Table 1. Comparison of our data and previously published data: incidence of submicroscopic deletions in leukemias with reciprocal translocations.

Disease	Reference
CML	**22/245 (9%): this study**
	23/250 (9.2%): Kolomietz *et al.*, 2001
	39/241 (16.2%): Huntly *et al.*, 2001
BCR-ABL positive ALL	**4/70 (6%): this study**
	1/13 (7.7%) Kolomietz *et al.*, 2001
	4/45 (9%) Specchia *et al.*, 2003
ALL with *MLL* rearrangements	**1/29 (3%): this study**
	3/18 children (17%): Barber *et al.*, 2004
AML with *AML1-ETO*	**4/112 (4%): this study**
	0/14 (0%): Kolomietz *et al.*, 2001
AML with *CBFB-MYH11*	**3/122 (2%): this study**
	5/15 (33%): Martinet *et al.*, 1997
	2/20 (10%): Kolomietz *et al.*, 2001
	6/42 (14%): Marlton *et al.*, 1995
AML with *PML-RARA*	**3/108 (3%): this study**
	0/30 (0%): Kolomietz *et al.*, 2001
AML with *MLL* rearrangements	**8/96 (8%): this study**
	0/22 children (0%): Mathew *et al.*, 1999
ALL and AML with *MLL* rearrangements	7/43 (16%): Kolomietz *et al.*, 2001

tions in 8% of our cohort with AML with different *MLL* rearrangements. This is in contrast to the results of Mathew *et al.*, who did not find submicroscopic deletions in 22 children with AML and *MLL* rearrangements.[7]

In conclusion, we found a similar incidence of 2-9% of submicroscopic deletions in a variety of leukemias. Although we analyzed a high number of patients we were unable to determine the prognostic impact of these deletions in acute leukemias (*data not shown*) because of the limited number of patients with submicroscopic deletions and the specific individual prognoses: AML with favorable balanced translocations have a good prognosis; *MLL* rearrangements in acute leukemias and *BCR-ABL* positive ALL show a poor outcome.

Submicroscopic deletions could represent a non-specific event in different types of leukemia without an association with a specific entity.[2] Polymerase chain reaction and Southern blot analyses of reciprocal breakpoints in ALL with *MLL* rearrangements and in CML showed a high rate of submicroscopic deletions at the molecular level.[8,9] The loss of tumor-suppressor genes due to submicroscopic deletions in CML might play a role in the worse prognosis of these patients.[1] Given the very low incidence of cases with deletions accompanying reciprocal translocations in AML and ALL, definitive conclusions on prognosis cannot be drawn.

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Key words: submicroscopic deletions, interphase fluorescence in situ hybridization, balanced translocations, AML, BCR-ABL positive ALL.

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Multiple Myeloma

Global real-time quantification/reverse transcription-polymerase chain reaction for detecting proto-oncogenes associated with 14q32 chromosomal translocation in multiple myeloma

A global real-time quantitative/reverse transcription-polymerase chain reaction technique for detecting the expression of six 14q32 chromosomal translocation-associated proto-oncogenes in marrow plasma cells was established and applied to myeloma specimens. This technique is an alternative method of detecting 14q32 rearrangements and allows investigation of the relationship between proto-oncogene expression and clinical features.

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Chromosomal translocations involving the immunoglobulin heavy chain gene (*IGH*) locus (14q$^+$) play important roles in multiple myeloma (MM).[1,2] The transcriptional acti-

vation of proto-oncogenes associated with the 14q+ chromosome such as *CCND1*, *FGFR3*, *c-MAF*, *MAFB*, *MUM1/IRF4*, and *c-MYC* may be involved not only in the development of MM but also in the treatment outcome.[3,4,5] We established a global real-time quantitative/reverse transcription-polymerase chain reaction (RQ/RT-PCR) technique that can be performed on clinical material.

