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Genetic Aberrations in Myeloma Cells

Thesis for the degree of Philosophiae Doctor

Trondheim, May 2009

Norwegian University of Science and Technology
Faculty of Medicine
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Genetiske avvik i myelomceller

Myelomatose (beinmargskreft) er kreft i plasmacellene i beinmargen. Det er den nest vanligste hematologiske kreftformen og rammer ca 300 personer i Norge hvert år. Kun 15% av pasientene er under 65 år. Det finnes ingen helbredende behandling for denne sykdommen, men i de siste årene har behandlingen blitt forbedret og i snitt lever pasientene nå i 5 år etter diagnosetidspunktet.

Genetikken i myelomatosecellene er ikke godt kartlagt, men grovt kan sykdommen deles inn i to genetiske grupper basert på antall kromosom og tilstedeværelse av translokasjoner i genet for den tunge immunglobulingenet (*IGH*). Den ene typen, hyperdiploid myelomatose, har 48-74 kromosom og sjelden *IGH*-translokasjoner, mens den andre hovedtypen, ikke-hyperdiploide myelomatose, har under 48 eller over 74 kromosom og ofte *IGH*-translokasjoner. Den hyperdiploide gruppen kjennetegnes ved at det ofte er tre kopier av minst fire av de åtte kromosomene 3, 5, 7, 9, 11, 15, 17, 19 og 21, men man vet enda ikke hva den grunnleggende genskaden som fører til kreftutviklingen er. Hos den ikke-hyperdiploide gruppen er det et fellestrekk at gen som er involvert i *IGH*-translokasjonen er den type gen som kan bidra i kreftutvikling.

Når man studerer myelomatose i laboratoriet bruker man vanligvis cellelinjer som er udødeliggjorte celler fra myelomatosepasienter. Alle til nå publiserte cellelinjer er ikke-hyperdiploide, og derfor har mesteparten av laboratorieforskningen på myelomatose blitt gjort på celler som bare representerer halvparten av pasientene.

I denne doktorgraden er det fokusert på genetikken til myelomatoseceller. Det er både lagt vekt på å beskrive cellelinjene i bruk på laboratoriet og å undersøke både generelle og spesifikke avvik i celler fra myelomatosepasienter.

Første artikkel er en beskrivelse av cellelinjen OH-2 som ble etablert i Trondheim i 1992. Det viktigste funnet er at cellelinjen OH-2 er den første hyperdiploide cellelinjen som er beskrevet. Cellelinjen har tre kopier av kromosom 3, 7, 15 og 21 og den har ingen *IGH*-translokasjon. Den har derimot en del av *IGK* (Immunglobulin lettkjede kappa-genet) satt inn mellom genene *MAFB* og *MYC* i en kompleks translokasjon.
Andre artikkel er en undersøkelse av Fibroblast vekstfaktor 3 (FGFR3). Cellelinjen INA-6 har ikke den vanlige translokasjonen mellom *IGH* og *FGFR3*, som er den eneste beskrevne årsaken til uttrykk av FGFR3 i myelomceller, men den uttrykker likevel FGFR3. INA-6 har i stedet en ekstra kopi av *FGFR3*, noe vi tror er skyld i uttrykket av FGFR3 i denne cellelinjen. Vi viser at uttrykket av FGFR3 er viktig i denne cellelinjen.
Tredje artikkel beskriver fosfatase i regenerativ lever 3 (PRL3), som tidligere er vist å være overuttrykt i metastaser i andre kreftformer. Det er også vist at ekstra kopier av *PRL3* genet er funnet i noen av disse metastasene. Vi undersøkte derfor om dette også var tilfellet i myelomatose. Det ser ikke ut til at ekstra kopier er utbredt i myelomatose, og det er mer sannsynlig at andre

faktorer enn ekstra genkopier er årsaken til høyt nivå i myelomcellene undersøkt.
Fjerde artikkel beskriver BCL3 som uttykkes i myelomceller som en respons på stimulering av forskjellige vekstfaktorer. *BCL3* genet er involvert i translokasjoner i andre kreftformer og vi fant også en translokasjon i *BCL3* locus i en pasient. Både PRL3 og BCL3 er høyt uttrykt i en undergruppe av pasienter med dårlig prognose, og begge genene/proteinene kan være viktige hos disse pasientene.
Femte artikkel er en foreløpig analyse av genetisk avvik og kliniske parametre som blant annet Beta-2-mikroglobulin og beinlesjoner hos nydiagnostiserte myelomatosepasienter i Norge. Vi fant ingen spesielle korrelasjoner mellom de ulike parameterne ved diagnose.

Arbeidet er utført i perioden 2006-2008 ved NTNU og St.Olavs hospital, med støtte fra Norges forskningsråd (NFR).

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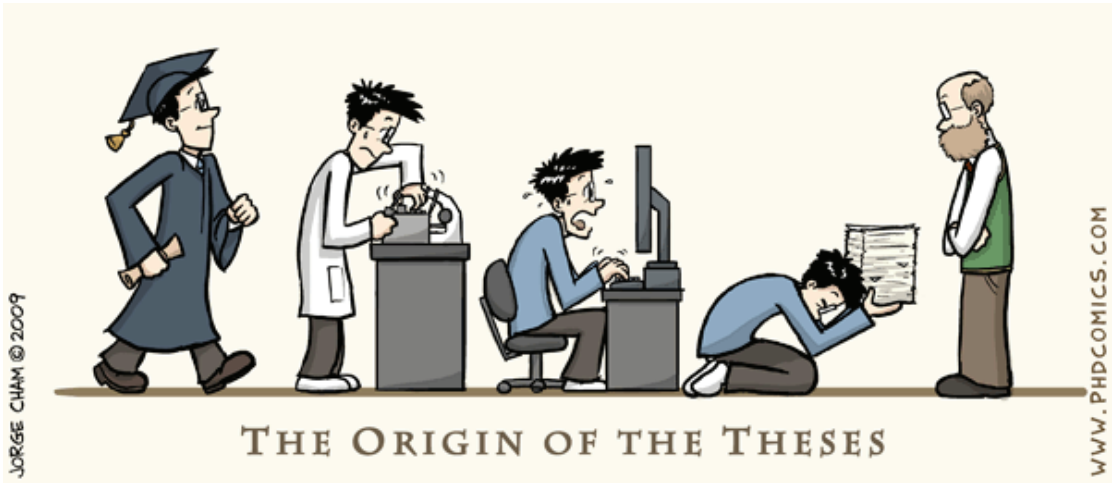

"A tribute to Charles Darwin", with permission Jorge Cham 2009, Piled higher and Deeper, www.phdcomics.com.

This work was carried out at the Department of Cancer Research and Molecular Medicine at NTNU in the period 2006-2008, and a smaller part at the Department of Pathology St.Olavs hospital in the same period.

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Trondheim, April 2009

Thea Kristin Våtsveen

LIST OF PAPERS

Paper I

Thea Kristin Våtsveen, Erming Tian, Stine H. Kresse, Leonardo A. Meza-Zepeda, Ana Gabrea, Oleg Glebov, Hong Yan Dai, Anders Sundan, W Michael Kuehl, Magne Børset. **OH-2, a hyperdiploid myeloma cell line without an *IGH* translocation, has a complex translocation juxtaposing *MYC* near *MAFB* and the *IGK* locus**
Accepted in Leukemia Research with minor modifications 02.03.2009

Paper II

Thea Kristin Våtsveen, Anne-Tove Brenne, Hong Yan Dai, Anders Waage, Anders Sundan, Magne Børset. **FGFR3 is expressed and important for survival in INA-6, a human multiple myeloma cell line without a t(4;14).**
Submitted manuscript

Paper III

Fagerli UM, Holt RU, Holien T, **Vaatsveen TK**, Zhan F, Egeberg KW, Barlogie B, Waage A, Aarset H, Dai HY, Shaughnessy JD, Jr., Sundan A, Borset M. **Overexpression and involvement in migration by the metastasis-associated phosphatase PRL-3 in human myeloma cells.** *Blood*. 2008;111:806-815

Paper IV

Anne-Tove Brenne, Unn-Merete Fagerli, John D. Shaughnessy Jr., **Thea Kristin Våtsveen**, Torstein Baade Rø, Hanne Hella, Fenghuang Zhan, Bart Barlogie, Anders Sundan, Magne Børset, and Anders Waage. **High expression of *BCL3* in human myeloma cells is associated with increased proliferation and inferior prognosis.** *European Journal of Haematology. 2009;82:354–363*

Paper V

Thea Kristin Våtsveen, Karin Fahl Wader, Lill-Anny G. Grøseth, Anders Sundan, Harald Aarset, Magne Børset, Anders Waage. **Genetic aberrations in Norwegian myeloma patients -a study based on interphase FISH on newly diagnosed patients from 2006-2008.** *Manuscript*

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3. SUMMARY OF PUBLICATIONS AND MANUSCRIPTS **37**

ABBREVIATIONS

AP	activator protein
BAC	bacterial artificial chromosomes
BM	bone marrow
CCND	cyclin D
CEP	centromere enumeration probe
CGH	comparative genomic hybridization
CH	IGH constant region
DEL	deletion
DER	derivative
DNA/RNA	deoxyribonucleic acid/ribonucleic acid
EMM	extra medulary myeloma
ECOG	Eastern Cooperative Oncology Group
FGFR	fibroblast growth factor receptor
FISH	fluorescence *in situ* hybridization
G-BANDING	giemsa banding
GEP	gene expression profiling
HGF	hepatocyte growth factor
HMCL	human myeloma cell line
HRD	hyperdiploid
IFM	Intergroup Francophone du Myelome
IGF	insulin like growth factor
IGH	immunoglobulin heavy chain
IGK	immunoglobulin light chain kappa
IGL	immunoglobulin light chain lambda
IκB	inhibitory κB
IL	interleukin
ITGB7	integrin β-7
IRF	interferon regulatory factor
ISS	international staging system
LSI	locus specific
MAF	musculoaponeurotic fibrosarcoma oncogene homolog C
MAFA	musculoaponeurotic fibrosarcoma oncogene homolog A
MAFB	musculoaponeurotic fibrosarcoma oncogene homolog B
MGUS	monoclonal gammopathy of undetermined significance
MM	multiple myeloma
MMGP	multiple myeloma genomic portal
MMSET	multiple myeloma SET domain
MUM-1	multiple myeloma oncogene-1
MYC	c-myc
MYEOV	myeloma overexpression gene
NF-κB	nuclear factor-κB
NHRD	non-hyperdiploid
OS	overall survival
PAC	plasmid artificial chromosome
PC	plasma cell
RB	retina blastoma
SKY	spectral karyotyping
SMM	smouldering multiple myeloma
TC	translocation and cyclin D
TLC	translocation
UAMS	University of Arkansas for Medical Science
VH	IGH variable region
WCP	whole chromosome paint

1. GENERAL INTRODUCTION

1.1 Multiple myeloma

1.1.1 Multiple myeloma epidemiology

Multiple Myeloma (MM) has probably existed for ages. Morse *et al.* described possible MM in skeletons from Indians (AD200-1300)[1]. The first published case of MM in the literature is Sarah Newburry, who was described by Samuel Solly in 1844[2]. Not until 1873 the term 'multiple myeloma' was introduced by the Russian von Rustizky[3]. The name came from the many tumors (greek, myelos=marrow, oma= tumor) that were present in the patient's bones.

24 500 persons in Norway are diagnosed with cancer each year. MM is the second most common haematological malignancy and 305 new cases were reported to the Cancer Registry in 2006. It is primarily a disease of the elderly and only 15 % of the patients are below the age of 65. MM is more common in men, with male:female ratio 1.5:1[4]. It is still an incurable disease but in the recent years with new therapy median survival is 5 years[5]. Also, some patients can live >10 years after diagnosis[6,7]. A Swedish study from 1973-2003 found that 1-year survival has increased and that survival after 5- and 10 years also have increased, but only in patients of age below 70[8]. In 2004 MM was responsible for 2.5% of all cancer-related deaths in Norway[4].

1.1.2 Clinical characterization

MM is a neoplasm of long-lived bone marrow plasma cells (PC)[9]. This accumulation of malignant plasma cells leads to monoclonal production of immunoglobulins (Ig). This can be measured in patient serum or urine, and excess secretion can lead to renal failure. The PCs will usually not be evenly

distributed in the bone marrow (BM), but be located in high numbers especially in the red bone marrow. In these locations the typical bone lesions arises, and bone pain is a common and debilitating symptom of MM. The international staging system (ISS) is the standard for staging of MM[10].

1.1.3 Prognostic factors

Several prognostic factors that identify groups of MM patients have been found. Not including genetic variations, which will be discussed later, predictors of survival are; age, ISS, hemoglobin level, creatinine, calsium, albumin, immunoglobulin subtype, and bone marrow infiltration[11].

1.1.4 The pathogenesis of multiple myeloma

MM, usually precedes by the pre-malignant tumor monoclonal gammopathy of undetermined significance (MGUS)[12]. MGUS is the most common lymphoid tumor and occurs in approximately 3% of persons over the age of 50[13]. MGUS is asymptomatic, and the line of a progression to MM increases by 1% each year[14]. Even though the prevalence of MGUS and MM is higher in African-Americans[15], and some evidence of familial clustering is seen[16-18], the effects of the genetics and the environment are not clear. An intermediate, usually asymptomatic, but more adverse pre-malignant stage is referred to as smoldering multiple myeloma (SMM). SMM progresses to MM at a rate of approximately 10-20% per year. It is however thought that not all MM cases progress through SMM[19] (also shown in figure 3).

1.2 Genetics in MM

Cancer is a genetic disease at the cellular level. The genetic disease can generally be divided into two scenarios of genetic events which contribute to transformation of the cell: Inactivation of genes by deletions, mutations, epigenetics, microRNAs or activation of genes by amplifications, translocations, mutations, epigenetics and microRNAs. The genes involved can be divided into three groups: oncogenes, tumor suppressor genes and DNA repair genes. Proto-oncogenes are genes that in healthy cells are involved in normal growth. Aberrations in proto-oncogenes lead to oncogenes that result in production of proteins that will enhance the cells' ability to growth and enhance cell divisions. Tumor suppressor genes are normal genes that codes for proteins that slow down cell growth and cell division or induce apoptosis. These genes might lead to malignancies if they are inactivated. DNA repair genes codes for proteins that remove mutations that arise during cell division. If these genes get mutated and loose their function, mutations in proto-oncogenes and tumor suppressor genes will not be repaired.

1.2.1 Genetic aberrations lead to dysregulation of several genes

To get a functional antibody repertoire it is crucial for the B-cell lineage to be able to rearrange its germ-line DNA. This is essential to create a functional adaptive immune system to fight infections. Hence, this is also a dual-edged sword and will occasionally lead to translocations of oncogenes to the Ig-loci. In all malignancies genetic aberrations are crucial for the development and transformation. In some malignancies there can be one or a few aberrations causing the transformation. In MM the initiating aberrations that lead to transformation are however still a mystery. It is unknown if there is any common genetic aberration in all MM patients. However, some aberrations are known and are shown to have impact on prognosis[20].

Classical cytogenetics has historically been used to define genomic aberrations by analyzing hundreds of patients. It has however been difficult to generate metaphases of patient tumor cells in MM because of the primary cells' slow proliferation rate. But recent studies using array comparative genomic hybridization (CGH) have revealed that virtually all MM patients have chromosomal abnormalities[21,22].

1.2.2 B-cell development

Normal B-cell development is illustrated in figure 1. During adaptive immune response, normal B-cells initiate variation (diversity) and joining (V(D)J) recombination. During primary immune response the immature B-cells migrate to secondary lymphoid organs to form pre-germinal center PCs. B-cells that enter the germinal center undergo affinity maturations by multiple rounds of somatic hypermutation of immunoglobulin heavy chain (*IGH*)- and immunoglobulin light chains κ/λ (*IGK/IGL*)- V region sequences. Cells that express high-affinity antigen receptors are selected for survival and undergo Ig switch recombination. A subset of activated B-cells then mature into memory B-cell differentiation into memory B-cells that can reside in the body for decades and then be activated upon exposure to the same antigen. Other B-cells form post-germinal center plasma cells that home to the bone marrow where they can be as differentiated, non-proliferating, long-lived PC for months and even years[23].

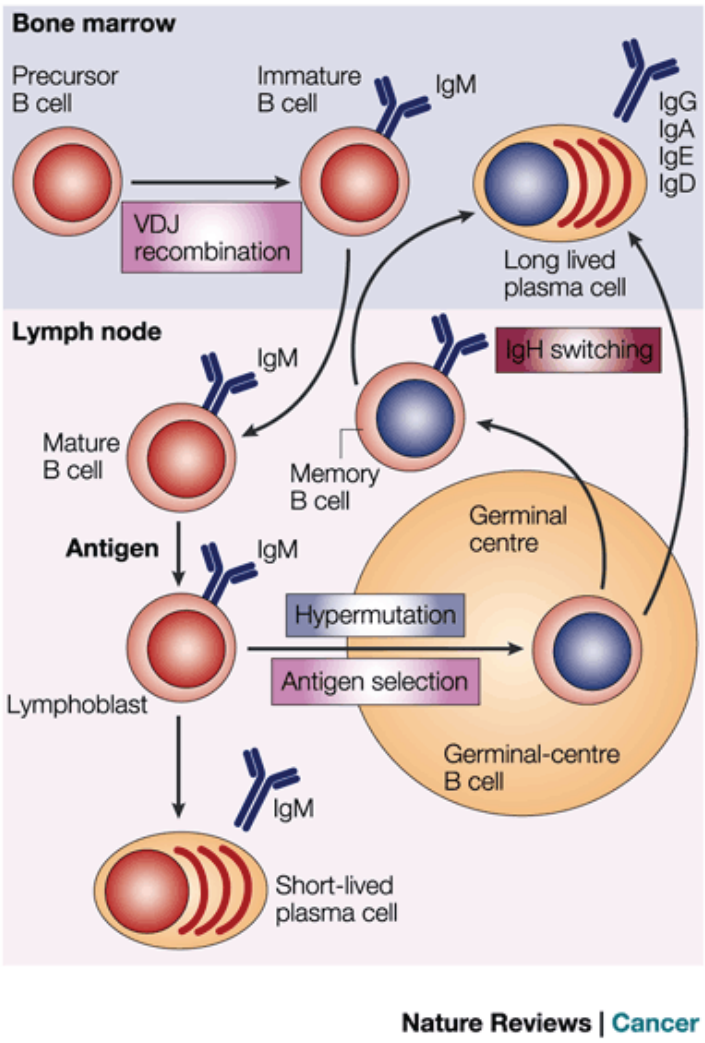


Figure 1
Normal B-cell development. Figure with permission[23]

1.2.3 Malignant B cell development: Translocations in MM involve three DNA modification systems

The translocations in the Ig genes are common in many B cell tumors, as well as in MM, and are thought to be caused by either: I) V(D)J recombination early in the B-cell development, II) somatic hypermutation in germinal center B cells or III) switch recombination in germinal center B-cells (figure 1). These processes can cause double strand DNA breaks in or near Ig, and it is thought that the same mechanism can occur near oncogenes, with the consequence of Ig translocations[24].

1.2.4 Primary translocations in MM

Primary translocations in MM are reciprocal translocations between the *IGH* gene at 14q32 and seven known genes. These translocations are termed primary since they are seen in MGUS, i.e. occur early and might initiate transformation. In primary translocations one of the strong *IGH* enhancers (Eμ, Eα1 and Eα2) is juxtaposed to an oncogene/proto-oncogene. The switch translocations in MM separate the strong 3`α- and μ-enhancers of the *IGH* onto different derivative (der) chromosomes. The enhancers can thereby turn on or up the transcription of the juxtaposed gene. The reciprocal partners to *IGH* are: 4p16, *MMSET/FGFR3*; 11q13, Cyclin D1; 12p13, cyclin D2; 6p21, Cyclin D3; 16q23, *MAF*; 20q12, *MAFB* and 8q24.3, *MAFA*. Translocations involving an *IGH* locus are present in nearly 50% of pre-malignant MGUS tumors, approximately 60% of fully malignant MM tumors, and nearly 90% of human myeloma cell lines (HMCLs)[25-28]. Figure 2 gives an overview of a normal switch recombination and an illegimate switch recombination resulting in a t(4;14)[23].

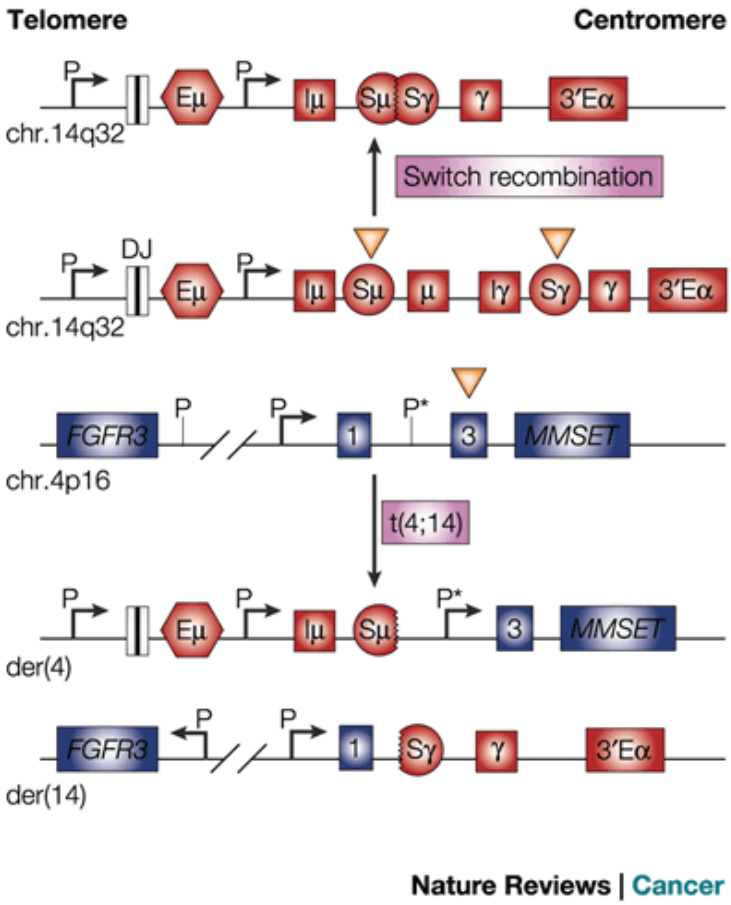


Figure 2
This figure illustrates a reciprocal translocation between *IGH* on chromosome 14 and *FGFR3/MMSET* on chromosome 4. The second line from the top shows germ line *IGH* gene, and the arrow up to the first line illustrates a normal switch recombination where the cells go from making IgM to IgG. The breakpoint leading to the translocation illustrated here is in the middle of the switch region and hereby dissociates the intronic enhancer (Eμ) and the 3'Eα. The double strand break dissociates the enhancers and the two ends are joined by another double strand breaks in another chromosome (here chr 4). By this the Eμ will be located on der(4) juxtaposed to *MMSET,* and can result in a hybrid mRNA transcript usually initiated from the Jh and the Iμ (intronic promoter). Also, the cryptic promoter (P*) on 4p16 can initiate transcription. The 3'Eα on der(14) dysregulates the expression of *FGFR3*. Illustration with permission[23].

t(4;14)

The t(4;14)(p16.3;q32) is one of the most common translocations and is present in 15% of newly diagnosed patients, but at a bit lower frequency in MGUS. The t(4;14) has not been described in other malignancies[29]. This translocation was also the first to show that *IGH* could dysregulate two genes at two derivative chromosomes, namely fibroblast growth factor receptor 3 (*FGFR3)* and multiple myeloma SET domain (*MMSET*)[30].

The translocations result in ectopic expression of functional FGFR3 in 70 % of the t(4;14) patients[31,32]. The reason for the lack of FGFR3 expression in the last 30% is mainly loss of der(14)[32]. FGFR3 is one of 4 high-affinity tyrosine kinase receptors for the FGF family of ligands. It is normally expressed in the lungs and kidneys, and it is expressed at high levels in the developing central nervous system, precursor bone cartilage rudiments, and resting cartilage at the end of growing bones[33]. FGFR3 is not normally expressed in PCs[34]. However, the chimeric *IGH/MMSET* gene fusion product is expressed in all t(4;14) patients[32,35]. The MMSET isoform RE-IIBP has been shown to be a histone methyltransferase with transcriptional repression activity[36]. MMSET has also been shown to be of importance in cellular adhesion, clonogenic growth and tumorigenicity[37], and knocking MMSET down has recently been shown to decrease the expression of genes that eventually decrease cell viability, adhesion and cell cycle progression[38].

t(4;14) has been associated with poor prognosis irrespective of FGFR3 expression[31]. Genetic studies show also that ~85% of t(4;14) patients have a deletion (del)13 (described later). These two abnormalities together show a worse prognosis than t(4;14) alone[29]. However, not all patients with t(4;14) have the same poor prognosis, and it might be correlated with other factors. This was found by the newest large study by Intergroup Francophone du Myelome (IFM), where t(4;14) patients with low β_2-microglobulin level showed longer survival compared to t(4;14) patients with high β_2-microglobulin level[20].

