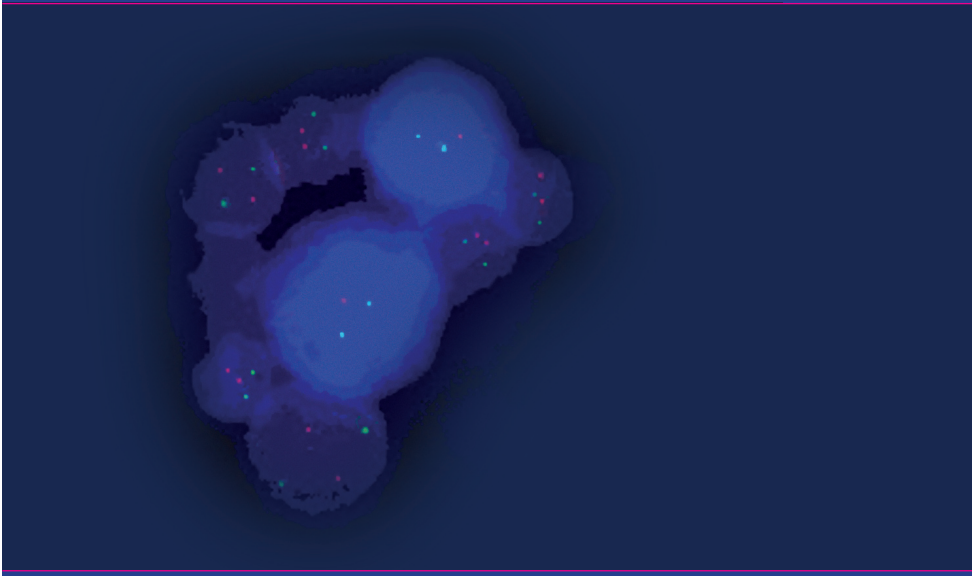

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cic
cell immunotherapy center

"Until There is a Cure... There is the Myeloma"