After obtaining written informed consent from patients, plasma cells were purified from mononuclear cells that had been derived from 1~2 mL of marrow aspirate by positive selection using anti-CD138 antibody-coated beads and an automatic magnetic cell sorting system (Miltenyi Biotec, Auburn, CA, USA).[6] One microgram of total RNA was reverse-transcribed to single-strand cDNA with a random primer and Superscript II (Invitrogen Life Technologies, Carlsbad, CA, USA) and the final volume was adjusted to 42 μL. The cDNA solution was diluted in ddH_2O at 1:128. RQ/RT-PCR was carried out in a total volume of 20 μL of reaction mixture containing 2 μL of diluted cDNA, 2 μL of FastStart DNA master SYBR Green I (Roche Molecular Biochemicals, Mannheim, Germany), and 0.5 μM of each primer with a LightCycler Quick System 330 (Roche Molecular Biochemicals). Table 1 shows the oligonucleotide sequences of the primers and PCR conditions used for amplification of each gene. For precise quantification of the transcripts, we assessed the expression level of β-actin mRNA as an internal control. The primer set for β-actin was purchased from Roche Molecular Biochemicals, and PCR was carried out according to the manufacturer's instructions. The standard curve for each gene was generated by evaluating serially diluted plasmid clones containing cDNA inserts of the gene as templates. The absolute expression level of the target gene was calculated from the standard curve. Quantitative assessment of the mRNA of interest was performed by dividing its expression level by that of β-actin mRNA to obtain the copy-number ratio. RT-PCR for the *IGH*-MMSET chimeric transcript was performed using primers IGJH1 (5'-CCCTGGTCACCGTCTCCTCA-3') and MMSET (5'-CCTCAATTTCCTGAAATTGGTT-3') as described by Chesi *et al*.[7] To test for mutations in codons 12, 13 and 61 of the N- and K-Ras genes at the mRNA level,[8] real-time PCR was carried out in a total volume of 20 μL reaction mixture containing 2 μL of diluted cDNA and 10 pmol of each primer (Table 1). A second PCR was carried out using 1 μL of the real-time PCR product. After purification, the PCR product was sequenced by an ABI3100 capillary DNA sequencer (Applied Biosystems, Foster City, CA, USA).

Both the sensitivity and reproducibility of our RQ/RT-PCR system were validated using serial dilutions of plasmid clones in ddH_2O ranging from 15 to 2.8×10^8 copies. These samples were amplified in triplicate. For each of the 6 genes, the plot of log concentration of the gene (x-axis) versus cycle number (y-axis) showed a linear relationship between 60 and 3.8×10^3 copies with minimal deviation (*data not shown*). We examined 19 MM cell lines for the expression status of the *CCND1*, *FGFR3*, *c-MAF*, and *MAFB* genes and found that the expression level was extremely high in MM cell lines that contained chromosomal translocations involving these gene loci (*data not shown*). However, the mRNA levels of *MUM1* and *c-MYC* were generally higher in the 19 MM cell lines than in fresh samples irrespective of the status of the chromosomal rearrangements involving these two gene loci (*data not shown*). This seemed to be due to the presence of a larger fraction of proliferating MM cells in MM cell lines than in fresh specimens.

Table 1. Primer sets and conditions of the real-time PCR assay.

Oligonucleotide Sequence	*Primer**	*$MgCl_2$*	*Condition***
CCND1			
Forward tacaccgacaactccatcc	0.5 μM	6 mM	95°C 180s
(5'-3')			95°C 15s
Reverse accaggagcagctccattt			54°C 10s
(5'-3')			72°C 10s
FGFR3			
Forward atcggcattgacaaggacc	0.5 μM	5 mM	95°C 180s
(5'-3')			95°C 15s
Reverse tctccatctcagacaccag			54°C 10s
(5'-3')			72°C 10s
cMAF			
Forward tcataactgagcccactcg	0.5 μM	5 mM	95°C 180s
(5'-3')			95°C 15s
Reverse gaacacactggtaagtacac			52°C 10s
(5'-3')			72°C 10s
MAFB			
Forward ttcaaccttgttggtgctg	0.5 μM	5 mM	95°C 600s
(5'-3')			95°C 15s
Reverse aatttgaccataagacaaggctgtagt			52°C 10s
(5'-3')			72°C 10s
MUM1			
Forward agccaagcataaggtctgc	0.5 μM	5 mM	95°C 600s
(5'-3')			95°C 15s
Reverse gctccttcacgaggatttc			54°C 10s
(5'-3')			72°C 10s
c-MYC			
Forward tctccttgcagctgcttag	0.5 μM	5 mM	95°C 600s
(5'-3')			95°C 15s
Reverse gtcgtagtcgaggtcatag			54°C 10s
(5'-3')			72°C 10s
K-Ras			
Forward ggcctgctgaaaatgactgaata	0.5 μM	5 mM	95°C 600s
(5'-3')			95°C 15s
Reverse cccacctataatggtgaatatct			54°C 10s
(5'-3')			72°C 15s
N-Ras			
Forward atgactgagtacaaactggtggtggttgga	0.5 μM	5 mM	95°C 600s
(5'-3')			95°C 15s
Reverse caaatgacttgctattattgatg			54°C 10s
(5'-3')			72°C 15s