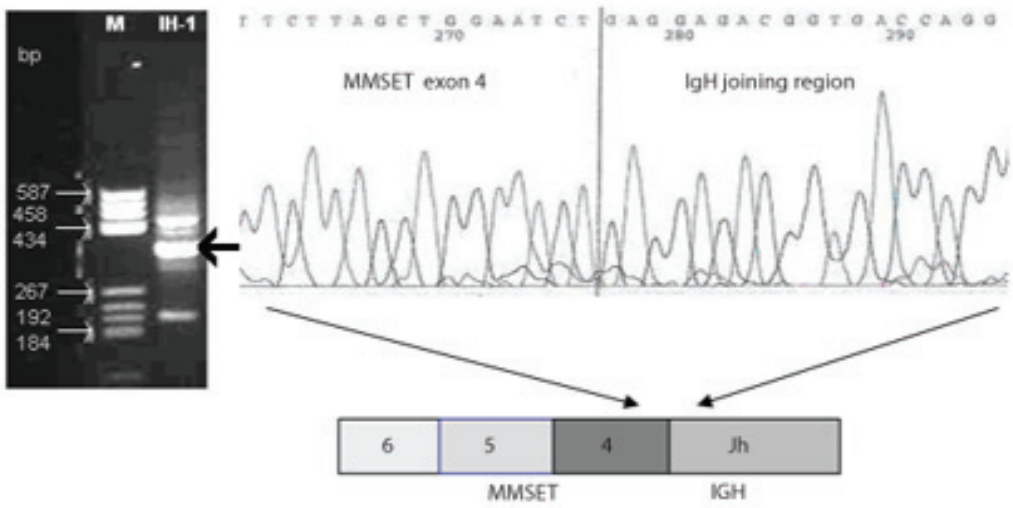


Figure 3
IH-1, a HMCL established here in Trondheim[39], has a t(4;14) (paper 2) and a fusion transcript detected by PCR with primers in Jh in *IGH* and exon 6 in *MMSET*. Left shows the gel with the specific fusion band next to the arrow. The joining area of *IGH* is fusioned to exon 4 of MMSET detected by sequencing the PCR product. The breakpoint in chromosome 4 is in the intron between exon 3 and 4 in this particular HMCL. Illustration prepared by author.

t(11;14)

The t(11;14)(q13;q32) is also one of the most common translocations in MM patients and is present in ~15% of newly diagnosed patients as well as in MGUS. Cyclin D1 (*CCND1*) at 11q13 encodes CCND1 which is over-expressed as a consequence of this translocation[40]. Myeloma over-expressed gene (*MYEOV*) is also in the same locus and has been shown to be over-expressed. The function of this gene is not well known[41]. So far the oncogenic role of the cyclin D1 over-expression is not known either, even though the cyclin Ds control entry of the cell cycle to the S (DNA-synthesis) phase by binding and activating cyclin-dependent kinases -4 and -6, which phosphorylate the retinoblastoma proteins, and thereby promotes the cell cycle [42]. Even though the cyclin Ds are involved in the cell cycle, the t(11;14) MMs are characterized by a low proliferation index[43]. Although an increased cyclin D1 expression does not cause increased proliferation, it might make the cells more susceptible to other proliferative stimuli from the microenvironment, e.g. BM stromal cells that express interleukin (IL)-6, insulin-like growth factor (IGF)-1 or other cytokines[25]. Early studies reported the t(11;14) patients to

show better survival[44]. However, later and larger studies did not confirm this better survival[20,45].

t(14;16), t(14;20) and t(8;14)

The t(14;16)(q32;q23) is present in ~5% of MM patients[46]. This translocation is not known in other malignancies than MM[29]. The t(14;20)(q32;q11) is even less common and is present in less than 1% of MM patients[47]. The translocations dysregulate musculoaponeurotic fibrosarcoma oncogene homolog (*MAF/c-MAF)* and *MAFB,* respectively. MAF proteins belong to the activator protein (AP)-1 superfamily of basic leucine zippers. MAFs work as transcription factors that positively regulate e.g. cyclin D2 (*CCND2*), Integrin β7 (*ITGB7*) and *ARK5* together with other *MAF*-related genes[26,48,49], that induce deregulation of cell cycle, cell-cell interaction and migration, respectively. Another feature is that MAF in oncogenesis has an ability to enhance the interaction between tumor cells and stromal cells[49]. Surprisingly, when compared to the translocation frequency, MAF was overexpressed in half of MM primary samples[49]. The mechanism behind this is not yet understood[50], and with GEP only 8-10% of the samples with overexpression of *MAFs* cluster together[51]. *MAFA (L-MAF)* on 8q24.3 has also been shown to be involved in MM, but at a much lower level (<1%) (mentioned in figure 5)[9]. The t(14;16) has been associated with short survival[28].

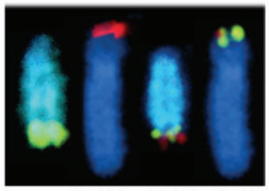
t(6;14) and t(12;14)

The t(6;14)(p21;q32) and t(12;14)(p13;q32) dysregulate cyclin D3 (*CCND3*) and *CCND2* and are present in ~2% and <1% of MM respectively[9]. The molecular consequences of these translocations are not well characterized.

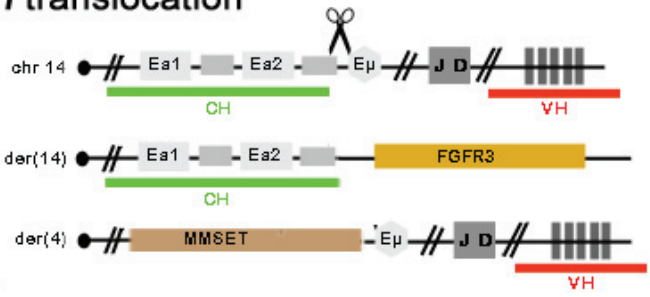
1.2.5 Structures of *IGH* translocations in MM

The structures of *IGH* translocations in MM tumors can be divided into four groups; a) the classical balanced translocations have no loss of DNA, and can be distinguished by Fluorescence *in situ* Hybridization (FISH) (described later) by dissociation of the *IGH*-constant region (CH) and *IGH*-variable region (VH) signals. CH is left on der(14) while VH is located on the telomere of the other derivative chromosome. This is the typical pattern found in the five primary *IGH* translocations; b) the variant translocations are distinguished by the whole *IGH* locus translocated to a derivative chromosome. Seen by FISH the CH and VH is colocalized on the derivative chromosome, and the der(14) has no CH/VH signal. It is therefore not possible to detect this variant translocation by conventional interphase FISH; c) the unbalanced translocations are described by a FISH pattern where there is either a der(14) CH signal or a VH signal, and the other derivative chromosome is lost; d) The fourth group of *IGH* translocations is the simple insertions. The CH signal is dissociated from the VH signal and is found internally on the recipients chromosomes. Usually, no whole chromosome paint (WCP)14 is detected together with the CH signal[23,52]. Figure 4 explains these kinds of translocations with metaphase FISH picture and chromosome illustration. By Spectral Karyotyping (SKY) it is only possible to detect some of the balanced translocations, and unbalanced translocations der(14). The other variants are hard to detect because of the relative small fragments of DNA that have been translocated. Hence, FISH with locus specific probes must be used.

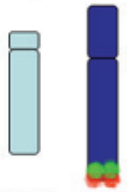
A) Classical *IGH* translocation



der(14) CH | der(4) VH | der(14) CH FGFR3 | der(4) VH MMSET

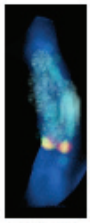


B) Variant *IGH* translocation



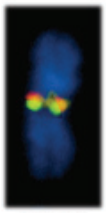
der(14) | der(8) +CH +VH

C) Unbalanced *IGH* translocation



der(14) CH CCND1

D) Simple insertion



der(11) CCND1 CH

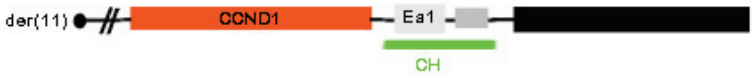


Figure 4. The different *IGH* translocations in MM: A) The classical *IGH* translocation is demonstrated by the t(4;14) in HMCL IH-1 (paper II). The two chromosomes to the left are shown with CH in green and VH in red, with VH translocated to chromosome 4. The two chromosomes to the right show the specific translocation with *IGH* in green and *FGFR3/MMSET* in red. A fusion signal between the labeled loci appears yellow. WCP in Aqua on chromosome 14. The chromosome drawing shows where the FISH probes cover the *IGH* gene. CH probe is labeled in green and VH probe is labeled in red. The der(14) and der(4) chromosome drawing depict the two derivative chromosomes and its translocation partner. The black small circles image the centromere. B) The variant *IGH* translocation is depicted by a figure because our laboratory does not have a HMCL with known variant *IGH* translocation. The der(14) to the left has lost the whole *IGH* which has been translocated to chromosome 8. This translocation results in a t(8;14) that dysregulates *MYC,* and can be found in e.g. HMCL XG-1[52]. The chromosome drawing to the right depicts the der(8). C) The unbalanced translocation is depicted by INA-6 where der(11) is lost, and der(14) with CH in green is translocated to chromosome 11 with *CCND1* in red. WCP aqua on chromosome 14. Chromosome drawing to the right depicts the only derivative translocated chromosome. D) CH in green has dissociated from VH and is inserted into *CCND1* in red on der(11) in U266. The chromosome drawing to the right depicts that a small part of CH is juxtaposed to *CCND1*. The black box depicts the rest of chromosome 11. The figure is made by the author after inspiration in article text from ref[23,52].

1.2.6 Secondary translocations

Secondary translocations occur during late stage of MM progression and do not involve B-cell specific DNA modification mechanisms. Secondary translocations also include the *IGL* gene (10%) and *IGK* (<1%). The secondary translocations are rarely reciprocal, and can also be complicated insertions of the genes[53]. Almost 20% of MM patients with *IGH* translocation involved a non-recurrent translocation partner, and many of these are secondary translocations[29,52].

MYC

Perhaps the most important secondary translocated gene in MM is *MYC* at 8q24. MYC was one of the first oncogenes identified, and has been linked to a spectrum of malignancies[54]. MYC is an important transcription factor that regulates cell growth, differentiation and apoptosis. In Burkitts lymphoma, *MYC* translocation to an Ig-gene is an early and unifying event[55]. This is in

contrast to MM where dysregulation of *MYC* apparently is caused by complex genomic rearrangements during late stages of MM progression involving B-cell specific DNA modification mechanisms[56]. In MM, *IGH-MYC* translocations are often a part of a complex rearrangement. Almost 90% of the HMCLs have *MYC* rearrangements and express high amounts of MYC detected on microarray (e.g. Paper I). Many *MYC* rearrangements involve an Ig locus, but the break point is only near the locus and not within the switch regions or the V(D)J sequence. This is similar to other *IGH* secondary translocations where the brake points are not involving J or switch region. The *MYC* translocations are mostly non-reciprocal or involve insertions, amplifications, inversions, and three chromosomes are often involved in the rearrangement[57]. In e.g. HMCL JJN-3 the *IGH-MYC* fusion was found on two different chromosomes [58]. And the HMCL RPMI-8226 has the *MYC*-region associated with *IGL* in a complex translocation to *c-MAF*[56]. This is very much like the *IGK* rearrangement in OH-2, where the *IGK3'*-enhancer is juxtaposed to *MYC* and *MAFB* (paper I). It was quite difficult to detect this *IGK3'*-enhancer by FISH, and similar translocations in other HMCLs and patients can easily be missed. *MYC* rearrangement has the same prevalence in both hyperdiploid and non-hyperdiploid tumors (explained later)[52]. *MYC* rearrangements are however rare in MGUS and SMM and are present in ~15 % of newly diagnosed MM tumors[59]. 40 % of the *MYC* translocations do not involve an Ig locus[57]. *MYC* translocation has no apparent impact on the progression of the disease[20], but it might have a yet hidden prognostic impact, since MYC is expressed also by other, yet unknown, mechanisms than translocations in the patients[20,60].

MYC has many target genes, and it is not known which are the most important in MM. Lately interferon regulatory factor 4 (IRF4, also known as multiple myeloma oncogene 1 (MUM1)) and MYC were found to positively reinforce the expression of each other in MM HMCL. IRF4 worked as a transcription factor that targeted *MYC* in myeloma cells, and at the same time *IRF4* was also a downstream target gene for MYC. Knocking out IRF4 was found to kill MM HMCLs, as did the knocking out of MYC[61].

1.2.7 Other genetic aberrations

Chromosome 13 deletion

Chromosome 13 deletion (del13), or more specifically 13q14 retinoblastoma (*RB)-1* deletion occurs in about half of MM karyotypes[62]. This abnormality is probably an early or primary event, since it is observed with similar frequency in MGUS and in patients with relapsed MM. The molecular consequences are not well known, and because most del13s are monosomies, many genes are lost and may be deregulated. GEP has revealed that this feature has a molecular signature[60,63]. Del13 was one of the first genetic markers to be used as a prognostic marker[64-66]. University of Arkansas for Medical Science (UAMS) published in 1995 the first findings of monosomy 13 having negative impact on survival[67], which was also confirmed later in their total therapy II study[68]. Chiecchio claims there is prognostic impact in del13 when found by conventional cytogenetics but not by interphase FISH only[69]. In the recent IFM study, del13 identified with FISH was not an independent prognostic factor, but was associated with poor prognosis related to the concomitant t(4;14) or del17p. Del13 may therefore not be a specific prognostic factor after all in MM, and may be considered a pseudomarker or a marker frequently associated with other more specific poor prognostic factors[20].

Deletion of 17p13

In MM it has more recently been found a deletion in chromosome 17p in 10% of newly diagnosed patients. *TP53* is located on 17p13 and down-regulation of the gene was correlated to the 17p13 deletion, which therefore makes *TP53* the top-target gene to be the important gene in the deleted locus. Mutation in *TP53* is associated with significantly shorter survival, with median survival only 1.5 years[70], and an IFM study found it associated with poor outcome[20]. Since TP53 is involved in apoptosis and induced by most chemotherapeutic agents, the loss or/and mutations in *TP53* may participate in the treatment resistance seen in del17 patients[29]. It appears that mutations

in *TP53* are rare, and it was reported in only 3% of the patients in a large study. Half of the cases with mutation were correlated to hemizygous loss of 17p13[70].

Chromosome 1

As we also show in paper I, for the OH-2 HMCL and primary cells, the 1q region is gained. This is also the case in about one third of MM patients[71]. UAMS reports poor outcome of patients with 1q abnormalities, with locus 1q21 especially in focus[72], but a Mayo study and IFM report that the prognostic value disappears when combined with other classical biological and genetic prognostic factors[20,29,73]. Since the correlation between the 1q amplification and prognosis is weak it is suggested that this is more of a marker of a clonally advanced and genomic unstable tumor that is more likely to have a faster progression[9].

Deletions in 1p are also common. A study on 1p32 shows that 15% of MM patients have a deletion of this locus harboring *CDKN2C.* Patients with hemi- or homozygote deletions had a shorter overall survival and the homozygote deleted cases were the most proliferative myelomas[74].

1.2.8 Ploidity

MM can be genetically classified in two: hyperdiploid (HRD) and non-hyperdiploid (NHRD) tumors. It has been established that both MM and MGUS can be separated by these two groups distinguishable by chromosome content[75-78]. Approximately 50% of tumors are hyperdiploid and contain 48-74 chromosomes. Although this classification appears somehow artificial, the HRD appears as a relatively uniform group, typically with non-random gains of at least four of eight odd-numbered chromosomes (3, 5, 7, 9, 11, 15, 19, 21). The remaining tumors are non-hyperdiploid, containing less than 48 and/or more than 74 chromosomes (near tetraploid). These two groups are further

classified by *IGH* translocations with the seven reciprocal partners described above. These are present in about 70% of NHRD tumors but only in about 15% of HRD tumors[79].

It appears that recurrent *IGH* translocations and HRD are primary events occuring early in pathogenesis. Secondary translocations, which include most *IGH* rearrangements not involving one of the seven recurrent partners, most *IGL* and *IGK* rearrangements, and *MYC* rearrangements, appear to contribute equally to progression of both HRD and NHRD tumor[52].

A lot has been published on NHRD MM with primary translocations, and virtually all HMCLs used in laboratories are derived from NHRD tumors. On HRD MM, however, there has just been a few publications, and only from more recent years. Two studies from Chng *et al* have exploited the GEP and the prognostic factors for the HRD tumors. They conclude that HRD patients have a better partial event-free survival and overall survival than NHRD patients. The presence of *IGH* translocations, especially those with unknown partners, has a negative impact on the HRD tumors. These translocations are perhaps a result of later secondary translocation events due to genomic instability in advanced tumors[80]. The GEP was able to identify four biologically relevant subtypes with prognostic implications. Cluster 1 is defined by overexpression of various cancer testis antigens and mitotic/proliferation-related genes. Since hypomethylation is implicated in the expression of cancer testis antigens in tumors, it is a possibility that the GEP is a surrogate marker for deregulated epigenetic mechanism. Cluster 2 is defined by overexpression of hepatocyte growth factor (HGF) and interleukin (IL)-6 genes. Deregulation of HGF induces migration, survival and growth via multiple downstream pathways. IL-6 mediates similar effects. Cluster 3 is defined by overexpression of genes involved in nuclear factor (NF)-κB-signalling. NF-κB is important in many downstream effects of MM–bone marrow interactions. Cluster 4 was less defined, except for the lack of high expression of the above genes, and with particularly low expression of HGF[81].

Another interesting finding is that HRD are more common in MGUS than NHRD. In addition, del13 is far more common in the HRD MGUS, in contrast to in MM where the del13 is more common with NHRD[82].

1.2.9 Molecular pathogenesis

The latest model for the molecular pathogenesis by Chng *et al* is shown in figure 5. The figure summarizes most of the events described above. Figure 5 shows the two pathways of pathogenesis; the NHRD and the HRD pathway. Four early events are described, for which the timing is yet unknown: *IGH*-translocations, HRD with multiple trisomies, loss of chromosome 13 sequences and dysregulation of a *CCND* gene. Later events include other Ig translocations, and secondary translocations involving *MYC*, mutations of *KRAS*, *NRAS* or *FGFR3* in t(4;14) tumors. Inactivation of *TP53* by various mechanisms is a progression event. Also, mutations to constitutively activate the nuclear factor (NF)-κB-pathway occur in half of MM tumors. This might induce independence from environmental factors necessary for activating pathways at earlier stages. Also, other secondary translocations, inversions, insertions, deletions, methylation of promotors or genes and additional inactivation of the RB1 pathway can occur at all stages[9].

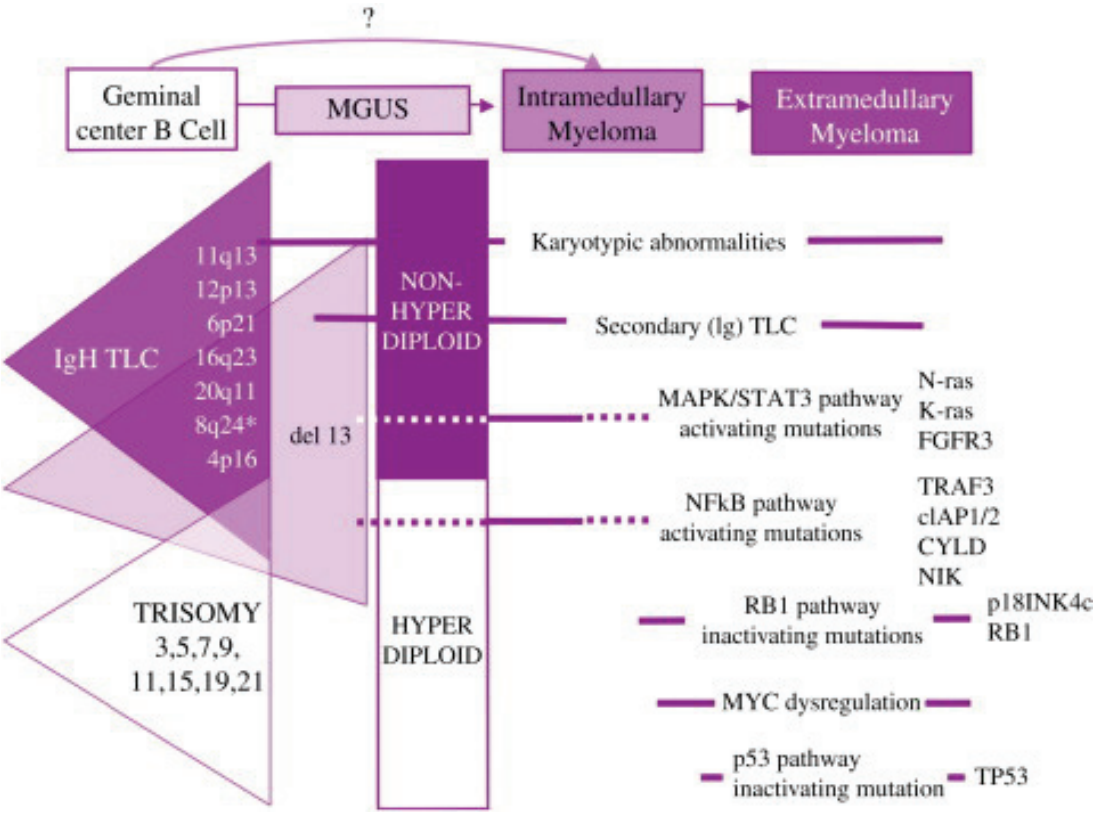


Figure 5
Description of disease stages, with molecular pathogenesis and timing of oncogenic events in the tumorigenesis of MM. The degree of overlap between triangles estimates the percentage overlap of genetic subgroups harboring coexisting genetic abnormalities. The translocation partners in the *IGH* translocation (TLC) group are ordered according to increasing frequency of coexisting del13. The different mutations activating signal or cell-cycle pathways are mutually exclusive, i.e. *RAS* and *FGFR3* always occur in different patients. *The 8q24 partner referred to here is *MAFA; MYC* is also located in this locus, but is usually a secondary *IGH* translocation. Illustration with permission [9]

1.3 Genetic and molecular classification systems of MM

Several MM research groups have made their own classification systems for MM. Since the survival period for patients varies from a few months to more than ten years it is important to be able to identify different risk groups. New advanced technologies have made it easier to look globally at genetic aberrations at both DNA and RNA levels. Perhaps some models are more useful for classifications of single patients than others. In the future, we hopefully have easily accessible technologies to do studies at the protein level, which might make it easier to see the full picture of the current classifications. I also miss a microRNA profile study in MM, but hopefully it will come in reasonable future. A classification system with high predictive power would hopefully contribute to tailored treatment for the MM patients.

1.3.1 Translocation and cyclin D (TC) classification

A characteristic feature of MM cells is the expression of *CCNDs*. Together with the occurrence of *IGH* translocations, *CCND* expression form the basis for the TC classification of MM. This classification of the patients is based on the observation that most tumor cells in MGUS and MM, have relatively high levels of *CCND* mRNAs. This, combined with the type of primary *IGH* translocation, gives the eight groups [listed a)-h)] described by Bergsagel and Kuehl (2005). The groups are: a) 11q13: with t(11;14) and *CCND1* expression, NHRD, (16 % of cases); b) 6p21: with t(6;14) and *CCND3* expression, NHRD, (3 %); c) D1: no t(11;14) but with *CCND1* expression, HRD, (34 %); d) D1+D2: As D1 but with additional *CCND2* expression, HRD, (6 %); e) D2: *CCND2* expression, HRD=NHRD, (17 %); f) none: express no cyclins, NHRD, (1 %); g) 4p16: with t(4;14) and *CCND2* expression, NHRD>HRD, (15 %) and h) MAF: with either t(14;16) or t(14;20) and *C-MAF/MAFB* and *CCND2* expression, NHRD, (7 %)[25].

This classification system has the advantage that it involves few markers that need to be measured, and the primary translocations can easily be detected with interphase FISH. However, the TC classification does not identify the HRD MM, and the majority of these patients will fall into the D1 and D1+D2 groups. There is no significant difference in survival for D1 and D1+D2[9,25,26].

1.3.2 Molecular classification based on gene expression profile

At UAMS, seven groups of MM patients were identified based on co-expression of unique gene clusters based on gene expression profile (GEP). The seven subgroups are classified by unique expression patterns. PR: proliferation; LB: low bone disease; MS: MMSET; HY: hyperdiploid; CD-1: cyclin D1; CD-2: cyclin D3; MF: MAF/MAFB. These groups also identify the primary *IGH* translocations[51]. This classification is relevant because it defines the high-risk groups, PR and MS. However, the study is made on a large set of genes and it might be difficult to use it clinically for single patients[9].

UAMS also identified a 17-gene model based on GEP, sufficient for defineing the high risk myeloma patient. Most of these genes are on chromosome 1 and the profile does not detect the different *IGH* translocation groups as in their gene cluster grouping[72]. Similar to this, the IFM proposes a 15-gene model to define the high risk myeloma patient. The two models do not share a single common gene. When applying both the 17- and the 15-gene model together, it is possible to identify subgroups that e.g. do not benefit from bortezomib[83]. Both these models might be very useful for tailored therapy.

1.3.3 Genetic factors and β-2 microglobulin based classification

The IFM has one of the newest models to predict prognosis. It uses genetic factors t(4;14) and 17p13 deletion detected by FISH combined with β-2 microglobulin level. The high risk group is defined by the presence of either t(4;14) or 17p13 deletion and β-2 microglobulin above 4, and it could dissect

the survival of patients in each ISS stage. This study shows that it is possible to find high risk groups with FISH and simple parameters like β-2 microglobulins[20]. This might be one of the most easy ways for classifying the patients, and manageable for most hospitals. However, this did not correlate with the Eastern Cooperative Oncology Group (ECOG) clinical trials. They saw no difference in survival comparing t(4;14) and del17 patients with β-2 microglobulin level. The IFM and the ECOG patients have been treated differently, and this factor might also be included to get the right picture of this way of classifying high risk patients[84].

1.3.4 Array Comparative Genomic Hybridization (CGH) based classification

The two main genetic groups, HRD and NHRD can be detected using array CGH to classify MM. The array study by Carrasco *et al.* also divided the HRD into two subgroups based on gain of 1q, del13 in the poor prognosis group and trisomy 11 in the better prognosis group. The NHRD could also be separated in two groups mainly by one of them having chromosome 1 abnormalities but with no significant difference in survival[21]. The finding on array CGH does not give more information than FISH regarding prognosis.

1.4 How to discover chromosomal abnormalities

In 1914 Theodor Boveri described in his book “Zur Frage der Entstellung maligner Tumoren“, an hypothesis that chromosomal abnormalities were the cellular change that lead to transformation. But at the first part of the 20th century it was difficult to prove his hypothesis. Not until Caspersson *et al*. introduced techniques to detect the chromosome bands, the cytogenetic analysis was revolutionized. Each and every chromosome could now be identified by its specific bands[85]. One of the famous chromosome aberrations is the Philadelphia chromosome, discovered by Nowell and Hungerford in 1960[86]. This t(9;22) was the first documented bona fide genetic signature of malignancy. The discovery that this was a t(9;22) and not a deletion of chr 22 was made because of improved techniques for chromosome banding, the Giemsa-banding (G-banding)[87]. Still Giemsa-banding is routinely used to visualize chromosome bands. Unfortunately the genomic aberrations needs to be more than 3Mb to be detected[88].

In situ hybridizations allow analysis of nucleic acids in its cytogenetic context on the chromosome, in the nucleus or in tissues. This technique was developed in the late 60ies using radioactive labeled probes. In the 80ies the radioactivity was replaced by the less harmful fluorescence dyes. Fluorescence *in situ* hybridization (FISH) was born[89].

1.4.1 Fluorescence *in situ* hybridization

FISH uses fluorescence labeled DNA probes that are homologous to the target gene or area. There are basically three different FISH-probes; locus specific probes (LSI) that cover one gene/locus, centromeric enumeration probes (CEP) and whole chromosome paint (WCP) probes. FISH on nuclei in interphase (interphase FISH), uses one to three LSI probes to e.g. discover chromosome translocations. CEP and LSI together are useful for detection of deletions or amplifications of genes and areas. The limitations here are that

you can only look at three to four colors at the same time, and it is necessary to have a hypothesis on what is wrong, in order to choose the right probes. In routine work, the detection of the primary *IGH* translocations and chromosome deletions in MM are done by interphase FISH on MM cells from patients. The *IGH* is labeled in e.g. green, and the other gene of interest, e.g. *FGFR3,* is labeled in red. A fusion signal, where green and red fluorescence are seen together, indicates that a translocation is present (Figure 6a). A rule of thumb is that in nuclei in interphase, a fusion signal between two probes means they are no more than 500kb apart.

Metaphases and FISH on metaphase chromosomes give some more information about a tumors' genetics. But because it is difficult to get informative metaphases from the low proliferative MM cells, this technique is therefore not suitable on a routine basis. However, FISH on metaphase chromosomes is a very useful technique for research purposes, especially when using HMCLs. The use of WCP, CEP and LSI probes together are often very informative for detecting new aberrations in the cell. Fig 6b shows HMCL OH-2 metaphase with both CEP and LSI probes. Doing the same experiment on interphase FISH would only give the number of *MYC* loci present compared to the number of chromosomes, and the fact that the extra copy was on the p-arm of chromosome 8 would have been missed.

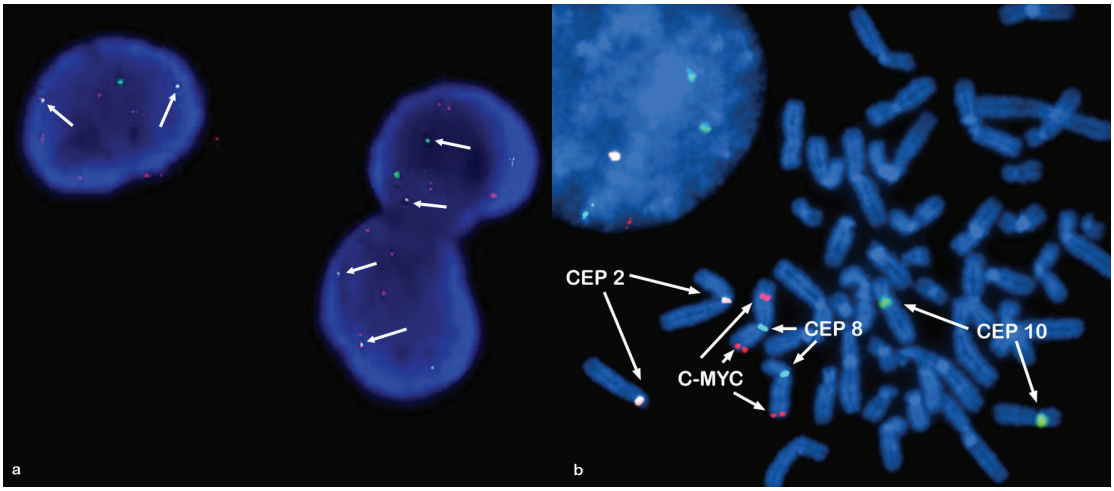


Figure 6
a) Interphase FISH on CD-138 separated MM cells from a patient. Arrows show fusion signals between *IGH* and *FGFR3/MMSET*. Green signals alone are normal *IGH* on chromosome 14, and red signals alone are normal *FGFR3/MMSET* on chromosome 4.
b) Metaphase FISH on HMCL OH-2. CEP probes help determine the different chromosomes and identified an extra copy of *MYC* at chromosome 8 (paper I). Pictures prepared by author.

1.4.2 Spectral Karyotyping (SKY)

SKY is the deluxe variant of metaphase FISH. With SKY each chromosome gets its own pseudocolor based on the combination of fluorescence-labeled probes. The advantage is that it is easy to visualize and detect novel deletions, translocations and amplifications. The resolution however, is not great. To be able to detect an abnormality it needs to be larger than 3-5Mb. It is also not possible to know which part of the chromosome which is involved in the abnormality without consulting e.g. corresponding Giemsa-banding or doing LSI FISH. Successful multicolor/SKY FISH was first available in the 90ies[90,91]. An example of SKY is given in section 4 in figure 7a of the HMCL RPMI 8226.

1.4.3 Array Comparative Genomic Hybridization

Conventional comparative genomic hybridization (CGH) is a technique where DNA is extracted from e.g. a tumor sample, labeled in one color and normal DNA is labeled in another color, and both samples are co-hybridized to normal metaphase chromosomes. Amplifications or deletions in the tumor sample will then be visualized on the corresponding normal chromosome, and it is possible to detect the specific area with aberrations. This technique has been improved by replacing the metaphase chromosomes by a microarray chip where DNA from clones or sequences has been spotted on a slide. The samples are labeled the same way as in conventional CGH but are co-hybridized to the array. The resolution is restricted only by the number of clones or spotted sequences. An array with overlapping clones will cover the whole genome[92]. An example of an array CGH of HMCL RPMI-8226 is shown in section 4 in figure 7b. This array CGH is made from bacterial artificial chromosomes (BAC) and Pi artificial chromosome (PAC) at a ~1 Mb resolution. In addition, it has a tiling path between 1q12 and the beginning of 1q25[93].

2. AIMS OF THE STUDY

The overall objective of this work was to study the genetics of myeloma cells. Especially we wanted to focus on not well known genetic aberrations linked to expression data in HMCLs and complement other studies in our myeloma group. But also a goal was to detect known genetic aberrations in Norwegian myeloma patients. More specifically the aims can be divided into the following aspects:

Characterization of HMCLs

It is important to choose the most relevant HMCLs to work with. Therefore it is important to characterize HMCLs both genetically and also how they respond to e.g. different stimulations of cytokines. First and foremost, the HMCLs established at our own lab were investigated. When we found OH-2 to be a hyperdiploid HMCL, it was important to characterize this cell carefully, so also others could fully enjoy the potential of it. Important methods have been, among others, FISH, array CGH, SKY, gene arrays, real time RT-PCR and proliferation studies.

Are there genetic aberrations that cause the over-expression of oncogenes, potential oncogenes or transcription factors in myeloma cells?

Many projects in our group are based on microarray data. Known putative oncogenes and known oncognes from MM and other cancers, as *FGFR3* and *PRL3*, and other potential oncogenes, as *BCL3,* were studied. Since MM often has translocations and genetic aberrations, it was a goal to see if the overexpression of some of these genes were caused by translocations or amplifications.

Study of genetic aberrations in Norwegian MM patients

Unfortunately MM is a very heterogeneous genetic disease. Interphase FISH is the preferred method to detect the known genetic aberrations. In addition to detect the most prevalent translocations, we wanted to look at the prevalence of some of the less common translocations in the Norwegian patient population, and to find out if the Norwegian patients differed from patients from other parts of the world. We also wanted to see if we could find correlations between genetic aberrations, and other parameters measured at time of diagnosis (e.g. β-2 microglobulin, M-component and PC%). Also, a part of the study was to make the preparation of cells and the FISH method as short, easy and reproducible as possible.

This is a prospective study, and it is not finished. The study started in 2006 and we decided to include 300 patients. We have only come half way in the patient inclusion process, and therefore the data on overall survival, response, time to remission and type of treatment has not yet been collected. The aim in the end is to see if there is any correlation between genetics, clinical findings at diagnosis, treatments, time to relapse and overall survival in the Norwegian MM patients. Because this is a prospective study we do not report other findings than what were measured at the time of diagnosis.

3. SUMMARY OF PUBLICATIONS AND MANUSCRIPTS

3.1 Paper I

OH-2, a hyperdiploid myeloma cell line without an IGH translocation, has a complex translocation juxtaposing MYC near MAFB and the IGK locus

Despite the heterogeneity, MM can be classified into two major groups: hyperdiploid HRD tumors with 48-74 chromosomes, which typically have extra copies of at least four of the eight odd numbered chromosomes 3, 5, 7, 9, 11, 15, 19, and 21; and non-hyperdiploid NHRD tumors, which usually have *IGH* translocations and 46< and >74 chromosomes. HMCL with a typical HRD phenotype was lacking, and this made the characterization of OH-2 even more interesting. The OH-2 HMCL is derived from extramedullary myeloma (EMM), and retains the same HRD phenotype as the EMM tumor, with extra copies of chromosomes 3, 7, 15, 19, and 21 as demonstrated by the array CGH. This provides a unique example of an HMCL and the corresponding primary tumor that share the same HRD phenotype. Spectral Karyotyping shows the same HRD phenotype in the HMCL. We also did a microarray of the HMCL to look at the expression of genes and compare it to a panel of 47 other HMCLs. High expression of cyclin D2, *MAFB* and *MYC* were striking findings, which were also confirmed by realtime RT PCR on the primary tumor material. This expression we eventually found out was caused by a complex translocation. The *IGK* enhancers had been translocated to *MYC* on chromosome 8 and also juxtaposing *MAFB* on chromosome 20 to the enhancers. The breakepoints were identified by high density array CGH. As a result of this analysis, we identified breakpoints, manifested by an approximately 30% decrease in copy number, involving all three loci: 685 kb telomeric of *MYC*; 138kb centromeric of *MAFB*; 10kb centromeric and 18 kb telomeric of the 3’ kappa enhancer. These results indicate that the 3’ kappa enhancer effectively is inserted between the *MYC* and *MAF* genes, so that both genes can be dysregulated by the same enhancer element.

3.2 Paper II

FGFR3 is expressed and is important for survival in INA-6, a myeloma cell line without a t(4;14).

FGFR3 is an oncogene dysregulated by the t(4;14) in myeloma cells, and is not usually expressed without this translocation. In INA-6 we discovered an amplification of the *FGFR3* locus by FISH. This amplification did not involve any immunoglobulin loci. By array CGH we discovered that the area of chromosome 4 from 4p15.32 to the telomere of the p-arm was amplified. This was in concordance with the extra copy found on metaphase FISH. This extra copy probably causes the expression of FGFR3 in the cells that we demonstrated by RealTime-PCR and Western blot. To find out if the FGFR3 was important for INA-6 we used the small FGFR3 inhibitors SU5402 and PD173074. Both decrease the proliferation and enhance apoptosis in INA-6, which indicate that FGFR3 not only can be present, but also be important even when the t(4;14) is not present in the cells.

3.3 Paper III

Overexpression and involvement in migration by the metastasis-associated phosphatase PRL3 in human myeloma cells

Numerous cytokines are known to support growth and survival of MM cells and there is likely to be redundancy in the signal. Attempts to block one signal will be compensated by other signals and we hypothesized that knowing the common genes upregulated by several cytokines would lead us to possible future targets. Preliminary microarrays showed that a limited number of genes were upregulated after cytokine stimulation in myeloma HMCLs OH-2 and IH-1. Phosphatases of regenerating liver (PRL phosphatises)-3 was one of these genes. PRLs constitute at class of small phosphatases with possible oncogenic activity. The PRL3 is known as a metastasis-associated phosphatase, and several reports show its importance in cancer cell invasion

and migration. Since cell migration is one of the processes fundamental to MM cell invasion and dissemination, we decided to look closer at PRL3 in MM. We show here for the first time, that PRL3 expression, demonstrated at mRNA and protein level, was increased in several MM HMCLs when stimulated with mitogenic cytokines. Also a large cohort of MM patients expressed *PRL3* at higher levels than normal PCs. Because amplification of *PRL3* has been associated with increased expression in other cancers, we used FISH to detect the copy number in the HMCLs. There are HMCL with many copies of chromosome 8/*PRL3,* but OH-2, which has the highest expression of *PRL3,* had an apparent normal chromosome 8/*PRL3* copy number. However, in RPMI-8226 we found an extra copy of the *PRL3,* which might be the cause of the expression of *PRL3* in this cytokine independent HMCL. The FISH results indicate that gene expression levels seem to be copy number independent. Amplification due to chromosome copy numbers does not correlate with gene expression levels in the MM cell lines. Most likely, other mechanisms are involved in most of the up-regulation of *PRL-3* expression. Immunohistochemistry on MM patients detected PRL3 protein in 18 out of 20 patients. In OH-2 the PRL3 was detected by anti-PRL3 staining to cycle between the cytosol and the nucleus in a cell-cycle dependent way. PRL3 was predominantly nuclear localized in G0/G1-phase and exclusively staining in the cytoplasm in the G2M phase. The same was also shown in patient sample. Downregulation of PRL3 expression by siRNA reduced SDF-1 -induced migration in the INA-6 cells, but had no effect on the cell cycle distribution or cell proliferation. Taken together these data suggest that PRL3 is one of the proteins translated as a response to several mitogenc cytokines and that it might have a role in migration also in MM cells. Several protein tyrosine phosphatases seem to be attractive drug targets in cancers PRL3 could be a molecular target in subgroups of patients with MM.

3.4 Paper IV

High expression of BCL3 in human myeloma cells is associated with increased proliferation and inferior prognosis.

This paper started with the same hypothesis as Paper III i.e. that intracellular signals generated by cytokines known to be important for growth and survival of myeloma cells, target common genes which may be important node molecules in myeloma pathogenesis. *BCL3* was one of a limited number of genes that were activated in the IH-1 and OH-2 cell lines in response to all cytokines analyzed. *BCL3* is located at 19q13, and is a putative oncogene encoding BCL3 that belongs to the inhibitory κB (iκB) family. The i-κB proteins modulate the DNA-binding activity of NFκB, a family of transcription factors involved in apoptosis and cell growth. Activation of NFκB is implicated as an important mechanism for the development of anti-apoptosis and drug resistance in MM. Depending on context, BCL3 either activates or inhibits NFκB-dependent gene transcription through interactions with homodimers of NFκB, p50 or p52. We found in a large cohort of myeloma patients, that Bcl-3 was over-expressed in MM cells from a subset of MM patients, and that high expression level on GEP by the time of diagnosis is associated with the proliferation subgroup of patients with an inferior prognosis. Furthermore, expression of BCL3 in HMCLs induced by growth promoting cytokines as IL-6, IL-21 and TNF-α is associated with increased proliferation of the cells. The array data was verified with RT-PCR in a small material with randomly selected patients, and BCL3 was also detected at the protein level, both with immunohistochemistry and Western blot. Because *BCL3* first was identified through its involvement in the t(14;19)(q32;q13) in B-cell chronic lymphocytic leukemia we also wanted to check if MM patients had an aberration in the locus of *BCL3*. We used a split probe and interphase FISH, and found that there was an unbalanced translocation in one out of the 19 randomly picked patients investigated. Also, four of the patients had an extra copy of the gene.
In conclusion, we here showed for the first time that BCL3 is present in a subset of myeloma patients and that high expression at diagnosis is associated with inferior prognosis. We have not shown that BCL3 is an independent adverse prognostic factor in myeloma; however we found that *BCL3* is a common target gene for several growth-promoting cytokines in myeloma cells. Taken together, this may indicate a potential oncogenic role for BCL3 in MM, but further studies are needed to clarify this.

3.5 Paper V

Genetic aberrations in Norwegian myeloma patients
-a study based on interphase FISH on newly diagnosed patients from 2006-2008.

This manuscript is a preliminary report of the findings in a so far three year long study. The aim has been to screen the Norwegian MM patients and look at genetic aberrations by interphase FISH. This is a prospective study, and we intend to include 300 patients before the final report will be given.

We analyze 139 MM patients *IGH* translocations by break-apart strategy and looked at the specific primary *IGH* translocations t(4;14), t(11;14), t(6;14) and t(14;16). All patients were also analyzed for del13 and del17. Up until now we have found an *IGH* translocation incidence that is a bit lower than some others have published (41% versus 60% in published studies). However, the prevalence of t(11;14) and t(4;14) is approximately the same as others have reported with an incidence of 16% and 12% respectively. The incidence for t(6;14) and t(14;16) are low, which might be caused by the lack of material for some patients. Deletion in chromosome 13 and 17 were present in 30% and 17% respectively. The del13 frequency is lower than others have reported, and del17 frequency is approximately the same as others have reported. There was no significant correlation between the specific *IGH* translocations and the deletions in chromosome 13 and 17. We did however find a significant correlation between del13 and del17. Del13 was seen in 12 patients (50%) of the 24 patients with del17, and in 31 (27%) of the 115 patients without del17. There were no significant correlations between clinical findings and genetic aberrations. The establishment of the method and an estimate of the prevalence of genetic aberrations in myeloma patients in Norway, may have significant implications for the future treatment strategy.

4. GENERAL DISCUSSION

4.1 Methodological considerations

4.1.1 HMCLs –Are they a good model for the MM disease?

Doing basic myeloma research usually means doing experiments with HMCLs. A lot of conclusions have been drawn based on observations from experiments with these HMCLs grown in monocultures in the laboratories. There are more than a hundred HMCLs around the world, and they are important tools in the study of MM [94]. Most of the genetic abnormalities in the HMCL are still the hidden secrets of the cells'. But the information of *IGH* translocation status and chromosome copy number is available for the most commonly used HMCLs [52]. Also the Multiple Myeloma Genomics Portal (MMGP) provides information from studies from different myeloma research groups (http://www.broad.mit.edu/mmgp/pages/publicPortalHome.jsf). GEP and CGH array results for a wide range of HMCLs and patients are available at MMGP.

HMCLs are not fully representative of MM. Some of the translocations are more common in HMCLs compared to MM patients. t(4;14) is present in ~25% of HMCLs versus ~15% of MM patients. t(14;16) is present in ~25% of HMCL versus only ~5% of MM patients[29]. This discrepancy may reflect the more aggressive disease conferred by this t(14;16) translocation, and thereby making it easier for the cells to grow *in vitro.* The difference between the prevalence of the translocations in the HMCL versus MM patients will however be smaller if one takes into consideration that only approximately half of the MM patients are represented with HMCLs. This is because most of HMCLs are derived from specimens from patients belonging to the NHRD MMs. The bias that most HMCLs are from NHRDs has to be considered when HMCLs are used as models of the human disease[29]. It appears that NHRDs more likely become independent of their environment compared to HRDs, and this confers a more aggressive disease allowing some NHRDs to grow *in vitro.*

The disconcordance between the HMCLs and the MM patients is a major concern. How well do the HMCLs represent the vide range of MM patients, especially since there is a lack of HRD HMCLs? And how well do the HMCLs represent the BM tumors in the MM patients.

To discuss the last question first: the HMCLs' originality. How much does the cell change when grown *in vitro* for several years-not to say decades? It is difficult to grow primary cells more than a couple of weeks in the lab, even with addition of stromal cells and mitogenic cytokines. Also, most HMCLs are from patients with relapse and/or extramedullary disease[94,95]. When establishing HMCLs there is also a clonal selection of one or more clone of the cells that proliferate more rapidly[96]. The HMCLs can therefore be considered to represent the most malignant end-stage PCs with multiple genetic abnormalities[21,22]. Also it is worth noticing that the same HMCLs used at different laboratories in the world might not be identical (personal communication M Kuehl). It is left to find out if this is due to for instance contamination, mislabelling or mutations during culture. However, on the positive side: our experiment with the HMCL OH-2 (paper I) showed only a really small difference between the primary cells and the HMCL on CGH array. The differences were a small deletion on 10q and an amplification on 8q in the OH-2 HMCL, which were not present in the primary cells. These aberrations might have occurred *in vitro,* but they could also have been present in a small fraction of the primary cells, and been selected during culturing.

There are hardly any HMCLs representing the huge hyperdiploid MM patient group, and not many HMCLs without *IGH* translocations. There have been attempts to describe hyperdiploid cell lines, and Li *et al.* described two stromal dependent hyperdiploid HMCLs without *IGH* translocations[97]. Although, I am a bit reluctant to call these two cell lines "true" hyperdiploid when looking at the SKY they present. Neither of the cells have more than two of the common triploid odd numbered chromosomes. This, and more, has also been criticized by others[98].

I find the fact that there are not available HMCLs for the HRD patient group somewhat a problem for the relevance of the MM research being done on HMCLs in labs all over the world. Looking at the publicly available array CGH at MMGP, it is easy to see that only a few HMCLs have the proper chromosome number to be an HRD HMCL. By looking deeper into the arrays I would only define the HMCL XG-2 (49 chromosomes) to be a HRD HMCL. The other HMCLs with HRD chromosome number (RPMI 8226, 60-62 chromosomes; JK-6L, 50 chromosomes; KHM-1B, 59 chromosomes; OCI-MY1, 49 chromosomes) I would not without more considerations put into the HRD group. I would like to use RPMI 8226 as an example. Figure 7 shows both SKY and array CGH of this HMCL. The HMCL has the correct number of chromosomes to be HRD, but when examining the chromosomes it is only chromosome 3, 19 (array CGH shows that a piece of one chromosome 19 is deleted) and 21 that are the odd number triploid chromosomes. The other chromosomes, having more than the normal two copies are even numbered chromosomes. Comparing the SKY and the array CGH gives a better picture of the real karyotype, because it is not only made from one or a few cells, but gives the global copy number in the whole HMCL population at the time of harvest. Another feature that gives this HMCL a pro-HRD karyotype, besides the total chromosome number, is the fact that it does not have one of the primary *IGH* translocations. It has a t(1;14)(q12;q32) which must be considered a non-recurrent *IGH* translocation, perhaps present at the same frequency in both HRD and NHRD [52]. The array CGH value zero on the Y-axis represents three chromosome copies, evident when compared with the SKY. There might be small differences between the SKY and array CGH that may also come from the fact that the HMCLs are harvested at different labs (Little Rock and Trondheim respectively).

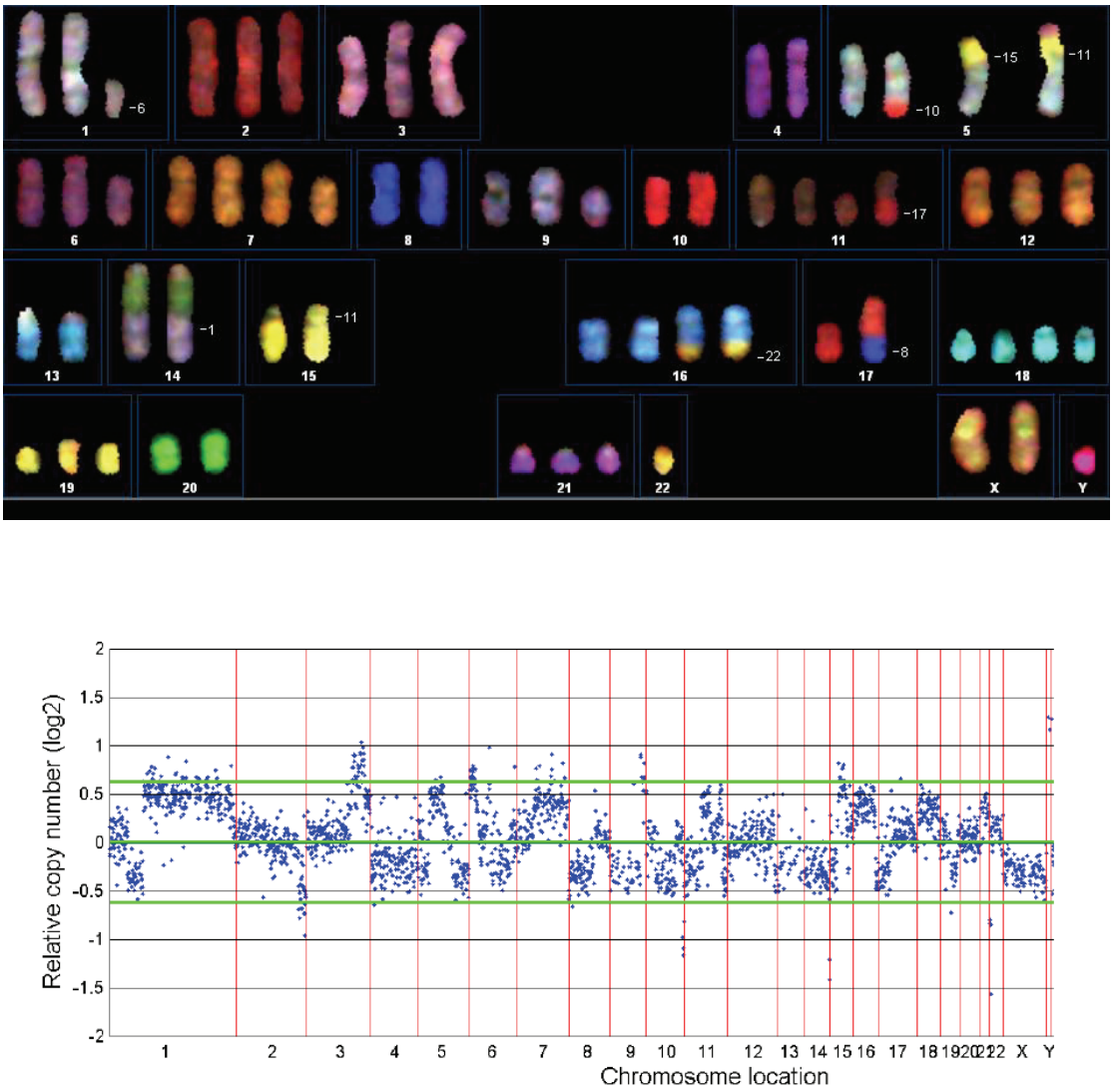


Figure 7. Upper figure shows a SKY of HMCL RPMI 8226 (with permission dr J Sawyer, UAMS, Little Rock). Lower figure shows an array CGH of RPMI 8226 (prepared by SH Kresse and L Meza-Zepada)

4.1.2 Isolation of primary MM cells from bone marrow aspirate from patients using anti-CD138 antibodies

Since HMCLs are what they are, a model system, it might be important to look at responses in freshly isolated primary MM cells. It is believed that experiments with primary MM cells *in vitro* are more relevant to the *in vivo* situation than HMCLs. Even though it is easy to argue that primary cells are better to use for experiments than HMCLs, there are also disadvantages. The cells have been harvested and have been kept together with e.g. EDTA or

heparin. There will also be a time gap between harvest and experiments. It is difficult to control if the mRNAs, microRNAs, epigenetics or proteins are not disturbed by this handling. Primary cells will always have the heterogeneity and it might be difficult to get the same result with samples from different patients. At least when the sample is from a newly diagnosed patient, we do not know anything about it. This is a good thing in that experiments become randomized. However, if the goal is to look at for instance FGFR3 responses, it will be more difficult.

Isolation of primary PCs has been done with anti-CD138 antibody from BM aspirates from MM patients. In Paper III and IV Macs CD138 coated Microbeads (Miltenyi biotech) were used and cells were separated sterile to be used also for long time cultures. For FISH and over-night cultures RoboSep (Stem Cell technology) automatic CD138 separation of BM aspirates was used. With both techniques purity of PCs were determined by May Grünvald-Giemsa staining to be on average >97%. With the RoboSep separations freshly drawn samples with more than 10 mill cells were usually >98% pure and samples sent to us by over night post 95-97% pure. Samples shipped over night with fewer cells were usually down to 90% pure, but this was still good enough to use for FISH analysis. Samples shipped over night were used for FISH analysis only.