**The concentration of each of the forward and reverse primers in the PCR reaction mixture is shown. **The conditions of the primary denaturation, denaturation, annealing and extension are shown.*

In a series of samples from 45 patients with MM and 3 with monoclonal gammopathy of undetermined significances (MGUS), 13 (27.1%), 9 (18.8%), 4 (8.3%) and 2 samples (4.2%) showed expression of CCND1, *FGFR3*, *c-MAF* and *MAFB*, respectively (Table 2). MM sample n. 21 showed *IGH-MMSET* expression but not *FGFR3* expression, resembling previously reported findings.[9] More interestingly, the expression of *CCND1* on the one hand and *FGFR3* or *c-MAF* or *MAFB* on the other were mutually exclusive in the same samples, whereas 3 of 10 samples that expressed *FGFR3* and/or *IGH-MMSET* coexpressed c-MAF or MAFB, partially contradicting a previous report.[10] *N-RAS* mutation was detected in five MM samples (10.4%). Our global *RQ/RT-PCR* technique performed on

Table 2. Proto-oncogene mRNA expression and Ras gene status in a series of MM and MGUS samples from patients.

No.	CCND1	FGFR3	RQ/RT-PCR c-MAF	MAFB	MUM1	c-MYC	RT-PCR IgH-MMSET	Ras
MM 1	999	–	–	–	79	–	–	–
MM 2	938	–	–	–	327	–	–	–
MM 3	84	–	–	–	514	–	–	–
MM 4	903	–	–	–	105	243	–	–
MM 5	7739	–	–	–	125	–	–	–
MM 6	1917	–	–	–	13	–	–	–
MM 7	196	–	–	–	30	2	–	–
MM 8	35	–	–	–	64	–	–	–
MM 9	735	–	–	–	–	–	–	–
MM10	42	–	–	–	57	–	–	–
MM11	78	–	–	–	29	4	–	–
MM12	437	–	–	–	79	–	–	–
MM13	–	284	–	–	249	–	1007 bp	–
MM14	–	548	–	–	108	–	1007 bp	–
MM15	–	2361	–	–	208	–	218 bp	–
MM16	–	839	–	–	65	–	1007 bp	–
MM17	–	239	–	–	122	14	218 bp	–
MM18	–	605	3060	–	170	–	218 bp	–
MM19	–	334	8960	–	114	126	1007 bp	–
MM20	–	378	–	11	415	45	218 bp	–
MM21	–	–	–	–	–	–	1007 bp	–
MM22	–	–	2067	–	593	387	–	–
MM23	–	–	94713	–	68	–	–	–
MM24	–	–	–	44	62	–	–	N-ras61
MM25	–	–	–	–	85	24	–	N-ras13
MM26	–	–	–	–	595	–	–	N-ras12
MM27	–	–	–	–	751	–	–	N-ras61
MM28	–	–	–	–	30	–	–	–
MM29	–	–	–	–	183	–	–	–
MM30	–	–	–	–	–	–	–	–
MM31	–	–	–	–	698	–	–	–
MM32	–	–	–	–	484	148	–	N-ras61
MM33	–	–	–	–	146	–	–	-
MM34	–	–	–	–	117	–	–	-
MM35	–	–	–	–	48	–	–	-
MM36	–	–	–	–	202	6	–	-
MM37	–	–	–	–	47	6	–	-
MM38	–	–	–	–	174	–	–	-
MM39	–	–	–	–	33	–	–	-
MM40	–	–	–	–	435	15	–	-
MM41	–	–	–	–	272	–	–	-
MM42	–	–	–	–	180	21	–	-
MM43	–	–	–	–	490	–	–	-
MM44	–	–	–	–	561	106	–	-
MM45	–	–	–	–	214	–	–	-
MGUS 1	168	–	–	–	92	–	–	-
MGUS 2	–	213	–	–	266	7	1007 bp	-
MGUS 3	–	–	–	–	310	–	–	-

RQ/RT-PCR: copy-number ratio ×10². The copy-number ratio defined as the absolute expression level × 10², is shown. IgH-MMSET: The size of the IgH-MMSET fusion transcript obtained by RT-PCR and confirmed by direct sequencing is shown, whereas – indicates negativity for IgH-MMSET fusion transcript. Ras: no mutation in codons 12, 13 and 61 of the N- and K-Ras genes.