4.1.3 FISH

In paper V interphase FISH was done on three different kinds of samples from patients. When using BM spreads and mononuclear cells it was important to positively select the PCs with antibodies for IGK and IGL. This cytoplasmatic (c)Ig-FISH works well to separate out the myeloma cells and to only score the PC. In some patients the cytoplasmatic staining can be weak, and if the PC% in addition is low, the FISH scoring is time consuming, and will not always give conclusive results. The introduction of CD138 separation of PCs the FISH procedure was considerably improved and less time consuming (Paper V).

4.2 Why is it difficult to establish HRD HMCLs?

As already stated, it is difficult to get primary MM cells to grow, and even more difficult to establish a HMCL, especially from HRD that miss one of the primary *IGH* translocations. One reason might be that tumors with HRD phenotype are more dependent on the microenvironment than cells with primary *IGH* translocations. As we explain in paper I, OH-2 is strictly dependent on mitogenic cytokines and on human serum to be able to grow, and even then it grows significantly slower than the other HMCLs in our lab. In our experience, other IL-6-dependent cell lines become IL-6-independent after culture for extended periods, but this has never been seen with OH-2. To be able to establish more HRD HMCL, we need to get primary MM cells to grow better *in vitro.* However, even when growing primary cells with human serum and a cocktail of mitogenic cytokines, we experience that the cells die after a few months.

Less than 10% of EMM are HRD[99,100]. It is not yet known what makes an intramedullary myeloma to become extramedullary. One genetic answer might be the acquisition of autonomous *CCND* dysregulation. One possible explanation is that HRD MM tumors, and especially those that ectopically express CCND1, are dependent on signals from the bone marrow microenvironment to enable bi-allelic expression of a *CCND* gene. If this is true, then progression to independence from the bone marrow microenvironment might require an alternative mechanism to dysregulate a Cyclin D gene. It has been proposed that *CCND* is dysregulated in all MGUS and MM, either by a translocation or by bi-allelically expression in many HRD tumors. The mechanism for this bi-allelically expression is still to be determined[26]. Perhaps OH-2, and also RPMI 8226 and XG-2, have accomplished CCND expression by a genomic rearrangement that dysregulates *MAF* or *MAFB*, both of which increase expression of *CCND2*. We need to answer at least two important questions regarding HRD MM tumors. First, what is the mechanism by which *CCND1* is bi-allelically dysregulated in HRD MM tumors? Second, why do HRD MM tumors so infrequently progress to EMM tumors or HMCL that express CCND1?

4.3 Is FGFR3 important in myeloma?

FGFR3 is one of the genes affected by the t(4;14). It was the finding of ectopically expressed FGFR3 in MM that gave the first indication that FGFR3 was a potential oncogene[101]. In MM, activating mutations in *FGFR3* are present in about 1/3 of t(4;14) patients and half of the HMCLs[102]. It is reported that *RAS* and *FGFR3* mutations are mutually exclusive. Since both RAS and FGFR3 activation, lead to MAPK activation, they may induce overlapping oncogenic signaling[103]. This is in concordance with our findings in HMCLs IH-1 and INA-6. Both IH-1 and INA-6 have no mutations in *FGFR3*, but *NRAS* is mutated in codon 12 in both HMCLs (unpublished data). In paper II we describe that INA-6 has expression of FGFR3 without t(4;14) and that FGFR3 might be important for the myeloma cells. Acidic-Fibroblast growth factor stimulated IH-1 and INA-6 have phosphorylated ERK1 and ERK2 detected by WB. Adding the inhibitors described in paper II the phosphorylation is only partly taken down compared to cells not treated with the inhibitors (unpublished data). This is expected with the mutated NRAS present. We believe that FGFR3 contributes to proliferation in INA-6 and IH-1 and it deserves to be more looked into.

4.4 The relevance of doing FISH analysis on every myeloma patient

Genetics as a prognostic tool is already established. NHRD patients have poorer survival than HRD and del17, t(4;14) and t(14;16) have poorer survival than other genomic aberrations[20,29]. We established FISH to be able to give the service to hospitals in Norway.

Some studies evaluate the genetic impact of prognosis and outcome after different treatment strategies. A study by Gertz *et al.* showed that when high dose therapy with stem cell support was used, t(4;14) patients had a progression-free survival of 8.2 months compared to 20.1 months for t(11;14)

patients. For patients with del17 progression-free survival was 8.7 months. This suggest that high-dose therapy with stem cell support might not be the best therapy for these patients[45]. In the UAMS total study II it was seen that patients with non-favorable cytogenetics/GEP have greatest benefit from thalidomide with a significantly higher overall and event free survival compared to favorable cytogenetics/GEP patients[104]. Also Chng *et al.*[105] suggested that the various genetic subtypes at diagnosis and relapse appear similar, but at the same time the prognosis of some genetic subtypes, like t(11;14), might be worse at relapse. Whereas for instance HRD patients appeared to behave similarly, both at diagnosis and at relapse. They therefore suggested that patients with t(11;14) should be treated more aggressively when relapsed. They also report that t(4;14) patients benefitted from bortezomib, improving the prognosis of these patients to that of genetic subgroups with better prognosis. All in all this states the fact that different treatment strategies may be needed for newly diagnosed and relapsed patients, even in the same genetic group[105]. Bortezomib also seemed to exacerbate some of the poor prognosis in del13 patients[106,107].

It is difficult to draw uniform conclusions from the studies that have been published until today. We have still not come so far that there are tailored treatments for patients with different translocations or other genetic aberrations. But it is a hope for the future that such treatments will come. I am hoping for a large study that correlates the outcome of treatments with genetic aberrations. Perhaps as Chng *et al.* reports that t(4;14) patients go from poor to a more average prognosis, treated with bortezomib, and perhaps patients with other genetic abnormalities like the del17 could benefit from some other drugs? I am looking forward to read about a large prospective study that incorporates genetic aberrations to treatment strategies, and I hope it will come soon. Some of the motivation, studying genetics in MM, is that it somehow eventually can help the patient to be treated fully, at least a longer event free survival and a better quality of life with managing the disease. So, I think is not unreasonable to do FISH on all patients, but I am not sure we know the full potential of it yet.

5. CONCLUSION AND FURTHER THOUGHTS

As with all research -the more you find out -the more questions are asked. This thesis answers some questions, but there are still more to be asked. In this study we provide further insight in the complex genetic field of the myeloma cells, particularly the HMCLs. More specifically, we have revealed the OH-2 cells' hidden hyperdiploid phenotype and thereby given a long sought hyperdiploid MM model system *in vitro*. We will however not stop here, and hopefully we will reveal why OH-2 HMCL are strictly dependent on human serum. We hope that an answer to that might help us to establish more HMCLs from HRD patients. We have also revealed that more specific genes like the *PRL3, FGFR3* and *BCL3* can be amplified or translocated, even though the specific mechanism behind it is not clear. It has also been interesting to screen Norwegian MM patients for genetic aberrations. Because this study is not completed, this will also be a study for the future. The biggest thoughts for future basic research are perhaps the questions I ask in the discussion about HRD MM, and I hope to be able to investigate the secrets of the HRD MM further.

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Paper I

Title: OH-2, a hyperdiploid myeloma cell line without an *IGH* translocation, has a complex translocation juxtaposing *MYC* near *MAFB* and the *IGK* locus

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Running title: OH-2, a hyperdiploid HMCL without an *IGH* translocation

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Abstract

Multiple myeloma (MM) can be classified into two groups: hyperdiploid (HRD) tumors (48-74 chromosomes), which typically have extra copies of chromosomes 3, 5, 7, 9, 11, 15, 19, and 21; and non-hyperdiploid (NHRD) tumors, which usually have immunoglobulin heavy chain (*IGH*) translocations. The OH-2 HMCL was derived from extramedullary myeloma, and retains the same HRD phenotype as the primary tumor, with extra copies of chromosomes 3, 7, 15, 19, and 21. This provides a unique example of an HMCL and the corresponding primary tumor that are shown to share the same HRD genotype. The OH-2 HMCL and the primary tumor cells both express high levels of Cyclin D2, but do not have an *IGH* or *IGL* translocation. Instead, both have a complex translocation in which a 28 kb sequence, including the *IGK* 3' enhancer, is inserted between *MYC* and *MAFB,* resulting in dysregulation of both oncogenes. These data, together with published results for two other HMCL, suggest that secondary *MAF/MAFB* translocations provide one mechanism that enables HRD tumors to overcome microenvironment dependence, enabling an intramedullary tumor to progress to an extramedullary phase from which an HMCL sometimes can be generated.

Introduction

Multiple myeloma (MM) is a neoplasm of long-lived bone marrow plasma cells [1]. Both MM and pre-malignant monoclonal gammopathy of undetermined significance (MGUS) can be separated into two groups that are distinguished by chromosome content [2,3]. Approximately 50% of tumors are hyperdiploid (HRD), and contain 48-74 chromosomes, typically with extra copies of at least four of eight odd-numbered chromosomes (3, 5, 7, 9, 11, 15, 19, 21). The remaining tumors are non-hyperdiploid (NHRD), containing <48 and/or >74 chromosomes. These two groups are further dustinguished by *IGH* translocations with five reciprocal partners (4p16, MMSET/FGFR3; 11q13, Cyclin D1; 6p21, Cyclin D3; 16q23, MAF; and 20q12, *MAFB*) that are present in about 70% of NHRD tumors but only about 15% of HRD tumors. It appears that recurrent *IGH* translocations and hyperdiploidy are primary events that occur early in pathogenesis. Secondary translocations, which include most *IGH* rearrangements not involving one of the five recurrent partners, most *IGL* and *IGK* rearrangements, and *MYC* rearrangements, appear to contribute equally to progression of both HRD and NHRD tumor [4].

Despite a low proliferation index, there is increased expression of one of the three CYCLIN D genes in virtually all MGUS and MM tumors, suggesting that this is a unifying and early oncogenic event. Primary translocations can directly dysregulate Cyclin D1 or Cyclin D3, or indirectly dysregulate Cyclin D2 (transcription target of maf proteins and unknown mechanism for *MMSET/FGFR3* translocation). The HRD tumors lacking a primary translocation mostly express increased levels of Cyclin D1 (~70%), Cyclin D1+Cyclin D2 (~10%), or Cyclin D2 (~20%) compared to normal plasma cells [5].

In contrast to intramedullary MM tumors, most (~60%) extramedullary (EMM) tumors have one of the recurrent *IGH* translocations, and less than 10% are HRD [6,7]. Similarly, human myeloma cell lines (HMCL) are derived almost exclusively from EMM tumors, mostly are NHRD and usually have one of the recurrent *IGH*

translocations (~75%), suggesting that intramedullary HRD tumors are less likely to develop into EMM tumors or HMCL [8]. There are no known examples of HMCL that have been shown to be derived from a primary tumor that has a HRD phenotype and does not have a recurrent *IGH* translocation [9].

OH-2 is a stroma-independent HMCL derived from an EMM tumor that developed following treatment and progression of an intramedullary MM tumor [10]. Previously, it was reported that the OH-2 HMCL, which has a doubling time of about four days, is completely dependent on interleukin (IL)-6, and human serum for stromal cell independent growth in culture. In this paper we report that the OH2 HMCL retains the same HRD genotype as the EMM tumor cells. We also characterize its chromosomal composition, some genetic anomalies, and some of its growth characteristics.

Material and Methods

Cell line, OH-2
The HMCL OH-2 was established in 1991 at St. Olavs University Hospital from pleural fluid of an MM patient in terminal stage of the disease [10]. The cell line is cultured in RPMI 1640 (Gibco, Paisley, UK) with L-glutamine (100 µg/ml) and gentamicine (20µg/ml), supplemented with 10 % human serum (AIT, St. Olavs University Hospital, Trondheim, Norway) and IL-6 2 ng/ml, in a humidified atmosphere containing 5 % CO_2 at 37°C. For an experiment to optimize growth condition for the HMCL, OH-2 was grown in various combinations, as denoted in legend to Fig 7.

Primary cells from the patient who gave rise to the OH-2 cell line
Cells from the pleural fluid of the patient who gave rise to the OH-2 cell line were separated by lymphoprep centrifugation and kept in RPMI 1640 with 10% DMSO and 30% fetal calf serum (FCS) in liquid nitrogen, frozen at the day of sampling. Cells were thawed, and RNA and DNA were isolated shortly after thawing. The cell suspension contained 90-95 % pure plasma cells. These cells are further called primary OH-2 cells. An epon-embedded biopsy taken 10 months before the pleura fluid harvest was unfortunately not applicable for either DNA analysis or immunohistochemistry.

Other HMCLs and culture conditions
JJN-3 [11], ANBL-6 (gift from Dr D Jelinek, Mayo Clinic, Rochester, MN) RPMI-8226 (from America Type Culture Collection, Rockville, MD) and INA-6 (gift from Dr M Gramatzki, Erlangen, Germany) are maintained as previously described [12,13].

Fluorescence in situ hybridization (FISH)
Probes for FISH were made from Bacterial Artificial Chromosome (BAC) clones containing the desirable genomic region covering loci 2p11, 4p16, 11q13, 14q32, 16q23, 20q12 and 22q11. In addition a break-apart probe for *IGK* was purchased from Dako (Dako Cytomation, Glostrup, Denmark). Supplementary Table A shows probe loci and clones. Probes for 4p16, 11q13, 14q32 and 16q23 were gifts from R. Fonseca, probes for 20q12 and *IGK* from M. Kuehl, the rest was purchased from Invitrogen. Whole Chromosome Paint (WCP) probes for chromosome 2, 14, 20 and 22 in aqua, and chromosome 8 in green (Applied Spectral Imaging, Micro System AB, Spånga, Sweden) were used to verify genes on their respective chromosomes.

CEP 2 and CEP 8 SpectrumAqua were used to label the centromeric region in the chromosomes (Vysis, Abbot Laboratories, Des Plaines, IL). FISH with WCP and in-lab LSI probes were used as follows: 2 µl LSI probe in water + 2 µl WCP with 6 µl LSI hybridization buffer (Vysis). For *IGK* 2 µl WCP 2 and 8 µl of *IGK* probe mix (Dako) were used. Metaphase spreads was made according to standard methods [14]. The metaphases were prepared at least one week prior to use without additional heating or chemical treatment. Method and microscopy as described earlier [12].

Karyotyping
Conventional cytogenetic methods Giemsa-banding (G-banding) and spectral karyotyping (SKY) were performed after standard procedure as described [15].

Microarray-based comparative genomic hybridization (array CGH)
The genomic microarray was done using 500 ng genomic DNA sample from OH-2 cell line and primary cells after methods as described [16,17].

Southern Blot for IGH, IGK and MAFB
Southern Blots were performed on genomic DNA from OH-2 to look for *IGH* illegitimate switch recombination fragments, as previously described [18].

Gene expression profiling
Total RNA was profiled and analyzed as described previously (8,24), using Affymetrix HG-U133_Plus_2.0 Chips (Affymetrix, Santa Clara, CA). Profiling data were available for 559 newly diagnosed MM tumors (GEO accession GSE2658; http://www.ncbi.nlm.nih.gov) and 46 HMCL (http://www.broad.mit.edu/mmgp/pages/publicPortalHome.jsf). The expression of individual genes was normalized to the median expression of that gene in the entire sample set of 559 MM tumors and 47 HMCL. A MAF index (MAFI) was calculated from the median expression of 50 genes that are up-regulated in the MAF group [19], and the samples in Table 1 were arranged in descending order, based on the value of the MAFI. Other probe sets used in Table 1 included: CCND1, 208711_s_at; CCND2, 200951_s_at; CCND3, 201700_at; FGFR3, 204379_s_at; MMSET, 223472_at; MAF is the median of 206363_at, 209347_s_at, 209348_s_at, and 229327_s_at; *MAFB* is the median of 218559_s_at and 222670_s_at; and *MYC* is 202431_s_at. A proliferation expression index, P(12), was calculated from the median expression of twelve genes that are included in a proliferation signature [8].

Cell proliferation assay
Cell proliferation assay was performed as previously described [20]. The cells were stimulated with one or several of these cytokines: 1 ng/ml recombinant human IL-6, 200 ng/ml a proliferation inducing ligand (APRIL), 200 ng/ml B-cell activating factor (BAFF), 100 ng/ml IGF-1, 10 ng/ml IL10, 20 ng/ml IL15, 10 ng/ml IL-21 (R&D Systems, Abingdon, UK), 10 ng/ml TNF (Genetech, South San Fransico, CA) and 100 ng/ml hepatocyte growth factor (HGF) purified in our own laboratory [21]. The counts per minute (cpm) obtained was normalized to the serum control sample.

Real-time RT-PCR on Cyclin Ds, and MAFB
Total RNA from OH-2, INA-6 and ANBL-6 cells cultured as described, was isolated using RNeasy Mini Kit (Qiagen, Crawley, UK), with DNase treatment. cDNA was made from RNA using SuperScript®III First Strand Synthesis System for RT-PCR

(Invitrogen). Cyclin D1-3 and *MAFB* TaqMan primers, (HS00765553_A1, HS00277041_A1, HS01017690_g1 and HS00271378_s1 respectively, TaqMan, Gene Expression Assays, Applied Biosystems, Foster City, CA), were used to detect cyclin Ds and *MAFB* expression. A control without reverse transcriptase added was used for the one exon gene *MAFB* to make sure there was no genomic DNA contamination. The comparative Ct-method was used for quantization with *GAPDH* (HS99999905_m1) as housekeeping gene.

Sequencing of TP53 and RAS
TP53, NRAS, HRAS or *KRAS2A/B* cDNA was PCR-amplified and sequenced to examine for nucleotide mutations. All RT-PCR reactions were purified with QIAquick PCR Purification Kit (Qiagen) and directly sequenced using BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA), DyeEx 2.0 Spin Kit (Qiagen) was used to purify the sequencing reaction that was analysed on ABI3100 Genetic Analyzer (Applied Biosystems). Details of primers and primer position are listed in Supplementary Fig B-C.

Results

OH-2 does not have an IGH translocation
Using a split probe strategy on metaphase spreads, there was no dissociation of the centromeric and telomeric probes for the *IGH*, *IGK*, or *IGL* loci (Figs 1a-c). OH-2 was also tested with dual fusion probes for the recurrent *IGH* translocation partner loci at 4p16, 6p21, 11q13, 16q23, and 20q12, but no cryptic translocation involving *IGH* with any of these loci was detected (not shown). WCP probes confirmed that the *IGH*, *IGK*, and *IGL* loci were on chromosomes 14, 2, and 22, respectively. However, it was noted that there was a complex translocation near the *IGK* locus on chromosome 2, with both *IGK* probes localizing to der(2) (Fig 1b). Southern blots identified 4.4 kb and 4.7 kb Hind III 5' switch mu/3' switch gamma legitimate switch recombination fragments, consistent with normal rearrangements involved in expression of IgG by the OH-2 tumor cells (not shown), but no illegitimate switch recombination fragments were detected.

Identical hyperdiploid chromosome content of OH-2 cell line and primary tumor
Conventional G-banding and SKY analyses (Fig 2a-b) revealed the presence of 48-51 chromosomes, with trisomies of chromosomes 3, 7, 15, 19, and 21, in the OH-2 HMCL. In addition to aneuploidy, ten translocations and one large interstitial deletion were identified (Fig. 2a-b). Unfortunately, karyotypic analyses are not available for either the initial intramedullary MM tumor or the EMM tumor. Therefore, array CGH was performed to check for additional chromosomal aberrations in OH-2, and to compare the OH-2 HMCL and the EMM tumor. The OH-2 HMCL and OH-2 primary tumor showed virtually identical array CGH patterns apart from a 10q deletion and amplification of a portion of 8q that were uniquely present in the HMCL (Fig. 2c). Significantly, array CGH analyses confirmed in both the cell line and tumor the extra copies of the five odd-numbered chromosomes, and also chromosome 14, which were confirmed by additional FISH analyses (not shown). Finally, the array CGH and karyotypic analyses also verified loss of one copy of chromosome 13, as well as loss of 1p and gain of 1q sequences.

OH-2 cell line and primary tumor cells express a high level of MAFB RNA

The gene expression profile of the OH-2 cell line was determined using an Affymetrix HG-U133_Plus_2 chip, and compared to results that are available for 46 other HMCL. Selected results are shown in Table 1. Strikingly, the OH-2 HMCL expresses *MAFB* RNA at a level that is higher than the three HMCL (SACHI, EJM, SKMM-1) that have t(14;20) translocations. Consistent with the expression of high levels of *MAFB*, the OH-2 cells express extremely high levels of Cyclin D2 RNA, low levels of Cyclin D3 RNA, and very low levels of Cyclin D1 RNA. Real time PCR assays confirmed that the OH-2 primary tumors cells also express high levels of *MAFB* and Cyclin D2 but low levels of Cyclin D1 RNA (Fig. 3). Significantly, the OH-2 HMCL has a *MAF* expression signature that places it among the 42 MM tumors and 14 other HMCL that express high levels of *MAF* or *MAFB* RNA (Table 1 plus Materials and Methods). In addition to Cyclin D2, some other apparent *MAF* targets with expression levels at least tenfold above the median of the tumors and cell lines include integrin beta7, *ARK5*, and *MRF-1*. Finally, the *MYC* RNA level in the OH-2 HMCL is in the highest quartile of the 47 HMCL (Table 1).

MAFB and MYC are juxtaposed by a complex translocation process involving IGK

In view of the high level of *MAFB* expression in OH-2, we suspected the translocations involving chromosomes 2, 8, and 20, as der(20)t(2;20)(p11;q12), der(8)t(8;20)(q24;q12)t(8;20)(q24;q12) or der(8)t(8;20)(q24;q12) and der(2)t(2;8)(p11;?q24) might be the cause. To determine if there is a rearrangement near *MAFB,* we performed FISH with a *MAFB* probe, as well as nearby centromeric and telomeric probes, in combination with WCP 2, 8, and 20, and also CEP probes for chromosomes 2 and 8 (Fig. 4c,e). *MAFB* is normally located at 20q11.2-q13.1 (http://www.genecards.org). There is one intact copy of chromosome 20 (Fig 4b), but the *MAFB* locus was also translocated to chromosome 8. There were two clones of the HMCL, with either der(8)t(8;20)(q24;q12)t(8;20)(q24;q12) (~30%) or der(8)t(8;20)(q24;q12) (~70%), each paired with a normal chromosome 8. The translocated *MAFB* was localized at the 8;20 junction on der(8), or on both 8;20 junctions on der(8)t(8;20)t(8;20) (Fig 4e-f). Probes that were about 400 and 700 kb centromeric to *MAFB* were present only on der(20), and a probe that was about 1100 kb telomeric to *MAFB* was present only on der(8) (Table 2). Thus the breakpoint on chromosome 20 apparently occurred less than 400kb centromeric to *MAFB*. A *MYC* probe hybridized to the 8;20 junction on der(8) or to both 8;20 junctions on der(8)t(8;20)t(8;20), but not to der(2)t(2;8) (Fig 4f-g). Therefore, *MYC* and *MAFB* are juxtaposed on both der(8). Despite the lack of a Vkappa/Ckappa split by conventional FISH analyses (Fig 1b) we suspected that a Ckappa enhancer was juxtaposed to *MAF* and *MYC*. By using a relatively small BAC that included intronic and 3' enhancer sequences from the *IGK* locus, we detected a hybridization signal on der (8)t(8;20)(q24;q12) (Table 2). A similar result was obtained with a pair of PCR probes that included 25 kb of sequences that encompass the intronic enhancer, the 3' enhancer, and the 3' kappa deleting element (Fig 4d) (Table 2). The co-localization of one or both kappa enhancers together with *MYC* and *MAFB* was confirmed, and is present in all t(8;20) junctions (Fig 4d). Interphase FISH analyses confirmed a *MAFB*/*MYC* fusion signal, both in the cell line and in primary tumor cells, thereby demonstrating that the close (<500 kb) juxtaposition of these two genes occurred in the patient (Fig 4f-g). The complex der(8)t(8;20)t(8;20) was also present in the EMM tumor cells (Fig 4f-g). These and additional FISH mapping studies are summarized in Table 2 and Fig 4.

Mapping breakpoints near MAFB, MYC, and kappa enhancer by high density array CGH. The colocalization of *MAFB*, *MYC*, and kappa sequences on both kinds of der(8) suggested that breakpoints should be localized telomeric of *MYC*, on both sides of kappa sequences, and centromeric to *MAFB* (Fig. 5). The rearrangements involving chromosomes 2, 8, and 20 appeared to be balanced in most cells, but a partial imbalance was suggested by the duplication of the t(8;20) junction in about 30% of OH2 cells. Therefore, high density tiling CGH arrays were designed in an attempt to identify breakpoints near the *MAFB*, *MYC*, and kappa enhancer sequences. As a result of this analysis, we identified breakpoints, as manifested by an approximately 30% decrease in copy number, involving all three loci: 598 kb telomeric of *MYC*, 148 kb centromeric of *MAFB*; 18.6 kb telomeric and 9.9 kb centromeric of the 3' kappa enhancer. These results (Supplementary Figure D) indicate that the 3' kappa enhancer effectively is inserted between the *MYC* and *MAF* genes, so that both genes can be dysregulated by the same enhancer element. The combined FISH and array CGH results are consistent with sequential translocations as depicted in Fig 5.

Additional genotypic and phenotypic characteristics of OH-2
The sequence of *KRAS* was normal, but there was a CAA (GLN) to AAA (LYS) mutation in codon 61 of *NRAS*. Although all but three (KMS-28BM; KMS-28PE; XG-6) of 46 HMCL either have a mutation in *TP53*, no or very low expression of *TP53*, or an increased level of *MDM-2* expression (M. Kuehl, unpublished), there were no sequence abnormalities of *TP53* or RNA expression abnormalities of *TP53* or *MDM-2* in OH-2. Similarly, although approximate 50% of the 46 HMCL have inactivated either p18*INK*4c or *RB1* [22], the RNA expression of both of these genes appears to be normal in OH-2 (data not shown). Surprisingly, despite an extremely slow rate of growth in culture (below), the OH-2 HMCL has a proliferation expression index of 18.9, the fourth highest of the 47 HMCL.

Proliferative response to mitogenic cytokines
We previously published the response of OH-2 to different cytokines, e.g. a synergistic response to TNFα and IL-21, an intermediate response to IL-10, IL-15, and TNFα alone, and a modest response to IL-2 and IGF-1 [20]. Although OH-2 has a low NF-kappaB index (MK, unpublished), we also wanted to test the TNF analogs BAFF and APRIL on OH-2 [23,24]. Both BAFF and APRIL gave a lower proliferative response, as measured with 3H-thymidine incorporation, and did not show any synergism with IL-21. This is depicted in Fig 6, which also shows that a variety of cytokines can enhance proliferation in OH-2.

Culture requirements for the OH-2 cell line
The growth of OH-2 is strictly dependent on IL-6 and human serum, although growth is extremely slow, with a doubling time of about four days. In an attempt to find a more effective way to culture OH-2 we modified ingredients of the medium as FCS with various cytokines, FCS concentrations, and advanced RPMI 1640. Fig 7 shows a representative selection of the cell counts measured before media was replenished. The only condition to keep the cells for long-term was to use IL-6 together with human serum. The cells with human serum without IL-6 showed better survival than cells with cytokines and FCS, but could not survive for extensive time under this condition either.

Discussion

Approximately 50% of MM tumors are HRD and do not have one of the five recurrent *IGH* translocations. However, this phenotype is found in less than 10% of reported EMM tumors [6,7]. Significantly, for the very limited number of HRD EMM tumors that have been reported, it is unknown whether the corresponding intramedullary MM tumor had the same HRD genotype. Similar to EMM, from which virtually all HMCL are derived, HMCL only infrequently are HRD in the absence of one of the five recurrent *IGH* translocations [4,25]. For example, of the 46 HMCL (excluding OH-2) in Table 1, only ten (22%) do not have one of the recurrent *IGH* translocations. Five (10% overall) of these HMCL have a HRD chromosome content (RPMI 8226, 60 chromosomes; XG-2, 49 chromosomes; JK-6L, 50 chromosomes; KHM-1B, 59 chromosomes; OCI-MY1, 49 chromosomes). Unfortunately, it is not known whether or not the corresponding intramedullary MM tumor or EMM tumor had the same chromosome number as these five HMCL.

As summarized above, the OH-2 HMCL, which was derived from an EMM tumor, does not have an *IGH* translocation and retains the same HRD phenotype as the tumor, with extra copies of chromosomes 3, 7, 15, 19, and 21. This provides a unique example of an HMCL and the corresponding primary tumor that share the same HRD phenotype. However it has a complex translocation involving the *IGK* locus, which is juxtaposed with both *MYC* and *MAFB*. The OH-2 HMCL and primary EMM tumor expresses very high levels of Cyclin D2 but only low levels of Cyclin D1, consistent with the phenotype of the 20% of HRD MM tumors that express increased levels of Cyclin D2 but not Cyclin D1 [8]. The OH-2 HMCL is similar to the RPMI 8226 HMCL in that both have dysregulated *MYC* through complex translocations events that juxtapose *MYC* with *MAFB* and IgK or MAF and IgL, respectively [4].

Other notable features of the OH-2 HMCL include the loss of 1p sequences, gain of 1q sequences, deletion of chromosome 13, and lack of trisomy 11, all of which are associated with the poorest clinic outcome amongst the hyperdiploid patient group (Carrasco et al). These genetic signatures correspond to the aggressive disease in the patient whose malignant cells derived OH-2 cell line [10]. Despite an extremely high expression proliferation index and dysregulation of *MYC*, the OH-2 HMCL has a doubling time of at least four days when grown *in vitro*. In this regard, it may be relevant that OH-2 is one of the few HMCL that does not have a mutation or markedly decreased expression of *TP53* or a substantial increase in *MDM-2* expression. It is also worth noticing the gene expression profile study by Chng et al. that classified different clusters of hyperdiploid myeloma patients, where one of the clusters is defined by the overexpression of *PRL-3, SOCS, HGF* and *IL-6* genes [26]. We have recently published that PRL-3 is highly over-expressed in OH-2 [12]. Other experiments in our lab have shown that IL-6 induces transcription of *HGF* in OH-2 (Hov personal communication).

It is a pertinent question why it seems more difficult to establish HMCL from tumors that are HRD and lack one of the five recurrent *IGH* translocations. A reasonable answer could be that tumors with this phenotype are more dependent on the microenvironment than cells with primary *IGH* translocations. The strict dependence of OH-2 on mitogenic cytokines and on human serum supports this. In addition it is worth noticing that OH-2 grows significantly slower than the other HMCLs in our lab.

In our experience, other IL-6-dependent cell lines become IL-6-independent after culture for extended periods, but we have never experienced this with OH-2. Most HMCLs reported in the literature grow in medium supplemented with FCS, but OH-2 cells are impossible to wean from their dependence on human serum, which is something that remains to be clarified. Anyway, one way of circumventing the problem with establishing similar cell lines might be to use human serum in the growth medium. An additional genetic answer to this might be the acquisition of autonomous Cyclin D dysregulation. In fact, it is remarkable that all of the six HDR HMCL (above) express high levels of Cyclin D2, whereas ~70% of HRD MM tumors express increased levels of Cyclin D1 (but not of Cyclin D2) compared to normal bone marrow plasma cells [8]. Significantly, of two recently reported stromal cell dependent HMCL, neither expresses Cyclin D1; instead, one expresses high levels of Cyclin D2 and the other expresses extremely low levels of *RB1*, perhaps eliminating the need for increased expression of a Cyclin D gene [27]. One possible explanation is that HRD MM tumors, and especially those that ectopically express Cyclin D1, are dependent on signals from the bone marrow microenvironment to enable bi-allelic expression of a Cyclin D gene. If this is true, then progression to independence from the bone marrow microenvironment might require an alternative mechanism to dysregulate a Cyclin D gene. Perhaps OH-2, and also RPMI 8226 and XG-2, have accomplished this by virtue of a genomic rearrangement that dysregulates *MAF* or *MAFB*, both of which increase expression of Cyclin D2.

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Authorship

TKV has done FISH, cytokine assays, PCR, designed and written the paper, ET has done microarray experiments, data minding and revised the paper, SHK has done array CGH and data minding, LMZ has done array CGH, AG has done FISH, OG has done high density array and sequencing of the breakpoints, AS has designed and revised the paper, WMK has designed, revised and written the paper, MB has established the cell line, designed, revised and written the paper.

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Titles and legends to figures

Figure 1. FISH detects intact immunoglobulin genes
a-c) Break-apart probes for *IGH*, *IGK* and *IGL*, a-c respectively, were used together with WCP for the corresponding chromosome 14, 2 and 22. The yellow co-localization signal for the immunoglobulin loci indicates co-localization of the probes. a) WCP 14 and *IGH* break-apart probe show no abnormalities. b) WCP 2 and *IGK* break-apart probe suggest no translocation within the *IGK* locus. On the two derivatives involving chromosome 2, both *IGK* probes remain together near the breakpoint on der(2) (Fig. 2b). c) WCP 22 and *IGL* break-apart probe show no abnormalities. (Original magnification 1000x).

Figure 2. The karyotype of OH-2 has a hyperdiploid profile
a) Representative G-band with karyotype; 48~51<2n>,XX,-1,
der(1)t(1;3)(q12;q13)del(3)(q24), der(2)t(2;8)(p11;?), +3, der(3)t(1;3)(p22;q21),
del(6)(q23), +7, der(8)t(8;20)(q24;?q12)t(8;20)(q24;?q12), der(10)t(8;10)(?;q26), -13,
der(13)(13qter→13p11::1q21→1q41::19?p13), +15, der(16)t(9;16)(?;?),
der(18)t(1;18)(q21;p11), der(20)t(2;20)(p11;q13), +21,
der(21)t(1;21)(q21;q22)dup(1)(q21q32)x2
b) SKY of the OH-2 cell line. The G-band and the SKY analyses demonstrate the aberrations in the OH-2 cell line. The chromosome number and the trisomy 3, 7, 15, 19 and 21 indicate a hyperdiploid karyotype. c) Array CGH shows the genomic aberrations of the OH-2 primary cells (black) and the OH-2 cell line (red) in the same plot. Log_2 ratios for each of the BACs and PACs are plotted according to chromosome position. Log_2 values ~ 0.6 indicate one extra copy of the chromosome area, and values ~ -1 indicate loss of one copy of the chromosome area. Smoothing of the mean of 15 clones is used in this plot. Detailed array CGH in supplementary Fig A.

Figure 3. Expression of *CCNDs* and *MAFB* mRNA in primary and HMCL OH-2
The expression patterns of OH-2 primary cells and OH-2 cell line are quite similar. Both have high expression of cyclin D2 and low expression of cyclins D1 and D3, but with a higher expression of cyclin D3 in the primary sample. INA-6 is a t(11;14) cell line and expresses high amounts of cyclin D1. ANBL-6 is a t(14;16) cell line and expresses high amounts of cyclin D2. *MAFB* is almost equally expressed in HMCL and primary OH-2 cells. The scale is in log10 and the values are relative quantitation based on the delta Ct method (see Methods). Cyclin D3 expression in OH-2 cell line was set as 1 and all values are normalized to this. Error bars show standard deviation of triplicates.

Figure 4. *MAFB* and *MYC* is juxtaposed in a complexed translocation
a) Normal chromosome 8 with WCP8 in green and *MYC* red; b) normal chromosome 20 with WCP20 in aqua and *MAFB* red; c) *MAFB* and probes centromeric to *MAFB* localize the breakpoint on der(20); d) Ck Enh is a PCR derived probe covering the enhancers in *IGK*, co-localizes with *MAFB* and *MYC* on both junctions on der(8); e)

der(8)t(8;20) with *MAFB* red; f) der(8) is replaced by der(8)t(8;20)t(8;20) in some cells, with *MYC* and *MAFB* juxtaposed at both 8;20 junctions. This is also detected in primary OH-2 cells giving two fusion *MYC*/*MAFB* (arrows) signals together with two CEP8 and one normal *MYC* and *MAFB* signals; g) Most of the cells have only one 8;20 junction. This is also detected in interphase nuclei with *MYC*/*MAFB* (arrow) fusion signal both in the HMCL and primary cells.

Figure 5. How did the complex translocation process occur?
There might have been an initial reciprocal translocation between chromosomes 2 and 20, which juxtaposed *MAFB* and the *IGK* locus on der(2), but without the usual split between the two *IGK* probes. A subsequent reciprocal translocation between der(2) and normal chromosome 8 would have generated der(8)t(8;20) and der(2)t(2;8), with the *IGK* sequences localized on der(2), but with co-localization of *MYC*, *MAFB*, and *IGK* enhancer sequences on der(8). The der(8)t(8;20)t(8;20) must have been generated subsequently by a rearrangement of der(8) that included duplication of the 8;20 junction. See Table 2 and text for further details.

Figure 6. OH-2 responds to a wide selection of cytokines.
Proliferation measured with methyl-[^{3}H]-thymidine incorporation in DNA in OH-2 cell line. Stimulation with different cytokines shows that OH-2 is responsive to a wide selection of cytokines, both alone and to different cytokines together with additive or synergistic effects. Error bars show standard deviation of triplicates.

Figure 7. Continuously growth of OH-2 HMCL
OH-2 is a slow growing cell line that is difficult to grow without human serum and IL-6. The chart shows a “stock experiment” in which different RPMI media, FCS and human serum, and different cytokines were used. 200 000 cells/ml were seeded at day 1. The cells were counted before media was replenished twice a week. After 24 days the only stock that survived was the one supplemented with human serum and IL-6. Fourteen different conditions were examined, but only eight representative conditions are shown in this chart.

Table 1. Selected HH-U133-Plus-2 expression results in 47 myeloma cell lines.
The values are normalized for 606 samples, including 559 untreated MM tumors and 47 HMCL. The sample order is in descending order based on a MAF index (MAFI), with 15 HMCL and 42 of 45 MM tumors in the MF group (not shown) included among the 57 samples with the highest MAF index. Translocation (TLC) targets are mainly associated with *IGH* but a few involve *IGL* (*), *IGK* (§) or no detectable Ig sequences (#). $ All HMCL express *MYC* except for PE-2 and U266, which express *MYCN* and *MYCL*, respectively (MK, unpublished). See Materials and Methods for additional details.

Table 2. FISH mapping of translocated chromosomes. The positions of probes relative to the *IGK* constant region and the 5’ ends of the *MYC* and *MAFB* genes are indicated in kb, with negative values indicating a location 5’ of the gene. *These smaller PCR (-1>+25kb) and BAC probes that were labeled with biotin and reacted with FITC-streptavidin uniquely detected *IGK* sequences at 8;20 junctions on both kinds of der(8).

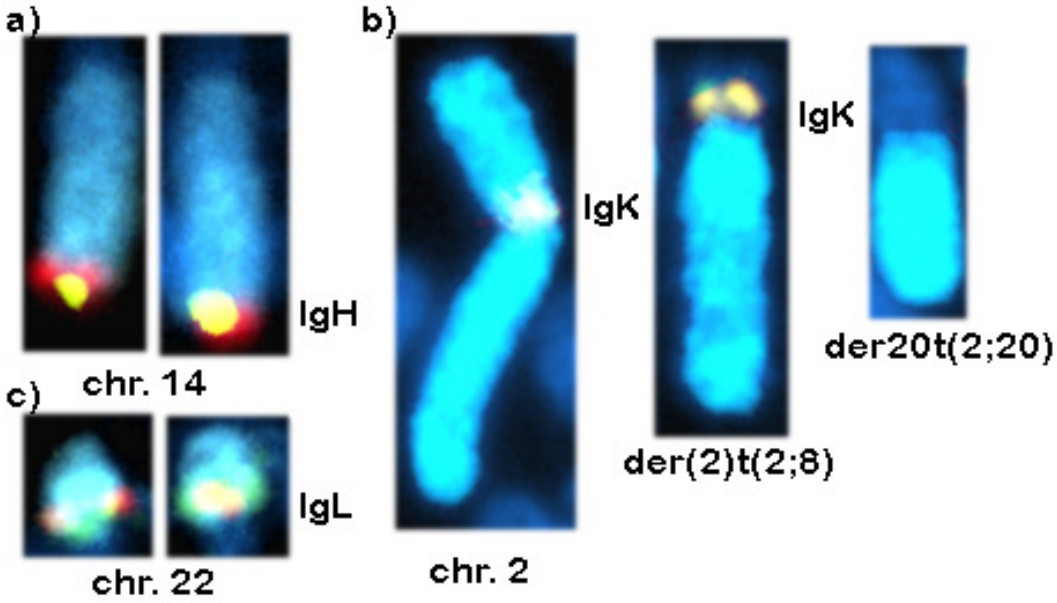
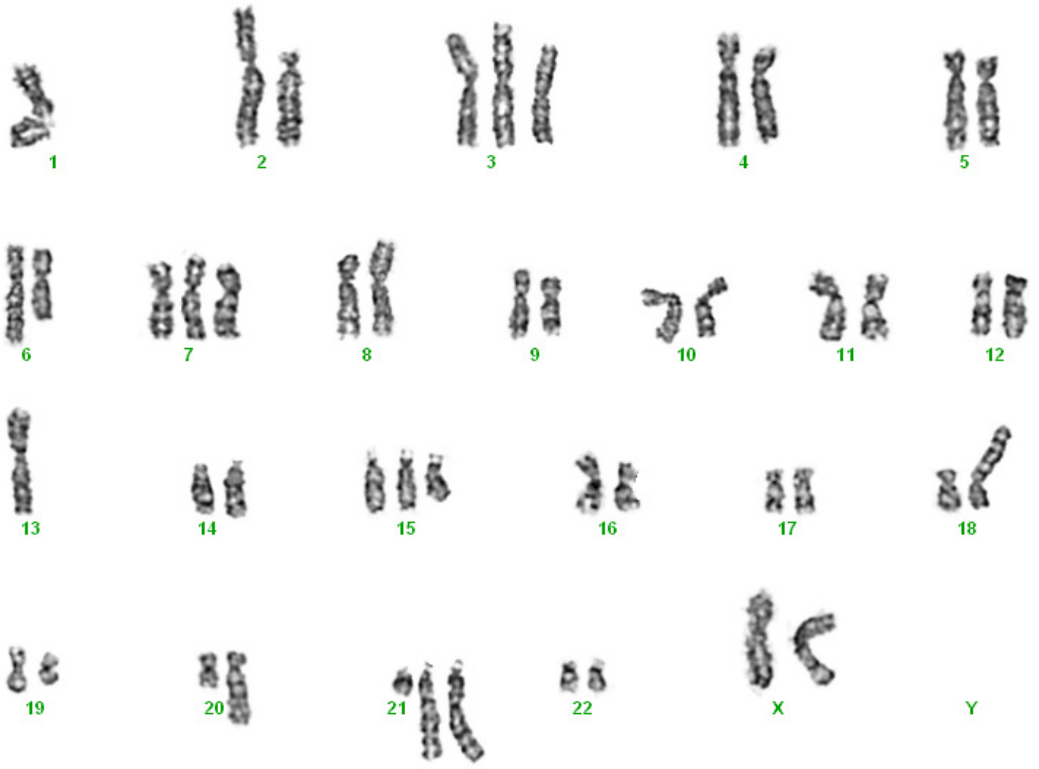


Figure 1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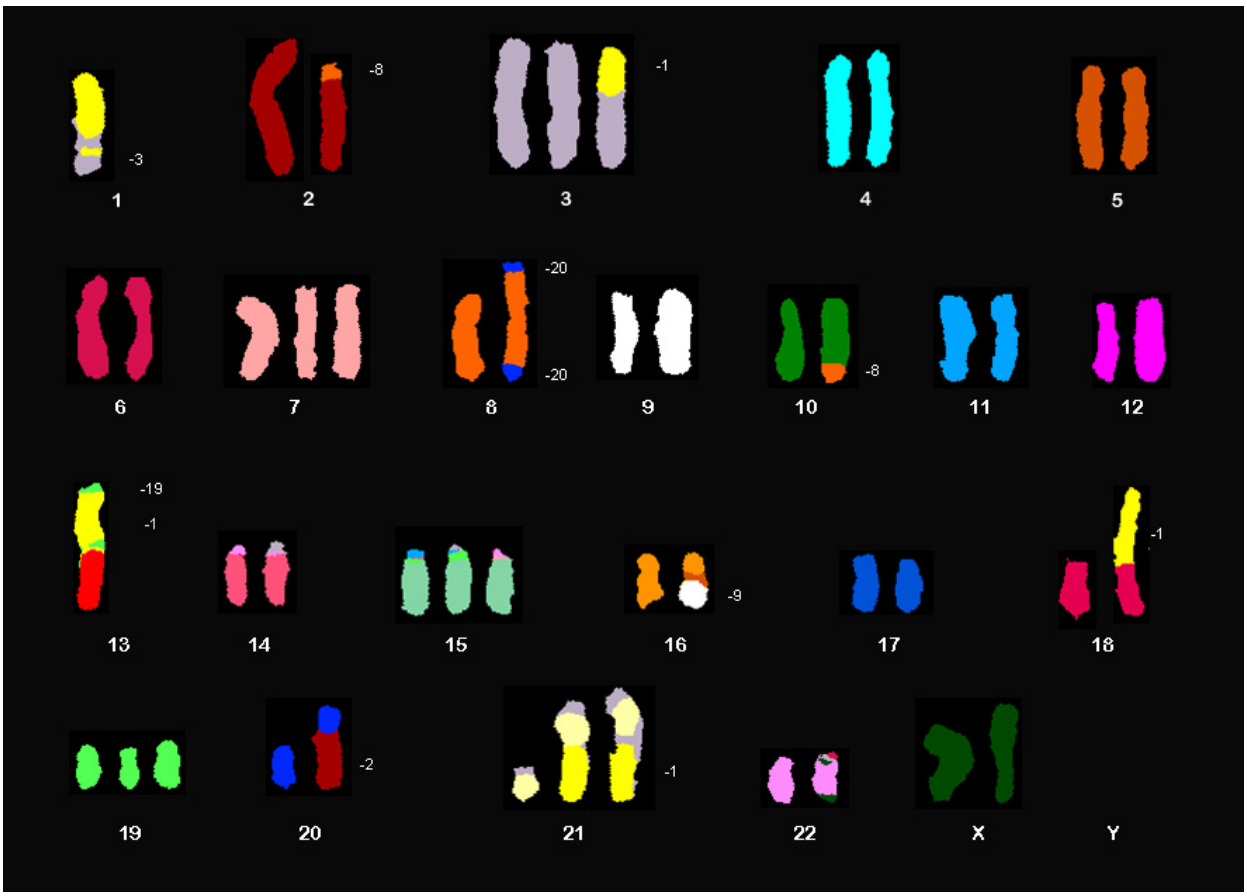
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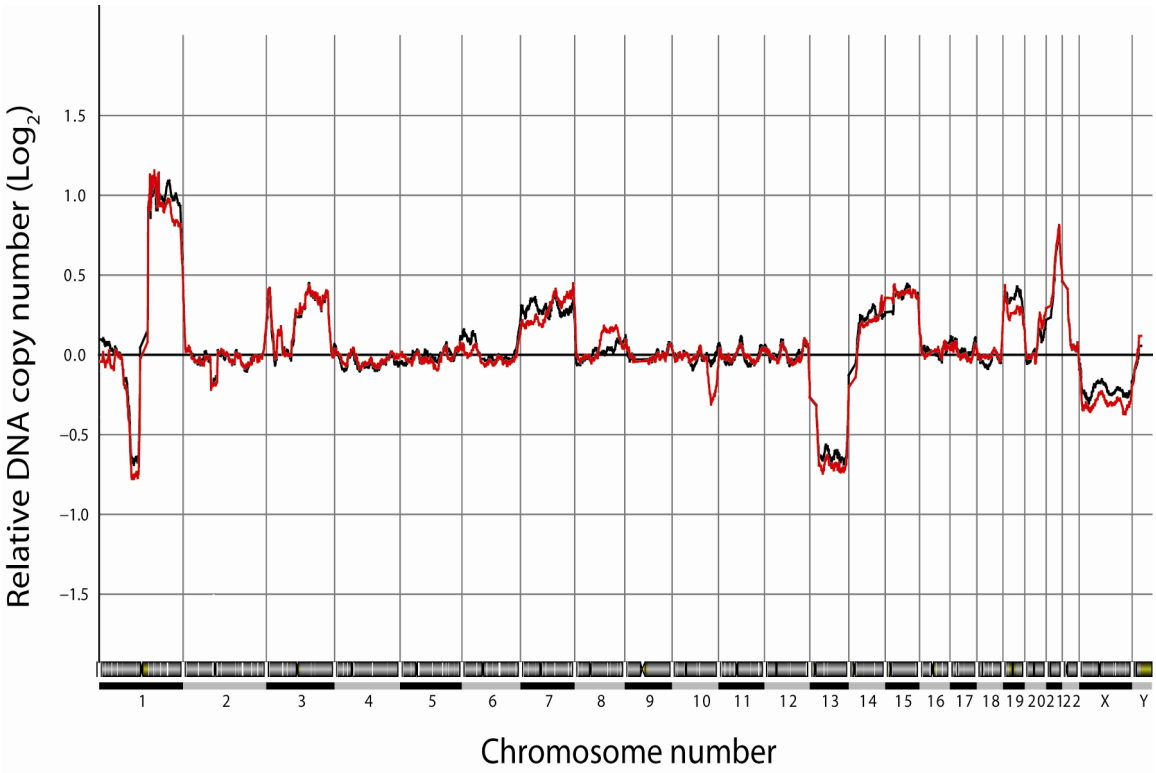
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Chromosome number

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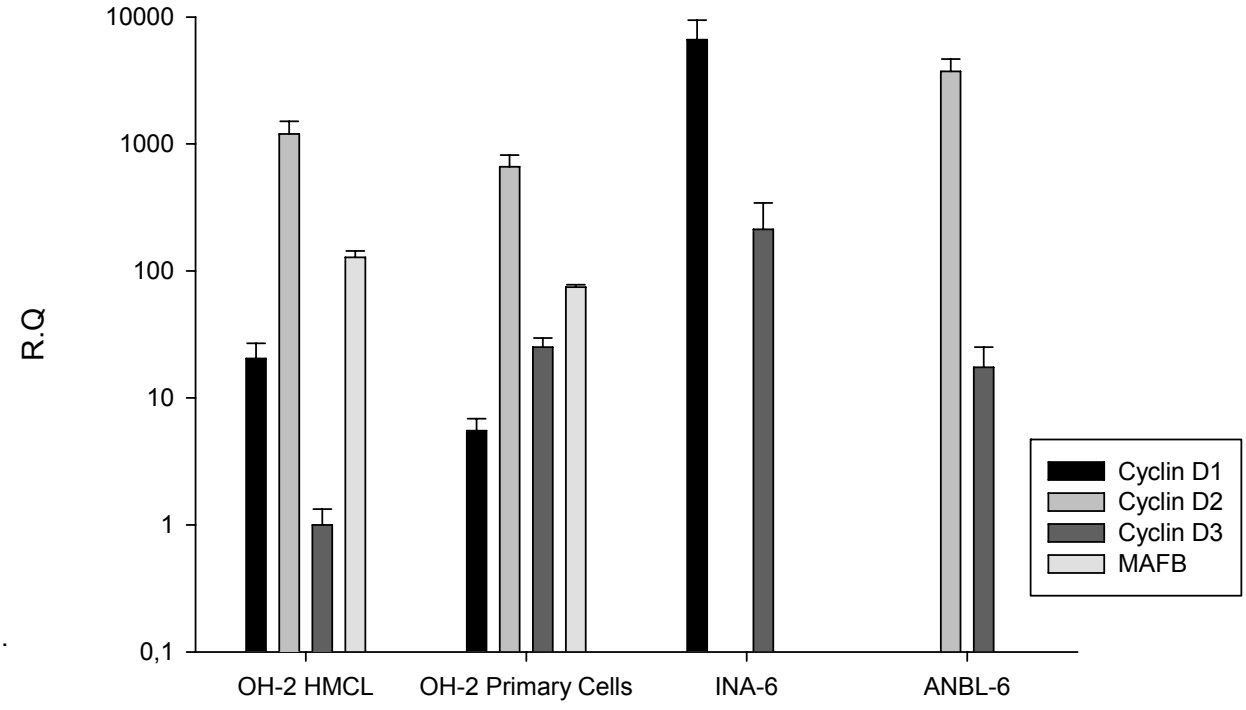
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OH-2 Primary Cells
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ANBL-6
Cyclin D1
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Cyclin D3
MAFB