RNA extracted from sorted plasma cells may be an alternative method of determining the expression of 14q32 chromosomal translocation-associated proto-oncogenes and can be easily performed on clinical samples. This technique may be more efficient and cost-effective than conventional cytogenetic fluorescence *in situ* hybridization.[4-6]

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Key words: multiple myeloma, proto-oncogene, 14q+ chromosome, Ras mutation, real-time polymerase chain reaction.

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Multiple Myeloma

Double versus single autotransplantation in multiple myeloma; a single center experience of 100 patients

One hundred patients with newly diagnosed multiple myeloma (MM) were treated with high-dose chemotherapy followed by single or double autologous stem cell transplantation (ASCT). Up-front treatment with a double ASCT tended to prolong progression-free and overall survival.

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High dose therapy (HDT) and autologous stem cells transplantation (ASCT) are superior to conventional chemotherapy in multiple myeloma (MM),[1] and double autografting may be even better.[2] A total of 100 consecutive patients with newly diagnosed MM were treated with HDT and ASCT between 2/1992 and 3/2003: 73 patients (upper age limit 70 years) received a single and 27 patients (<61 years; 4 older patients) received a double translant (Table 1). Informed consent was obtained, and the double ASCT protocol was approved by the local Ethics Committee. After initial debulking therapy, usually 3-4 cycles of VAD, stem cells were mobilized with 2-4 g/m^2 of cyclophosphamide + granulocyte colony-stimulating factor. Details of the HDT are shown in Table 1. All patients received a blood graft. The EBMT response criteria were used.[3] A very good partial response (VGPR) was included and was otherwise similar to complete response (CR) but serum/urine immunofixation was positive. Overall survival (OS) and progression-free survival (PFS) were calculated from the first transplant to death.

Table 1. Patients' characteristics.

	Single ASCT	*Double ASCT*
Age; years, median (range)	59 (37-73)	55 (45-66)[1]
Sex (female/male)	32/41	12/15
Myeloma type		
IgG	42 (58%)	14 (52%)
IgA	12 (16%)	1 (4%)
IgD	1 (1.3%)	0
Light chain	16 (22%)	11 (40%)
Plasmacytoma	1 (1.3%)	1 (4%)
Nonsecretory	1 (1.3%)	0
Stage I/II/III	7/37/29	2/10/15
B2microglobulin >4 mg/L	18 (38%)[2]	5 (26%)[3]
Treatment line I/II/III	54/16/3	22/2/3
Local radiation therapy	7 (9%)	3 (11%)
T from onset Ther to Tx I; months, median (range)	6 (3-12.5)	5 (3-11)
T from onset Ther to Tx II; months, median (range)		11 (6-18)
HDT: Melphalan 140 mg/m^2 + TBI 12 Gy	20 (27 %)	1 (2 %)
Melphalan 200 mg/m^2	54 (73 %)	53 (98 %)[4]
Interferon (IFN) maintenance therapy	42 (57 %)	11 (41 %)
Duration of IFN therapy; months, median (range)	11 (1-101)	16 (2-45)

[1]4 patients of more than 60 years of age: [2]not available for 27 patients; [3]not available for 8 patients: [4]total number of HDT (incl. 1st and 2nd transplants) in the double ASCT group.

HDT supported by both the single and double ASCT was well tolerated. There was only one transplant-related death in the single transplant group. Organ-specific toxicities and engraftment kinetics were comparable between the first and second transplant procedures in the double ASCT group. The rate of good responses (CR + VGPR) increased from 18 to 71% (CR rate from 4 to 41%) with the single ASCT, and from 7 to 70% (CR rate from 0 to 52%) with the double ASCT. All patients responded to double autografting whereas there were three patients (4%) in the single ASCT group who did not. The median follow-up time from HDT is 51 (4-138) months in the single ASCT group and 46 (10-78) months in the double transplant group. For these groups, the median PFS was 29 (0-112) and 72+ (5-75) months (p=0.098), and the median OS 60 (0-138) and 78+ (10-78) months (p=0.078), respectively (Figure 1).

This non-randomized comparison between single and double autotransplantation as an up-front treatment of patients with MM shows that double autografting tends to