Figure 3

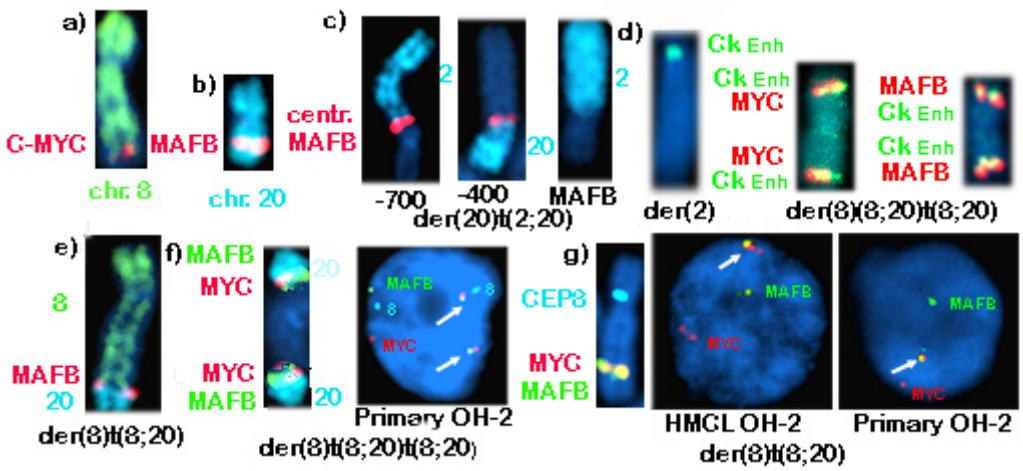
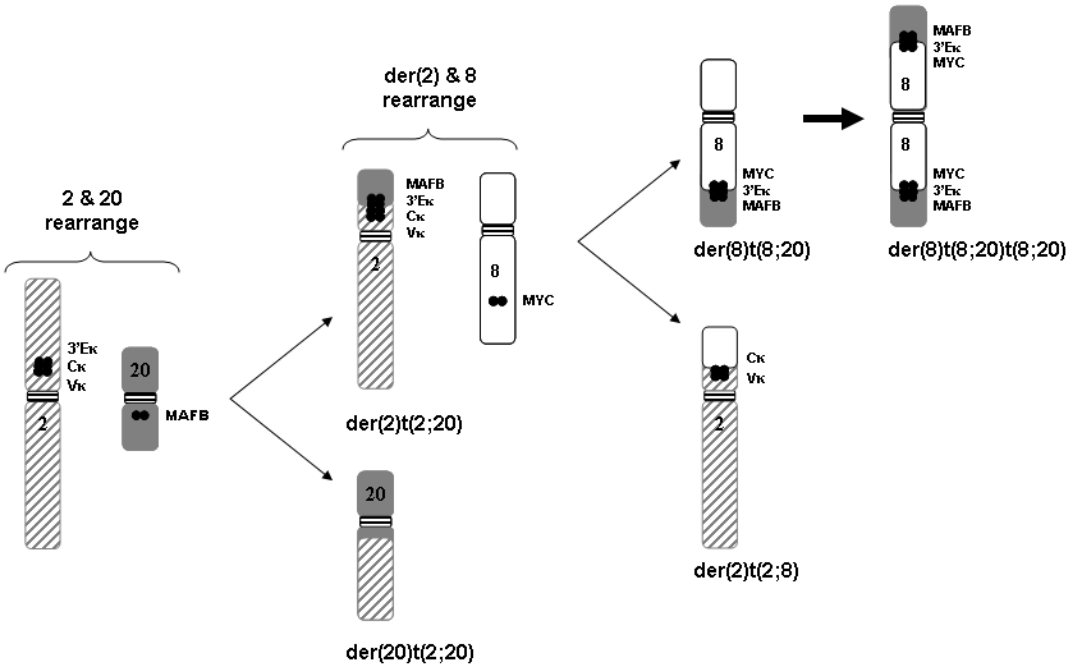


Figure 4



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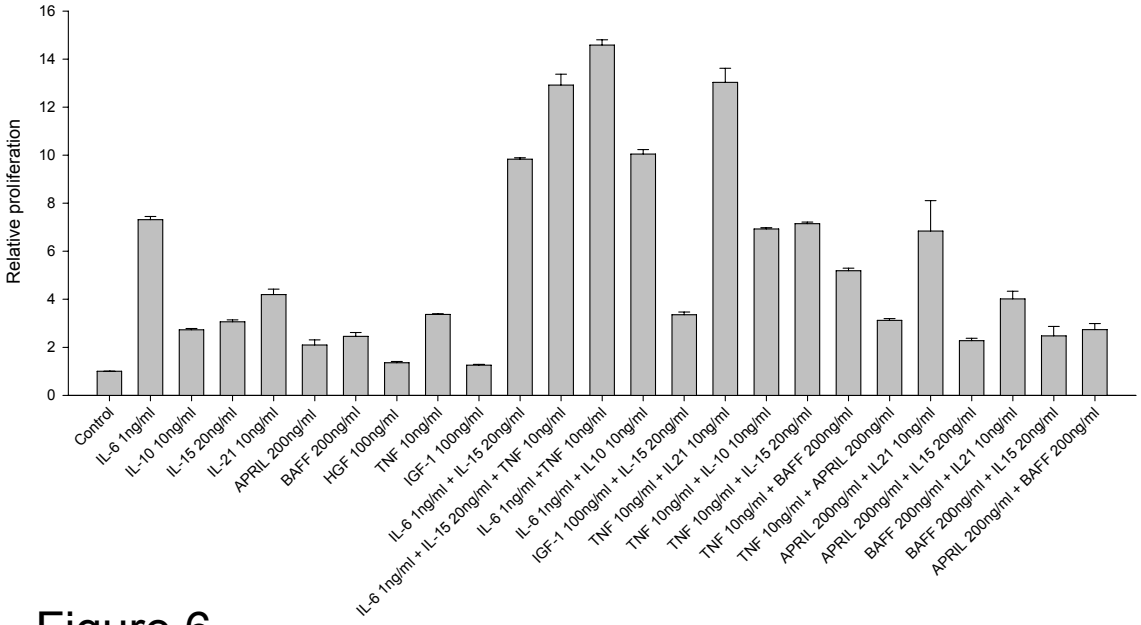

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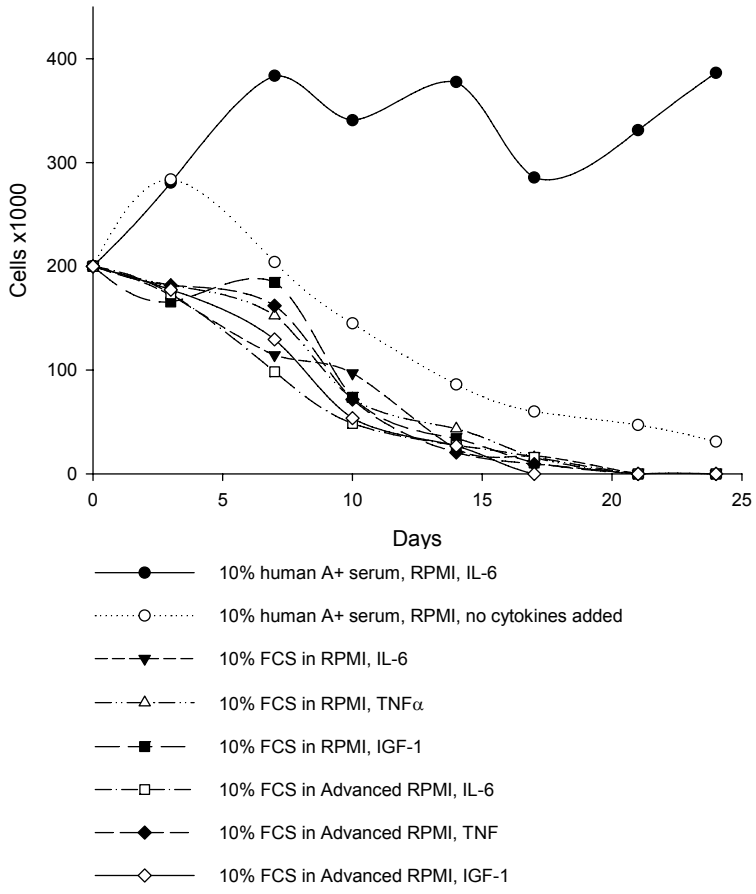


Figure 7

Figure A. Array CGH of primary and HMCL OH-2.
Genome-wide ratio plot of OH-2 primary cells (upper panel) and OH-2 cell line (bottom panel) by array CGH.

Figure B. Sequencing results, primer position and sequences of the *TP53* primers.
There were no mutations in *TP53* in the cell line. Figure B I and B II show the position and sequence of the primers, respectively. OH-2 has the amino acid prolin in codon 72.

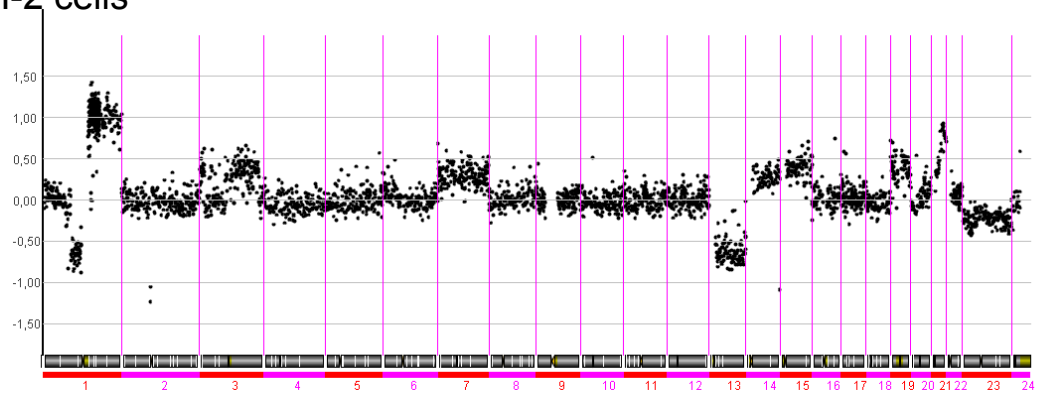
Figure C. Sequencing of *RAS* in OH-2 cell line.
OH-2 has a mutation in codon 61 in *NRAS*. There is no expression of *HRAS*, and *KRAS2B* is wild-type.

Figure D. **Array comparative genomic hybridization identifies duplicated sequences and breakpoints in OH2 cell line.**
Copy number of OH2 genomic DNA sequences normalized to placental male DNA was determined by segmental analysis on Agilent custom arrays for chromosome 8 (A), chromosome 2 (B), and chromosome 20 (C). Affected translocation targets: *MYC* locus, 3'-enhancer of *IgK*, and *MAFB* locus are indicated by vertical arrows, and distances from targets to the breakpoints are shown above horizontal arrows.
Abscissa: position for corresponding chromosome, bp
Ordinate: log2 ratio of Cy5 to Cy3 signal

Supplementary

Primary

OH-2 cells



OH-2
HMCL

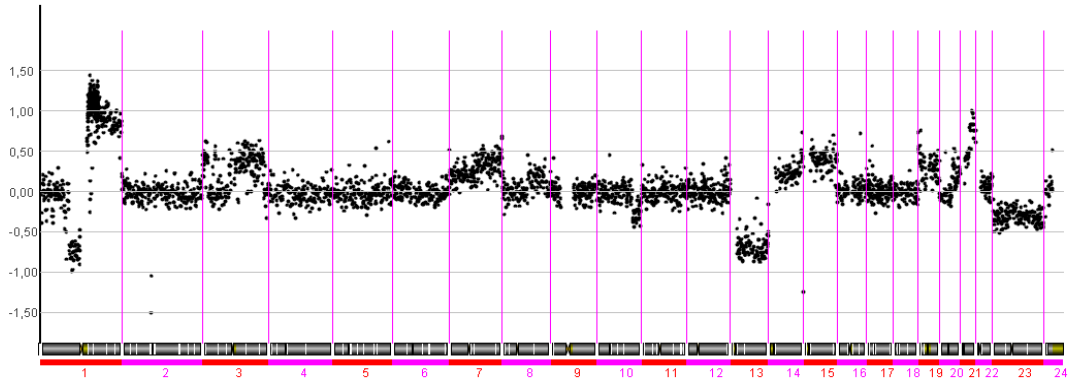
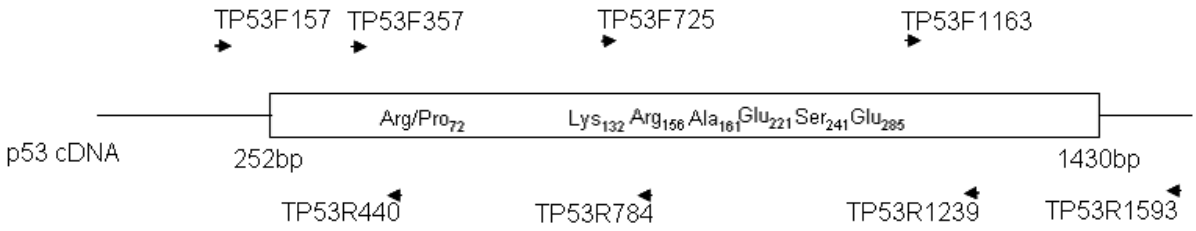


Figure A



B I

Primer	Sequence
TP53F157	5'-CCGGGGACACTTTGCGTT-3'
TP53F357	5'-CCGTCCCAAGCAATGGTA-3'
TP53F725	5'-CGCCATGGCCATCTACAA-3'
TP53F1163	5'-TAAGCGAGCACTGCCCAA-3'
TP53R440	5'-GCCTCTGGCATTCTGGGA-3'
TP53R784	5'-CTGAGCAGCGCTCATGGT-3'
TP53R1239	5'-ACGCCCACGGATCTGAAG-3'
TP53R1593	5'-AACACCAGTGCAGGCCAA-3'

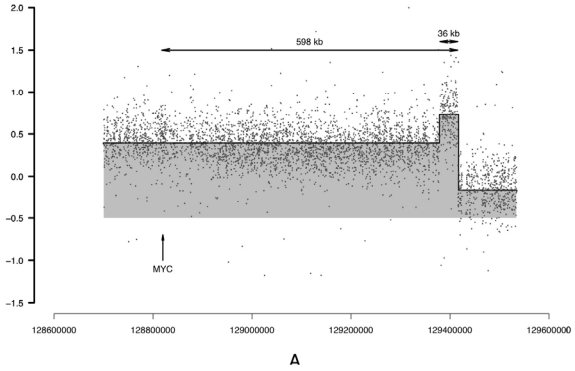
B II

Figure B

	N-Ras			H-Ras			K-Ras2b		
Codon	12	13	61	12	13	61	12	13	61
wt	**GGT Gly**	**GGT Gly**	**CAA Gln**	**GG C Gly**	**GGT Gly**	**CC A Gln**	**GGT Gly**	**GGC Gly**	**CAA Gln**
OH	**GGT Gly**	**GGT Gly**	**AAA Lys**				**GGT Gly**	**GGC Gly**	**CAA Gln**

Primer	Sequence
N-RasF	5'-CCAAAGCAGAGGCAGTGGA-3'
N-RasR	5'-TGCAGCTTGAAAGTGGCTCTT-3'
H-RasF	5'-GCAGGCCCCTGAGGAGC-3'
H-RasR	5'-TVAGGAGACCACACACTT-3'
K-RasF	5'-GGGAGAGAGGCCTGCTCAA-3'
K-Ras2aR	5'-GAAGGCATCATCAACACCCAG-3'
K-Ras2bR	5'-GAAGGCATCATCAACACCTGT-3'

Figure C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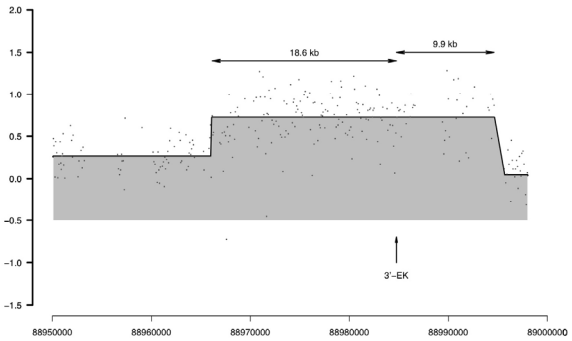
A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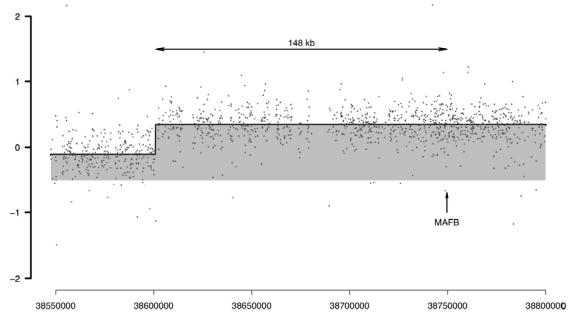
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C

Figure D

Paper II

FGFR3 is expressed and important for survival in INA-6, a human myeloma cell line without a t(4;14)

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(2) Department of Pathology and Medical Genetics, St. Olavs University Hospital, Trondheim, Norway.
(3) Department of Hematology, St. Olavs University Hospital, Trondheim, Norway.
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Running title: FGFR3 is important in t(4;14)^{NEG} HMCL INA-6

Acknowledgement

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Keywords: FGFR3, SU-5402, PD173074

Abstract

Fibroblast Growth Factor Reseptor 3 (FGFR3) is an oncogene that is dysregulated by the t(4;14) in multiple myeloma (MM) cells, and which is usually not expressed in MM cells without this translocation. By fluorescence *in situ* hybridization (FISH) we discovered an amplification of the *FGFR3* locus in INA-6, an MM cell line without t(4;14). There was also no involvement of the other Ig loci in translocations with the *FGFR3* gene. By PCR and Western blot we demonstrated expression of FGFR3 mRNA and protein in the cells, probably caused by the extra copy of the gene. The FGFR3 inhibitors SU5402 and PD173074 decreased the proliferation of INA-6, which indicates that FGFR3 may play a role also in cells without the t(4;14).

Introduction

MM is a genetically unstable malignancy of long-lived plasma cells and accounts for 10% of all haematological malignancies. Characteristic of this neoplasm is accumulation of a malignant clone within the bone marrow. Multiple and complex chromosomal abnormalities are common in MM, and translocations involving the immunoglobulin heavy chain (*IGH*) locus on 14q32 are present in 40-60 % of patients. The five primary *IGH* translocations include 11q13, 4p16, 16q23, 6p21 and 20q12, targeting *CCND1, FGFR3/MMSET*, *C-MAF*, *CCND3* and *MAFB* respectively (1).

Switch translocations in MM separate the strong 3'α- and μ enhancers of *IGH* onto different derivative chromosomes. The primary t(4;14)(p16.3;q32) chromosomal translocation results in dysregulation of genes on both derivative chromosomes. On der4 Multiple Myeloma SET-domain (*MMSET*) and on der14 fibroblast growth factor receptor 3 (*FGFR3*) is the target gene involved. The translocation results in ectopic expression of functional (FGFR3) in 70 % of the t(4;14) patients. The IgH/MMSET fusion product is expressed in all t(4;14) patients (2). The MMSET isoform RE-IIBP has recently been shown to be a histone methyltransferase with transcriptional repression activity (3). MMSET has also been shown to be of importance in cellular adhesion, clonogenic growth and tumorigenicity (4). t(4;14) implies a poor prognosis and shorter survival after traditional MM treatment (5), whereas novel treatment regimens seem to be particularly effective in this patient group and conceal the prognostic impact of t(4;14).
FGFR3 is one of four transmembrane tyrosine kinase receptors for the FGF family of ligands. Normally, FGFR3 is expressed in the kidneys and lungs and during the development of the nervous system and not in normal plasma cells (6). It has been demonstrated that FGFR3 can function as an oncogene, and contribute to tumor progression in MM (7, 8). It has also been shown that ectopic expression of FGFR3 promotes cytokine independence and that inhibition of the FGFR3 promotes apoptosis (7, 9).

It is uncommon for MM cells to express FGFR3 without a t(4;14) (10, 11). On this background, we here describe that the human myeloma cell line (HMCL) INA-6 has FGFR3 expression without the t(4;14) or any other Ig locus involvement. We also

demonstrate that the expression of the FGFR3 is important for survival of this particular cell line.

Materials and Method:

Cell lines

The cell lines INA-6 (gift from Dr Gramatzki, Erlangen, Germany), OH-2 (12) and IH-1 (13, 14) were maintained as previously reported. All three cell lines are IL-6 dependent (15).

Fluorescence *in situ* Hybridization

The probes used for fluorescence *in situ* hybridization (FISH) were made from Bacterial Artificial Chromosome clones containing the desirable regions (gift from R. Fonseca). For the detection of the translocations we used a fusion strategy employing two sets of probes, one that hybridizes to 14q32 and one that hybridizes to the desired region, 4p16.3 or 11q13 (Table of clones in supplementary material A). The probes were labelled according to standard nick translation protocol (Vysis, Abbot laboratories, Des Plaines, IL). Centromere enumeration probe (CEP) 4 SpectrumGreen (Vysis) and Whole Chromosome paint (WCP) 14 in aqua (Applied Spectral Imaging (ASI), Micro System AB, Spånga, Sweden) were used for assessing the locus-specific probes to their chromosomes. Metaphase spreads were made according to standard methods (16). The metaphase spreads were air-dried at least over night, but not chemically treated in any way before hybridization. Hybridization was done after standard Vysis protocol. Cells were scored using a NIKON ECLIPSE 90i epifluorescense microscope with PlanApo VC 60x/1.4 oel (Nikon Instruments Europe B.B, Badhoevedorp, The Netherlands), and software from Applied Imaging (CytoVision version 3.7 Build 58, San Jose, CA).

Sequensing

The four *FGFR3* regions extracellular (EC) domain, transmembrane (TM) domain, tyrosine kinase (TK) domain and stop codon (SC) are known to be hot spots for activating mutations. To determine if the receptor in our cell lines was mutated, the receptor was amplified by reverse transcription (RT)-PCR. Total RNA was isolated using RNeasy Mini Kit (Qiagen, Crawley, UK) from INA-6 and IH-1 cells grown in standard condition. cDNA was made from RNA using SuperScript®III First Strand Synthesis System for RT-PCR (Invitrogen). Primer sequences were obtained from Chesi et al (7) (Supplementary material B). Each PCR reaction was purified with QIAquick PCR Purification Kit (Qiagen), and then directly sequenced using BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). The sequencing reaction was purified with DyeEx 2.0 Spin Kit (Qiagen) and analyzed on ABI3100 Genetic Analyzer (Applied Biosystems).

Real Time RT-PCR

RNA was isolated and cDNA made as previously described (15) from cells directly from stock. *FGFR3* and *MMSET* TaqMan primers (HS00179829_m1 and HS00370212_m1 TaqMan, Gene Expression Assays, Applied Biosystems, Foster City, CA), were used to detect the FGFR3 and MMSET expression. The comparative Ct-method was used for

quantization with *GAPDH* (HS99999905_m1) as housekeeping gene on the StepOnePlus Real-Time PCR system (Applied Biosystems).

Western Blot Analysis
Samples of IH-1, OH-2 and INA-6 were made as previously described (15). Membranes were blocked with non-fat dried milk (5%) in Tris-Buffered Saline supplemented with Tween-20 (0.05%) and incubated with antibody against FGFR3 (rabbit polyclonal IgG anti-FGFR3 (H-100), Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. GAPDH was used as loading control. Antibody binding was visualized after staining with horseradish peroxidise-conjugated secondary antibodies (DAKO Cytomation, Copenhagen, Denmark) and detection of chemiluminescense (ECL, Amersham, UK) by photographic film (Amersham).

Cell Proliferation Assay
Two small-molecule receptor tyrosine kinase inhibitors were employed in this study, each of which function in a similar manner by competing with ATP for the specific binding site at the catalytic domain of the fibroblast growth factor receptor. SU5402 (3-[(3-(2-carboxyethyl)-4-methylpyrrol-2-yl)methylene]-2-indolinone) (17) and PD173074 (1-tert-Butyl-3-[6-(3,5-dimethoxy-phenyl)-2-(4-diethylaminobutylamino)-pyrido[2,3-d]pyrimidin-7-yl]-urea) (18) were both obtained from Pfizer Global Research and Development. The compounds were dissolved in DMSO and stored at -80°C. Cells at a density of 2.0 x 10^4 INA-6 and IH-1, and 4.0 x 10^4 OH-2, were seeded in 200 µl RPMI supplemented with 10 % FCS with or without cytokines in 96-wells plastic culture plates (Corning Costar, Corning, NY, USA). Cells were starved for 4h, then SU-5402 at concentrations from 3,125 µM to 60 µM or PD173074 at concentrations from 6,25 nM to 200 nM was added. After 30 min, 20 ng/ml aFGF + 100 µg/ml Heparin (compounds and concentrations from (18, 19)) were added. The control was only provided with 10 % FCS and the appropriate concentration of DMSO. Proliferation was measured as previously described (15). The counts per minute (cpm) obtained were normalized to the serum control sample and shown in figure 3.

Viability test
Flow cytometry was used to determine the percentage of viable cells with annexin V-FITC binding and PI uptake (Apoptest-FITC kit, Nexins Research, Kattendijke, Netherlands). 1 x 10^6 cells treated as above were incubated with 0.06 µg annexin-FITC for 1 h and 0.5 µg PI for 5 min on ice in PBS containing 0.1 % bovine serum albumin, before analysis.

Results and Discussion

INA-6 has an abnormal pattern of 4p16
Most reported cases of FGFR3 expression in MM are correlated to the t(4;14)^{POS}. The expression is caused by the *IGH* enhancer or a mutated FGFR3 believed to be caused by somatic hypermutation (20). Here we describe a HMCL, INA-6, that has expression of FGFR3 without involvement of the usual t(4;14)^{POS}. To verify the chromosomal abnormalities in the cell lines, metaphase FISH was used, and any *IGH* rearrangement

was detected using fusion strategy. INA-6 had an unbalanced t(11;14)^{POS} (21) with two der(14) chromosomes and no der(11) (Fig. 1a). Figure 1b shows that INA-6 has triploid chromosome 4 with two of the chromosomes apparently normal, and one chromosome 4 with an extra copy of locus 4p16.3, which includes both *FGFR3* and *MMSET* on the 4p arm. There was no difference between the signal pattern when using the probe covering *FGFR3* or the probe covering *MMSET* alone. The aberration on chromosome 4 was verified by array-based comparative genomic hybridization showing that the area spanning from 4p15.32 near the LIM domain binding 2 gene to the 4p telomere was amplified (data not shown). This is in concordance with the array comparative genomic hybridization data on INA-6 from Mayo Clinic public available at: http://www.broad.mit.edu/mmgp/pages/portalHome.jsf#. Because secondary translocations to the other Ig genes are known to dysregulate genes in MM, *IGL* and *IGK* were also checked with FISH on metaphase chromosomes. The light chain loci where shown not to be rearranged (data not shown). In figure 1c we show that IH-1 has a classical t(4;14)^{POS} with *FGFR3* on der(14) and *MMSET* on der (4) and is used as positive controls in the assays. OH-2 is used as a negative control because it does not have an *IGH* translocation (Våtsveen, manuscript) and does not express FGFR3.

Wild type FGFR3 is expressed in both HMCLs

To check the expression of FGFR3 in IH-1 and INA-6, Real Time RT-PCR and Western blots (WB) were made. Figure 2a shows Real Time data. INA-6 expressed less FGFR3 mRNA than IH-1. OH-2 was used as a negative control that did not express FGFR3. The WB showed that both IH-1 and INA-6 expressed FGFR3, also here IH-1 has a higher expression of FGFR3 but not to the same extent as seen by the PCR. Figure 2b shows the WB bands of the FGFR3 in the cell lines with GAPDH used as loading control. Because t(4;14)^{POS} is associated with both increased expression and activating mutation of the *FGFR3* (22), sequencing was done, with no mutation detected in *FGFR3* in either INA-6 or IH-1 (data not shown).

Inhibition of FGFR3 leads to lower DNA synthesis and apoptosis in INA-6 and IH-1

Inhibition of FGFR3 in MM cell lines blocks growth and is associated with apoptosis (19). The FGFR3 inhibitors SU5402 and PD173074 were therefore tested on INA-6, IH-1 and OH-2. IH-1 is used as a positive control in the experiments because of its t(4;14)^{POS} and unmutated *FGFR3*. OH-2 is used as a negative control because it has no *IGH* translocations or FGFR3 expression (Vatsveen submitted manuscript). The proliferation assay measuring DNA synthesis showed decreased proliferation of unstimulated INA-6 and IH-1 in the presence of the inhibitor SU5402 at concentrations as low as 3,25 µM and 15 µM, respectively (figure 3a), and with the inhibitor PD173074 at 25 nM and 50 nM respectively (supplementary figure Ia). On the basis of the titration curve 15 µM SU5402 and 100 nM PD173074 was used further to show the decrease in proliferation with 20 ng/ml aFGF + 100 µg/ml heparin added to the cells. IH-1 was more responsive to the inhibitor when aFGF was added, whereas INA-6 responded in approximately the same way both in the presence and in the absence of aFGF (Figure 3b). PD173074 in concentration at 100nM gave almost identical results as with 15µM SU5402 (PD173074 results in supplementary figure Ib). In our experiments OH-2 showed no change in proliferation by addition of SU5402 or PD173074, not even at concentrations 60µM (figure 3a) or 200nM (supplementary figure Ia) respectively, which indicates that the

inhibitors were not toxic at the concentrations used. We also testes effects of the inhibitors in a viability assay based on annexin V binding. Figure 3c shows the viability of aFGF-stimulated cells with and without SU5402 inhibitor. In INA-6, the percentage of viable cells with the inhibitor was almost zero. IH-1 had also a decline in the percentage viable cells, but not to the same degree as INA-6, paralleling the relative responses of the two cell lines in the proliferation assay (viability data for PD173074 in supplementary figure Ic). OH-2 had no significant change in the viability with inhibitor added. It has earlier been demonstrated that even at 72-hour stimulation, PD173074 is not cytotoxic to myeloma cells (23). We thereby assume that the decreased proliferation and apoptosis seen in IH-1 and INA-6 was due to specific inhibition of the FGFR3. For INA-6 the extra copy of *FGFR3* and the abnormal position of the locus can possibly explain the FGFR3 detected on WB and the ability the inhibitors have to lower proliferation and enhance apoptosis. It also shows that the expression of FGFR3 is important for the survival in these cell lines.

Because expression of FGFR3 in t(4;14) ^{NEG} only has been reported in a few cases (10, 11, 24), it is likely that FGFR3 expression is not very common in t(4;14)^{NEG} patients. Even though FGFR3 expression is rare in t(4;14) ^{NEG}, INA-6 can be a good model to elucidate the role of *FGFR3* in MM cells. Since it does not express *MMSET* at a high level, contrary to all the t(4;14)^{POS} cell lines (supplementary data II), and, hence, lacks the *IGH/MMSET* fusion transcript, it might be useful as a model system for investigating *FGFR3's* role in MM cells independently of the interaction of high levels of *MMSET*.

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Figure legends

Figure 1. Fluorescence *in vitro* hybridization on metaphase spreads is used to identify chromosomal abnormalities in the HMCLs.
a) INA-6 has an unbalanced t(11;14) with two copies of der(14). *IGH* is labelled in green and 11q13 *CCND1* in red. Four copies of chromosome 11 is found in the HMCL; b) Probes for *FGFR3* and *MMSET* red shows a extra copy of the 4p16.3 loci on one of the chromosome 4 p-arm. CEP4 green confirms that the genes are present on chromosome 4; c) IH-1 has a classical t(4;14). *IGH* is labelled in green *FGFR3/MMSET* in red.

Figure 2. Real Time RT-PCR and Western Blot confirm FGFR3 expression in INA-6
a) Real Time RT-PCR shows FGFR3 expression in both INA-6 and IH-1 with a higher expression in IH-1. Delta Ct-method with GAPDH as control is used for the relative quantisation. Western Blot shows IH-1 and INA-6 expresses FGFR3, with OH-2 as a negative control with no FGFR3 expression.

Figure 3. SU5402 and PD173074 similarly decrease proliferation measured as DNA synthesis and viability in HMCLs expressing FGFR3
a) HMCL OH-2, INA-6 and IH-1 were treated with the FGFR3 inhibitor SU5402 in increasing concentrations from 3,25μM to 60μM and plotted against relative proliferation where 1 was set as proliferation in untreated (10% FCS and DMSO only) cells. Proliferation is measured as DNA synthesis by ^{3}H-Tymidin incorporation. The proliferation decreased significantly in both INA-6 and IH-1 in as low concentrations as 15μM, whereas OH-2 was unaffected by the inhibitors even at 60μM where both IH-1 an INA-6 had a very low proliferation. Error bars represent standard deviation of triplicates.
b) The HMCLs where also grown with either 20ng/ml aFGF+100μg/ml heparin or in the presence of only serum and DMSO. Each condition was treated with or without the FGFR3 inhibitor SU5402 at 15μM. In both INA-6 and IH-1 aFGF + heparin induced cell proliferation, and, SU5402 led to a decrease in DNA-synthesis in both unstimulated and aFGF + heparin stimulated cells. In IH-1 the decrease in proliferation is greater when treated with aFGF compared to untreated control. OH-2 do not express FGFR3, and inhibition of the FGFR3 has no effect in this cell line. Error bars represent standard deviation of triplicated data. (Counts per minute were normalized to 1 to the HMCLs sample with serum, for simplification)
c) OH-2, INA-6 and IH-1 with the same conditions as in b) were labelled with PI and annexin to measure the viable cells with flow cytometry. 15μM SU5402 with 20ng/ml aFGF+100μg/ml heparin is shown as an example. The inhibitors drastically decreased the viability in INA-6. In IH-1 the overall viability are a bit higher than in INA-6, but is also decreased with the inhibitors. OH-2 has as expected no significantly difference in viability with or without the inhibitors. The low % of viable cells in untreated sample OH-2 and INA-6 is what we always experience when the cells are grown for more than over night without IL-6. Error bars represent standard deviation of duplicated flow data. All data shown in figure 3 are representative for at least two separate experiments. The results with FGFR3 inhibitor PD173074 is shown in supplementary figure I.

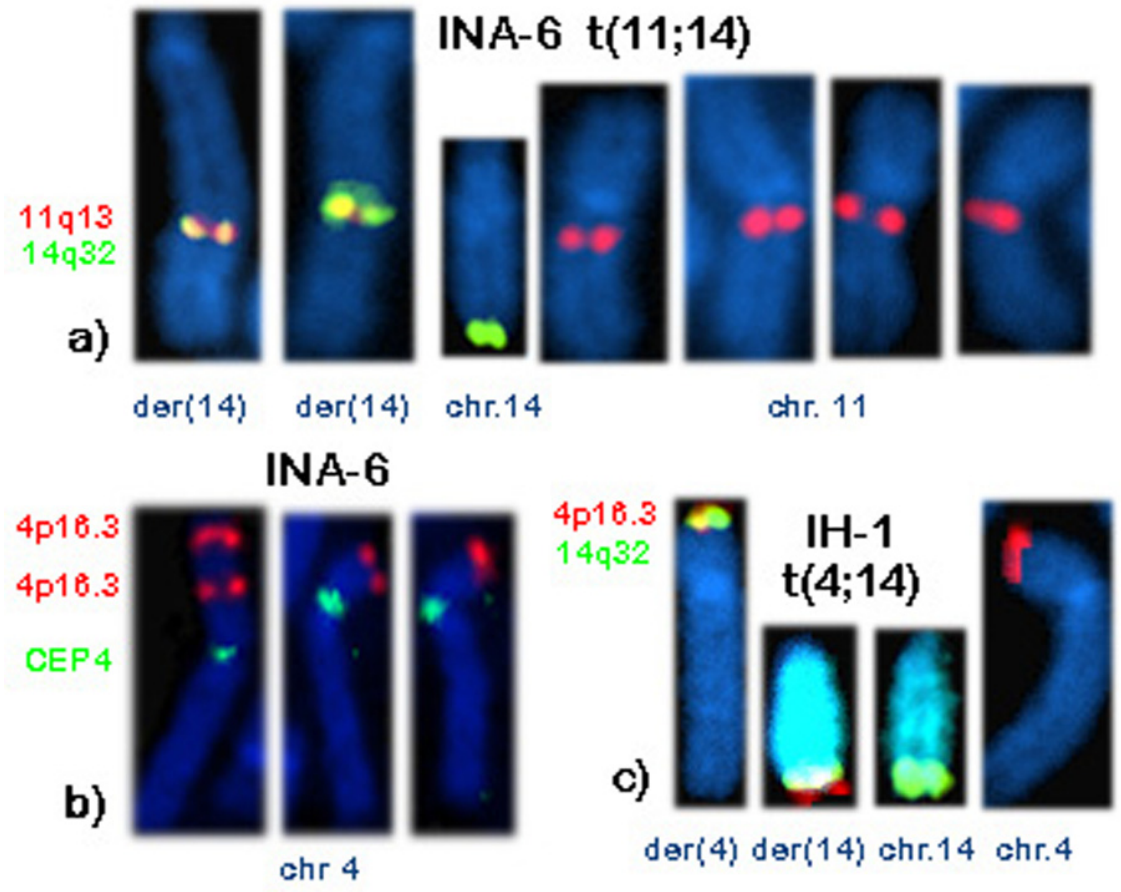
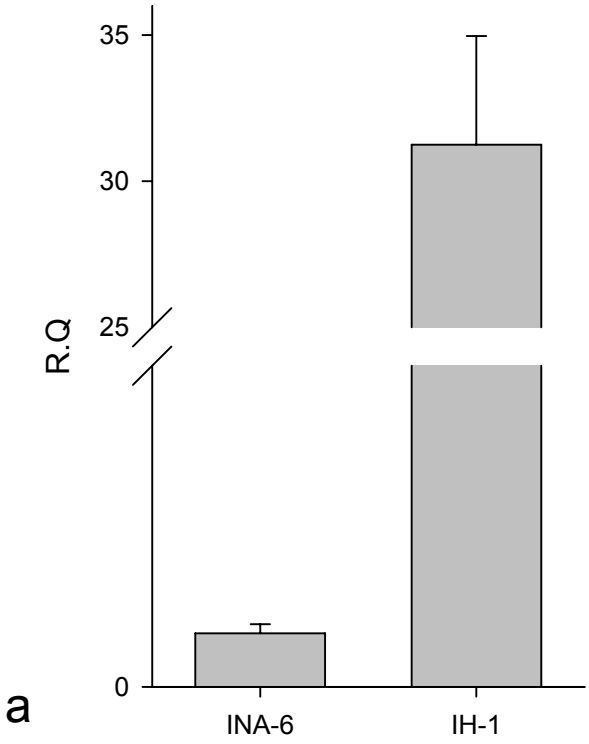

INA-6 t(11;14)
11q13
14q32
a)
der(14)
der(14)
chr.14
chr. 11
INA-6
4p16.3
4p16.3
CEP4
b)
chr 4
4p16.3
14q32
IH-1
t(4;14)
c)
der(4)
der(14)
chr.14
chr.4

Figure 1



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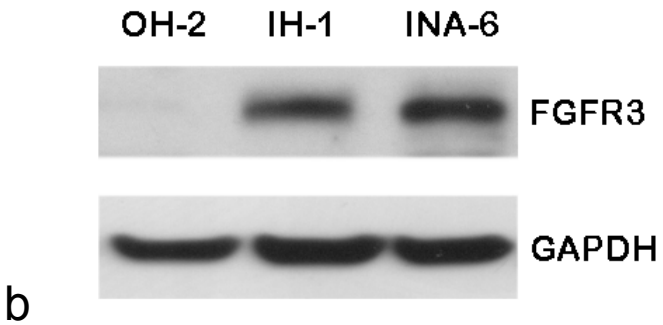
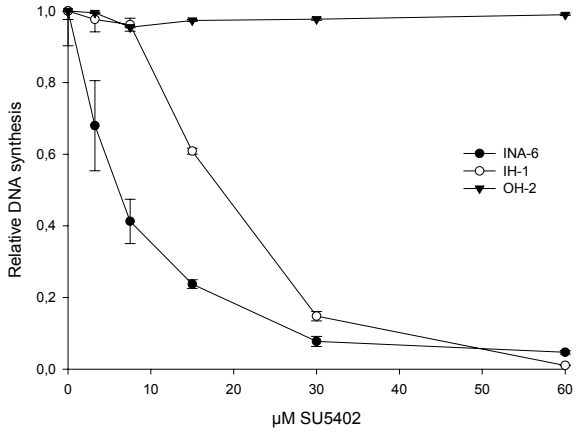
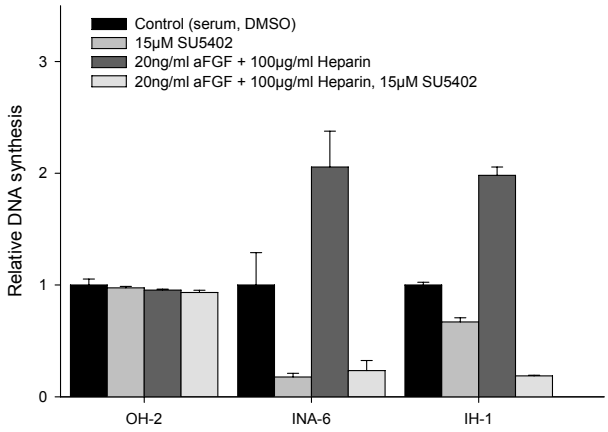


b

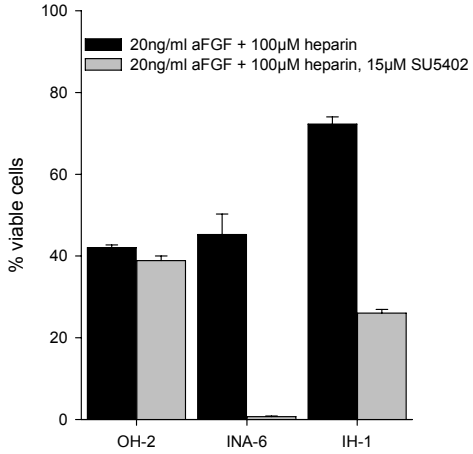
Figure 2


Relative DNA synthesis
1,0
0,8
0,6
0,4
0,2
0,0
0
10
20
30
40
50
60
µM SU5402
INA-6
IH-1
OH-2

a


Control (serum, DMSO)
15µM SU5402
20ng/ml aFGF + 100µg/ml Heparin
20ng/ml aFGF + 100µg/ml Heparin, 15µM SU5402
Relative DNA synthesis
3
2
1
0
OH-2
INA-6
IH-1

b


20ng/ml aFGF + 100µM heparin
20ng/ml aFGF + 100µM heparin, 15µM SU5402
% viable cells
100
80
60
40
20
0
OH-2
INA-6
IH-1

c

Figure 3

Supplementary figure legends

Supplementary Table A:
FISH probes are made from isolated Bacterial Artificial Clones covering the desired regions listed below.

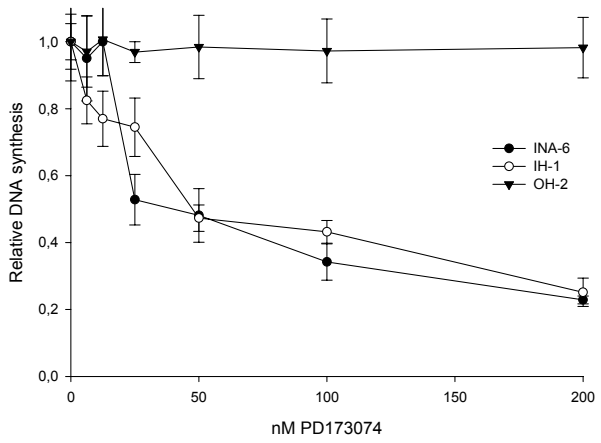
Supplementary Table B:
Primers for sequencing the FGFR3 receptor are listed below.

Supplementary figures:
Figure Ia: HMCL OH-2, INA-6 and IH-1 were treated with the FGFR3 inhibitor PD173074 in increasing concentrations from 6,25 nM to 200nM and plotted against relative proliferation where 1 was set as proliferation in untreated (10% FCS and DMSO only) cells. Proliferation is measured as DNA synthesis by ^{3}H-Tymidin incorporation. The proliferation decreased significantly in both INA-6 and IH-1 in as low concentrations as 25nM and 50nM respectively, whereas OH-2 was unaffected by the inhibitors even at 200nM where both IH-1 an INA-6 had low proliferation. Hence the concentration used in later experiments is not toxic to the cells. Error bars represent standard deviation of triplicates.
Figure Ib:
The HMCLs where also grown with either 20ng/ml aFGF+100µg/ml heparin or only serum and DMSO. Each condition was treated with or without the FGFR3 inhibitor PD173074 at 100nM. In both INA-6 and IH-1 aFGF + heparin induced cell proliferation, and the inhibitor it led to a decrease in DNA-synthesis in unstimulated and aFGF+ heparin stimulated cells. In IH-1 the decrease in proliferation is greater when treated with aFGF compared to untreated control. OH-2 do not express FGFR3, and inhibition of the FGFR3 had no effect in this cell line. Error bars represent standard deviation of triplicated data. (Counts per minute were normalized to 1 to the HMCLs sample with serum, for simplification)
Figure Ic:
OH-2, INA-6 and IH-1 with the same conditions as in figure II were labelled with PI and annexin to measure the viable cells with flow cytometry. The PD173074 drastically decreased the viability in INA-6 and IH-1. OH-2 has as expected no significantly difference in viability with or without the inhibitors. Error bars represent standard deviation of duplicated flow data. All data shown in figure 3 are representative for at least two separate experiments.

Figure II
Relative Quantitative Real Time PCR. TaqMan probes for *MMSET (*Hs00370212_m1, TaqMan) was used or detecting relative *MMSET* expression in OH-2, INA-6 and IH-1 cells directly from stock. The comparative Ct-method was used for quantization with *GAPDH* (HS99999905_m1, TaqMan) as housekeeping gene. INA-6 had approximately double expression of MMSET compared to OH-2 which could be explained with the fact that INA-6 has four copies of the gene whereas OH-2 has two copies. IH-1 which has a *IGH/MMSET* fusion transcript (not shown) has more than four times as much *MMSET* as INA-6, and is regulated by the *IGH* enhancers.

Supplementary data

a



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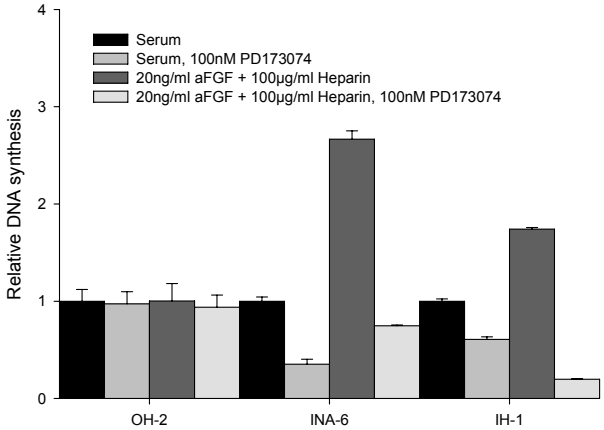

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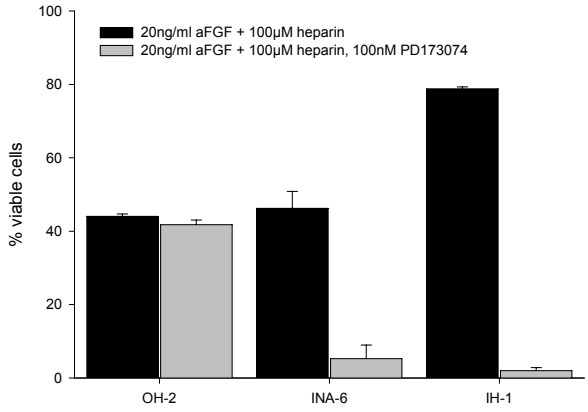

Figure I

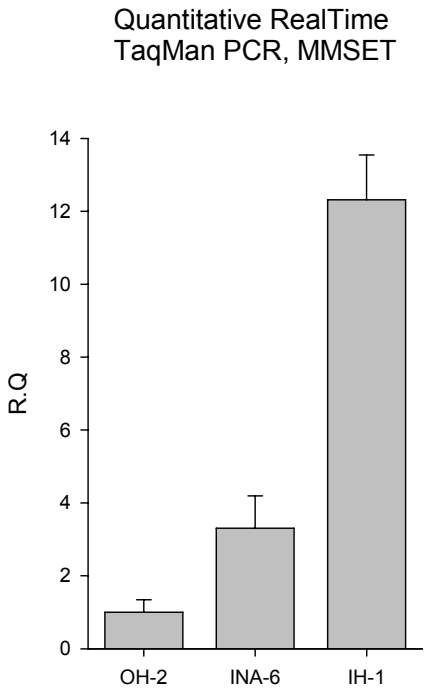
Quantitative RealTime
TaqMan PCR, MMSET
R.Q
14
12
10
8
6
4
2
0
OH-2
INA-6
IH-1

Figure II

Supplementary Table A: Probes for FISH

Gene	Locus	Probe name/BAC-clone
IgH	14q32.33	CH Ig6 U2-2 364 VH
IgKc **IgK v**	2p11.2	RP11-15J7 RP11-433C18
IgLc **IgLv**	22q11.22	CTD-2194H2 RP11-22M5
RB-1	13q14	RP11-305D15 RP11-174I10
TP53	17p13	RP11-89D17
Myeov **Cyclin D1**	11q13	1144 505 365 J C E B
MMSET **FGFR3**	4p16.3 4p16.3	184D6 293022

Supplementary Table B: Primer sequences for *FGFR3*

FGFR3 region	Base pair	Primer	Sequence 5'-3'direction
EC	117	F:o5666 R:o5706	CGG CAG ACG TAC ACG CTG CTT GCA GTG GAA CTC CAC GTC
TM	442	F:o5580 R:o202	GCG CTA ACA CCA CCG ACA AG CTC CCC TGA GGA CAG CCT TGC GAT
TK	120	F:o5724 R:o5703	ATG AAG ATC GCA GAC TTC GGG GTA GAC TCG GTC AAA CAA GGC
SC	313	F:o66 R:o5725	CTC CCA GAG GCC AAC CTT CAA GCA G ATC TGC ACT GAG TCT CAT GCC

F: forward primer, R: reverse primer

Paper III

Is not included due to copyright

Paper IV

Is not included due to copyright

Paper V

Genetic aberrations in Norwegian myeloma patients

-a study based on interphase FISH on newly diagnosed patients from 2006-2008.

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Acknowledgement

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Abstract

Multiple myeloma (MM) is a genetically unstable malignancy of long-lived plasma cells (PC), and complex chromosomal abnormalities such as translocations involving the immunoglobulin heavy chain (*IGH*) are common. The easiest way to detect genetic aberrations in MM patients is by interphase fluorescence *in situ* hybridization (FISH).
In this study we wanted to use interphase FISH to find the prevalence of the most common primary translocations and deletions in chromosome 13 and 17 in Norwegian MM patients. This study includes 139 bone marrow samples from newly diagnosed MM patients. The incidence was: 57 (41%) patients had an *IGH* translocation, 42 (30%) patients had del13 out of which 20 had del13 as only known aberration, 24 (17%) patients had del17 and 54 (38%) patients had none of the aberrations we looked for. Of the *IGH* translocations 23 (16%) patients had t(11;14), 17 (12%) patients had t(4;14), 2 (1.5%) patients had t(6;14) and 1 (1%) patients had t(14;16). 14 (10%) patients had an unknown *IGH* translocation. Clinical information was collected in a subset of 65 patients. There was no significant correlation between clinical findings and genetics in these 65 patients.

Introduction

Multiple myeloma (MM) is a genetically unstable malignancy of long-lived plasma cells (PC) in the bone marrow (BM) and accounts for 10% of all haematological malignancies. Multiple and complex chromosomal abnormalities are common in MM, and translocations often involve the immunoglobulin heavy chain (*IGH*). Primary translocations involving an *IGH* locus are reported to be present in nearly 50% of pre-malignant monoclonal gammopathy of undetermined significance (MGUS), approximately 60% of fully malignant MM tumors, and nearly 90% of human multiple myeloma cell lines. In primary translocations one or all of the strong *IGH* enhancers are juxtaposed to an oncogene/proto-oncogene. The switch translocations in MM cells separate the strong 3`α- and μ-enhancers of the *IGH* onto different derivative chromosomes. The enhancers can thereby turn on the transcription of the juxtaposed genes. The reciprocal partners to *IGH* are: 4p16, *MMSET/FGFR3*; 11q13, Cyclin D1; 12p13, Cyclin D2; 6p21, Cyclin D3; 16q23, *MAF*; 20q12, *MAFB* and 8q24.3, *MAFA*[1-4].

The t(4;14)(p16.3;q32) and t(11;14)(q13;q32) are the most common translocations and are present in 15% of newly diagnosed patients. The t(4;14 has not been described in other malignancies[5]. This translocation was also the first example to show that *IGH* could dysregulate two genes at two derivative chromosomes, namely fibroblast growth factor receptor 3 (*FGFR3)* and multiple myeloma SET domain (*MMSET*)[6]. The translocation results in ectopic expression of functional FGFR3 in 70 % of the t(4;14) patients, but is associated with poor prognosis irrespective of FGFR3 expression[7,8]. However, the chimeric *IGH/MMSET* gene fusion product is expressed in all t(4;14) patients[8,9]. t(4;14) patients with low β_2-microglobulin (B2M) level have shown longer survival compared to those with high levels. Genetic studies also imply that ~80-85% of t(4;14) patients have a deletion (del)13. These two

abnormalities together show a worse prognosis than t(4;14) alone[10]. It has, however, been reported that it is the t(4;14) and not the del13 that is the important adverse prognosis factor at least in relation to autologous stem cell transplantation[11]. The t(11;14)(q13;q32) involves cyclin D1 at 11q13 encoding the cyclin D1 protein is over-expressed as a consequence of this translocation[12]. Early studies reported the t(11;14) patients to have longer survival[13]. Later and larger studies did not confirm this better survival[10,14]. The t(6;14)(p21;q32) dysregulates cyclin D3 and is present in ~5% of myeloma. The t(14;16)(q32;q23) dysregulates *MAF* and is present in ~5% of MM patients[15]. This translocation is also unique for MM patients and has an unfavourable prognosis[5]. The t(14;20)(q32;q11) dysregulates MAFB, and is present in less than 1% of MM patients[16].

Del13 was one of the first genetic markers to be demonstrated to predict a negative outcome[17-19], and is found in about half of MM karyotypes[20]. In the recent Intergroup Francophone du Myelome (IFM) study, del13 identified with interphase fluorescence *in situ* hybridization (FISH) was not an independent prognostic factor, but was associated with poor prognosis related to the concomitant t(4;14) or del17p. Del13 may be considered a pseudomarker or a marker frequently associated with other more specific poor prognostic factors [10]. In MM it has more recently been found a deletion in chromosome 17p in 10% of newly diagnosed patients. *TP53* is located on 17p13 and down-regulation of the gene expression was correlated to the 17p13 deletion, which therefore makes *TP53* likely to be the most important gene in the deleted locus. Del17 is associated with poor outcome[10].

Secondary translocations occur during late stage of MM progression and do not involve B-cell-specific DNA modification mechanisms. Secondary translocations also include the *IGL* gene (10%) and *IGK* (<1%)[21]. The secondary translocations are rarely reciprocal, and can also be complicated insertions of the genes. Almost 20% of MM patients with *IGH* translocation involved a non-recurrent translocation partner[5,22].

According to previous studies using interphase fluorescence in situ hybridization (FISH), translocations and deletions have been associated with unique biological, clinical, and prognostic features[4,5]. We aimed to investigate the prevalence of the following genetic aberrations at the time of diagnosis in Norwegian MM patients; *IGH* translocations, t(4;14), t(11;14), t(6;14), t(14;16), del13 and del17. Furthermore, we wanted to investigate a possible correlation between these genetic aberrations and clinical stage, β_2-microglobulin (B2M), type of immunoglobulin and bone disease in patients at time point of diagnosis. A further motivation for the study is the emerging differentiated treatment according to cytogenetic aberration.

Material and methods

Patient samples

From January 2006 to December 2008, 161 BM aspirates were investigated by interphase FISH. The patient material represents approximately 1/5 of the patients diagnosed with MM in Norway during the same period. After

exclusions as shown in figure 1, we report results from 139 newly diagnosed patients. The female to male ratio in the MM patient group was 1:1.8 and the median age at sampling was 68 (range 29-90 years) years. 80% of the samples were shipped with over night post from hospitals outside Trondheim. Clinical information about the myeloma patients was obtained from the patients records. Clinical information was available for 65 patients. Registered information was: stage according to Durie Salmon (DS) and International Scoring System (ISS), type and concentrations of serum and urine M-protein, PC percentage in bone marrow aspirate, serum β_2-Microglobulin and bone lesions. Bone lesions were scored semi-quantitatively from x-ray findings, and assigned into three groups: 0 = no bone lesions, 1 = ≤ 5 bone lesions, and 2 = more than 5 bone lesions. The median age of these 65 MM patients at sampling was 65 (range 37-90 years) years and female to male ratio 1:1.8. The distribution of serum Ig-class was IgG type in 24 patients (40%), IgA in 17 patients (26%), other Ig isotypes in 1 (2%) patients, only light chain secretion in 14 (22%) patients, and non-secretary myeloma in 1 (2%) patients. For 8 (12%) patients no information was available. 20 (31%) patients were in stage 1 according to the ISS, 17 (26%) patients in stage 2 and 14 (22%) in stage 3. For 13 (20%) patients no information was available.

2-3 ml of bone marrow was aspirated from the crista iliaca into a EDTA-tube (Vacuette, Greiner Bio-One GmbH, Austria). The bone marrow aspirate of in-house samples and shipped samples were received at the Laboratory of Hematology St. Olav's University Hospital. Mononuclear cells (MNC) were enriched using Cell Preparation Tubes with citrate (CPT tubes, BD Vacutainer, Franklin Lakes, USA). Three methods for PC evaluation were used, BM smears, cytospins of MNCs or CD138 separated cells. Before March 2008, FISH was performed with cytoplasmatic-immunoglobulin-(cIg)-FISH on cytospins from MNC or on BM smears. cIg-FISH uses a AMCA- (7-amino-4-methylcoumarin-3-acetic acid) conjugated goat anti-human Igλ and Igκ (Vector laboratories, Brulingame, CA) which bind the light chains in the cytoplasm of the PC allowing specific detection of the PC in the samples. After March 2008 the PC were separated from MNC by immunomagnetic cell selection with CD138 monoclonal antibodies using the RoboSep (StemCell Technologies, Vancouver, BC, Canada) cell separation device. Cytospin with ~10-30 000 of the CD138 positive selected MM and with 50 000 of MNC were used. One slide was always stained with May Grünwald-Giemsa to verify the PC % and the purity of the cells. With CD138 separation, cells from freshly drawn samples with more than 10 mill MNC were usually >98% pure and samples sent to us by over night post >95% pure. Samples shipped over night with fewer than 10 mill MNC might be down to 90% pure, but this is still good enough to use for FISH analysis. All samples were fixed in 100% EtOH for 5 min, room temperature, before storing in -20°C until FISH was performed.

FISH probes

The probes used for the primary *IGH* translocations were made from Bacterial Artificial Chromosome (BAC) clones containing the desirable region (gift from R. Fonseca). BACs for probes covering *CCND3*, *MYC*, *TP53* and *RB1* were purchased from BACPAC resources (BACPAC resources, Children's Hospital Oakland, CA). All clones are listed in supplementary table A. The probes were

labeled with SpectrumGreen or SpectrumOrange dUTPs (Vysis, Abbot Laboratories, Des Plaines, IL) according to standard nick translation protocol (Vysis). Before the start of the study the probes were hybridized to normal cells for control. All probes had an hybridization efficiency higher than 95%. For some samples commercial IGH brake apart probe, t(4;14), t(11;14), LSI RB-1 and LSI TP53 were used (Vysis). The strategy for identifying the *IGH* translocations is by IGHc/IGHv break apart probe before looking for the specific *IGH* translocation using dual fusion strategy.

FISH procedure

The cells were fixed in methanol/acetic acid (1:3 vol/vol) (-20°C, 40 min) and air dried. Probe mix (100 ng labelled DNA probe in 3 µl H_2O + 7µl LSI hybridization Buffer (Vysis)). 2,5 µl probe mix was used for cytospin samples and 10 µl for BM smears. The probe mix was sealed with cover glass and rubber sement. Hybridization was done in a Dako hybridizer (Dako Cytomation, Glostrup, Denmark). Program: Denaturation (10 min, 75°C) Hybridization (10-18 h, 37°C). Posthybridization wash was performed in 0.4 x SSC/0.3% NP-40 (pH 7.0-7.5, 72°C, 2 min), then 2xSSC/0.1% NP-40 (pH 7.0-7.5, RT, 6-60 s). For cIgFISH, the slides were directly put in 1x phosphate buffer saline (PBS) for 5 min then incubated with goat serum (15% in 1xPBS) (Invitrogen, Carlsbad, CA) for 5 min. The serum was poured off and incubated with AMCA conjugated goat-IgG anti-human Igλ/Igκ (1:200 in 3% BSA in 1x PBS) (Vector laboratories) for 10 min. The signal was amplified with a second AMCA labeled anti-goat IgG antibody (Vector laboratories). Slides were washed for 2x 10 min in 1x PBS, then air-dried and anti-fade added (Vectashield hard-set mounting medium without DAPI, (Vector Laboratories). All steps in the immunostaining were done at RT. For CD138 separated cells slides were air dried after the posthybridization wash and anti-fade with DAPI applied (Vectashield hard-set mounting medium with DAPI, (Vector Laboratories)). FISH was scored using NIKON ECLIPSE 90i epifluorescence microscope with PlanApo VC 100x/1.4oel (Nikon Instruments Europe B.V, Badhoevedorp, The Netherlands), and software from Applied Imaging (CytoVision, version 3.7 Build 58, 2005, San Jose, CA).

Scoring of FISH

All slides were scored by the same person. For every probe set 100 interphase nuclei were scored. Cut-off levels were used according to the European Myeloma Network[23]: deletions ≥ 20% of evaluated cells; split signals and translocations ≥10% of evaluated cells. Slides with <95% hybridization efficacy in non-PC cells were not used. For cIgFISH all evaluated cells must have positive immunostaining and the typical morphology of the PC seen by May Grünwald-Giemsa staining. The percentage PC in the samples were determined by May Grünwald-Giemsa staining. For samples where less than 25 nuclei were possible to score, the specimens were considered non-informative. If only 25-50 cells were possible to score more than 50% of the cells must have a given aberration, to be considered positive for the genetic aberration. If more than 90% of the 25-50 cells had normal signal pattern the samples were considered normal, if less than 50% of the cells had a given abnormality or less than 90% were normal the specimen were considered non-informative. For cells with informative

interphase nuclei between 50 and 100 cells normal cut-off levels were used. For non-informative samples a new sample, if available, was used to redo the experiment, and if e.g. cytospin of MNC gave non-informative result a BM smear could be used. After the introduction of CD138 selection of cells, the procedure was considerably improved, and all samples gave informative result.

Statistics

SPSS Statistical Software version 14.0 was used for statistic calculations (SPSS Inc., Chicago, IL). Pearsons' χ^2 or Fisher's exact tests were used for between-group comparison of discrete variables, and Mann Whitney U test was used for continuous variables. The level of statistical significance was set at $p < 0.05$. P-values were 2-tailed.

Ethics

The work presented is a part of the Norwegian national biobank for multiple myeloma (4.2005.1438), and this particular study was approved by the Regional Ethic Committee (4.2007.933). All patients sign an informed consent form according to the Declaration of Helsinki.

Results

Genetic aberrations and clinical disease parameters

Of the 147 samples from patients with reported MM, 139 were analyzed after excluding MGUS, non-MM samples and not newly diagnosed patients. The most common aberration found was as expected *IGH* translocations and del13. 57 (41%) patients had an *IGH* translocation, 42 (30%) patients had del13 out of which 20 had del13 as the only detected aberration, 24 (17%) patients had del17 and 54 (38%) patients had none of the aberrations we looked for. Of the *IGH* translocations 23 (16%) patients had t(11;14), 17 (12%) patients had t(4;14), 2 (1%) patients had t(6;14) and 1 (1%) patients had t(14;16). 16 (11%) patients had an *IGH* translocation with an unknown partner. Half of the patient samples with unknown translocation testing was only done for t(4;14) and t(11;14), because of lack of material. The prevalence of the abnormalities was the same in all age categories of the patients. There was significant correlation between del13 and del17. Del13 was seen in 12 (50%) of the 24 patients with del17, and in 30 (26%) of the 115 patients without del17, odds ratio 2.83, 95% confidence interval (1.15, 6.98), $p = 0.02$. There was no significant correlation between del13 or del17 and the *IGH* translocations. Only 41 % of the t(4;14) patients had del13 simultaneously. The various combinations of aberrations are summarized in a Venn diagram in figure 2.

For 48 patients, B2M levels were known. There was no significant correlation between B2M levels and the *IGH* translocations del13 or del17. However, when patients with any of these genetic aberrations were analyzed together as a group, the B2M levels were significantly higher than in patients with no detected aberration, with median B2M 4.3 mg/L (range 1.6-50.9) and 2.8 mg/L (range 1.0-11.2), respectively, $p = 0.02$.

There was no significant correlation between genetic aberrations and bone disease, ISS or DS stage, type of immunoglobulin or light chain at diagnosis.

Discussion

MM cells are characterized by genetic instability and chromosomal abnormalities[24]. In this FISH study we found that *IGH* translocations were the most frequent chromosomal abnormality. We found it at lower frequency than in many other studies, which reports ~60% in MM[25,26], however, two studies from the UK find *IGH* translocations in 44-45% of their patients[27,28]. Table 1 gives an overview of genetic aberrations reported from different countries. The studies indicate that the prevalence of *IGH* translocations in MM is lower in the Northwestern part of Europe although a random variation of the studies cannot be ruled out. The specific translocations t(11;14) and t(4;14) are present in approximately the same frequency as in other studies[29]. Is might be reasonable to speculate if the "correct" number primary translocations, and lower overall *IGH* translocations are due to the fact that all patients are newly diagnosed in the present study? If the patients included are diagnosed at an earlier stage of the disease than in other countries, the secondary *IGH* translocation might not yet have arisen or they are present below the cut-off level. Others have shown that aberrations increase during the progression of the disease[30]. For the t(14;16) and t(6;14) the results are lower than others have published[29]. The explanation for this is most likely that for eight of the 16 t(?;14) patients there was not enough material left or the quality of the material left was poor. Hence, the test for t(6;14) and t(14;16) was not done on these patient samples. The del13 was present in the same frequency as in a Danish study[30], but at lower level than in other studies[19]. For del17 the frequency was almost as others have found[31].

There was a significant correlation between del13 and del17. This is an interesting and novel finding. There was, however, no significant correlation between neither del13 nor del17 and the primary *IGH* translocations, which was quite surprising, given that others have published that 80-85% of t(4;14) patients have a del13[20,32]. Also there was no correlation between del13 and *IGH* translocations as a group, whereas others have found that they are correlated[20,33].

The level of B2M has in many studies been demonstrated to correlate with prognosis[34], and it is also a factor in the ISS staging. There was no significant difference in B2M between the specific genetic groups. However, we found a higher level of B2M in the patients with genetic aberrations analyzed as a group.

There was no significant correlation between genetic aberrations and bone disease, stage according to ISS or DS, type of immunoglobulin or light chain at diagnosis. A correlation between genetic aberrations and stage and prognosis has been shown by others[31]. They did, however, not use samples from newly diagnosed patients which could explain the difference between our findings. There was also no significant correlation with the genetic aberrations

and type of immunoglobulin or type of light chain, even though a correlation has been shown by others[13].

The detection of genetic abnormalities can be important to predict prognosis in patients and to predict responses to treatment. Recently bortezomib was shown to be beneficial for t(4;14) patients[35] and it also seemed to overcome some of the poor prognosis in del13 patients[36,37]. In the University of Arkansas for Medical Science total therapy study II patients with non favourable cytogenetics/gene expression profile (GEP) group benefited the most from thalidomide, and they had a significantly better overall and event free survival compared to the same non-favourable cytogenetics/GEP control group. Conversely, it was noted that patients without cytogenetic aberrations/favourable GEP did not have the same benefit from thalidomide treatment[38].

The establishment of the method and estimate of the prevalence of genetic aberrations in MM patients in Norway may have significant implications for future treatment strategy

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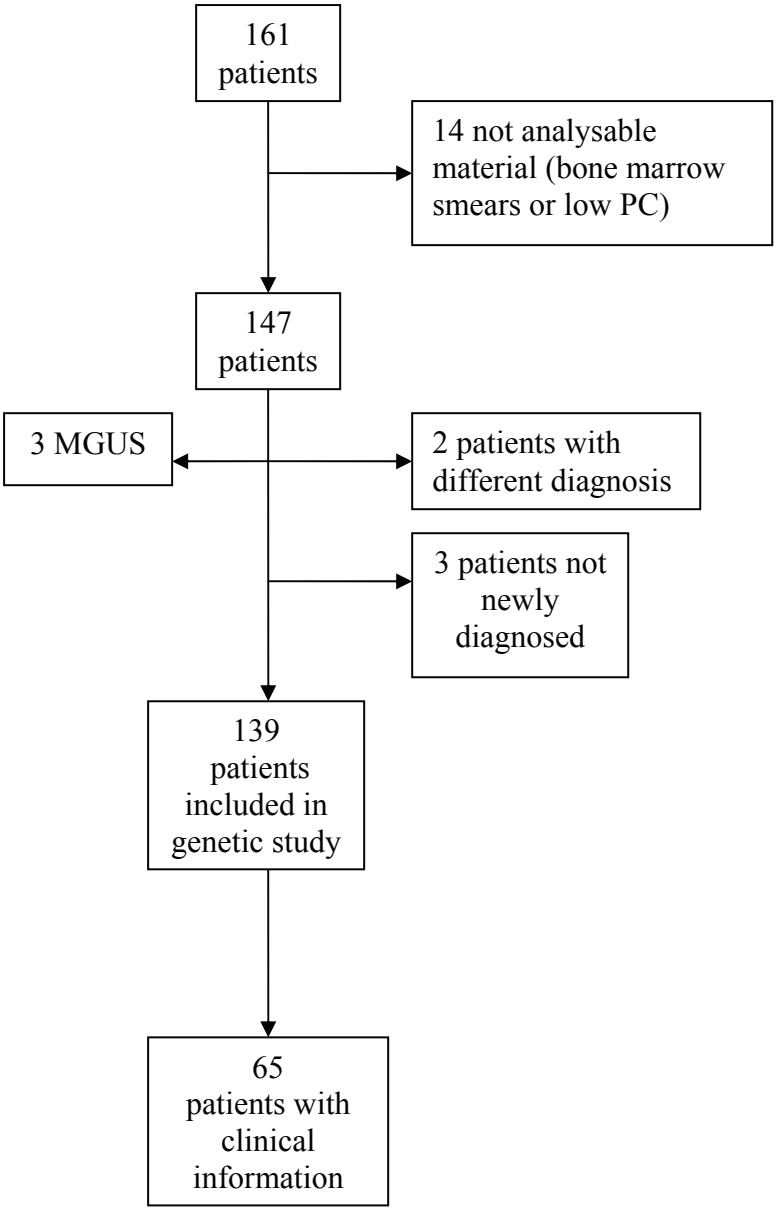

Figure 1. Flow chart of the included patients samples of the study

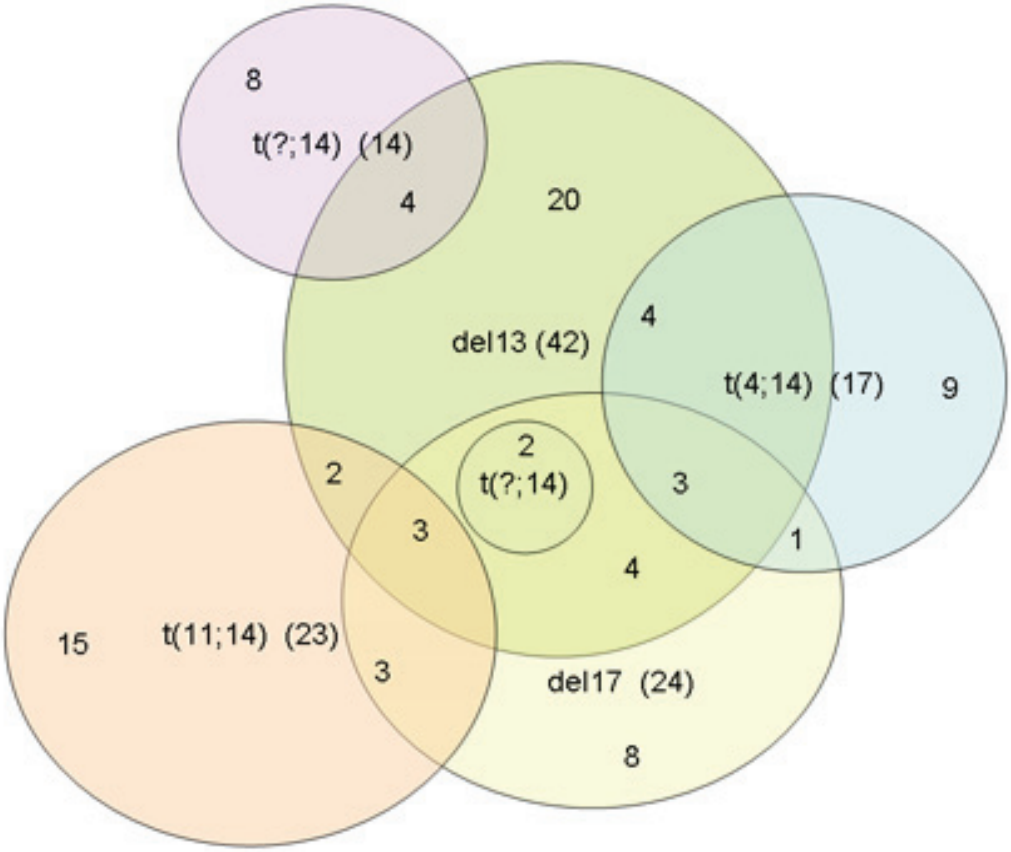

Figure 2. Venn diagram of the most common aberrations found in the study of MM patients in Norway.
The most common aberration is the *IGH* translocation (57 of 139 patients). 54 patients with *IGH* translocation are shown, 2 patients with t(6;14) and 1 patient with t(14;16) are not drawn in the diagram. 42 patients had a del13, in which 20 the del13 was the only known aberration. For del17 2/3 of the patients had the del17 together with another known aberration. 23 patients had a t(11;14), in which 2/3 had no other known aberration. For t(4;14) only half of the patients had no other known aberration. The only aberrations that are significantly correlated are del13 and del17.

Table 1. The prevalence of different genetic aberrations in some countries

Country	Number of patients	IGH split	t(4;14)	t(11;14)	t(6;14)	t(14;16)	t(?;14)	del13	del17	reference
Denmark	40	19 (47.5%)	2 (5%)	8 (20%)	NR	NR	9 (24%)	11 (28%)	6 (15%)	Christensen et al [30]
UK	195	86 (44%)	21 (11%)	30 (15%)	NR	7 (4%)	28 (15%)	81 (42%)	8 (4%)	Ross et al[27]
UK	729	325 (45%)	85 (12%)	107 (15%)	NR	NR	NR	348 (48%)	63 (9%)	Chiecchio et al[28]
Japan	23	10 (43%)	1 (4%)	5 (21%)	NR	4 (17%)	NR	12 (52%)	NR	Takimoto et al[33]
USA	84-196	NR	17%	17%	NR	NR	NR	52%	11%	Gertz et al[14]
France	532-936	NR	14%	21%	NR	NR		48%	11%	Avet-Loiseau et al[10]
Norway	139	57 (41%)	17 (12%)	23 (16%)	2 (1%)	1 (1%)	14 (10%)	42 (30%)	24 (17%)	

Abbreviations: NR not reported

Supplementary Table A: Probes for FISH

Gene	Locus	Probe name/BAC-clone
IgH	14q32.33	CH Ig6 U2-2 364 VH
IgKc **IgK v**	2p11.2	RP11-15J7 RP11-433C18
IgLc **IgLv**	22q11.22	CTD-2194H2 RP11-22M5
RB-1	13q14	RP11-305D15 RP11-174I10
TP53	17p13	RP11-89D17
Myeov **Cyclin D1**	11q13	1144 505 365 J C E B
MMSET **FGFR3**	4p16.3 4p16.3	184D6 293022
MAF	16q23	10205 10206
Cyclin D3	6p21	RP11-720D9 RP11-298J23

Dissertations at the Faculty of Medicine, NTNU

1977

1. Knut Joachim Berg: EFFECT OF ACETYLSALICYLIC ACID ON RENAL FUNCTION
2. Karl Erik Viken and Arne Ødegaard: STUDIES ON HUMAN MONOCYTES CULTURED *IN VITRO*

1978

3. Karel Bjørn Cyvin: CONGENITAL DISLOCATION OF THE HIP JOINT.
4. Alf O. Brubakk: METHODS FOR STUDYING FLOW DYNAMICS IN THE LEFT VENTRICLE AND THE AORTA IN MAN.

1979

5. Geirmund Unsgaard: CYTOSTATIC AND IMMUNOREGULATORY ABILITIES OF HUMAN BLOOD MONOCYTES CULTURED IN VITRO

1980

6. Størker Jørstad: URAEMIC TOXINS
7. Arne Olav Jenssen: SOME RHEOLOGICAL, CHEMICAL AND STRUCTURAL PROPERTIES OF MUCOID SPUTUM FROM PATIENTS WITH CHRONIC OBSTRUCTIVE BRONCHITIS

1981

8. Jens Hammerstrøm: CYTOSTATIC AND CYTOLYTIC ACTIVITY OF HUMAN MONOCYTES AND EFFUSION MACROPHAGES AGAINST TUMOR CELLS *IN VITRO*

1983

9. Tore Syversen: EFFECTS OF METHYLMERCURY ON RAT BRAIN PROTEIN.
10. Torbjørn Iversen: SQUAMOUS CELL CARCINOMA OF THE VULVA.

1984

11. Tor-Erik Widerøe: ASPECTS OF CONTINUOUS AMBULATORY PERITONEAL DIALYSIS.
12. Anton Hole: ALTERATIONS OF MONOCYTE AND LYMPHOCYTE FUNCTIONS IN REALTION TO SURGERY UNDER EPIDURAL OR GENERAL ANAESTHESIA.
13. Terje Terjesen: FRACTURE HEALING AND STRESS-PROTECTION AFTER METAL PLATE FIXATION AND EXTERNAL FIXATION.
14. Carsten Saunte: CLUSTER HEADACHE SYNDROME.
15. Inggard Lereim: TRAFFIC ACCIDENTS AND THEIR CONSEQUENCES.
16. Bjørn Magne Eggen: STUDIES IN CYTOTOXICITY IN HUMAN ADHERENT MONONUCLEAR BLOOD CELLS.
17. Trond Haug: FACTORS REGULATING BEHAVIORAL EFFECTS OG DRUGS.

1985

18. Sven Erik Gisvold: RESUSCITATION AFTER COMPLETE GLOBAL BRAIN ISCHEMIA.
19. Terje Espevik: THE CYTOSKELETON OF HUMAN MONOCYTES.
20. Lars Bevanger: STUDIES OF THE Ibc (c) PROTEIN ANTIGENS OF GROUP B STREPTOCOCCI.
21. Ole-Jan Iversen: RETROVIRUS-LIKE PARTICLES IN THE PATHOGENESIS OF PSORIASIS.
22. Lasse Eriksen: EVALUATION AND TREATMENT OF ALCOHOL DEPENDENT BEHAVIOUR.
23. Per I. Lundmo: ANDROGEN METABOLISM IN THE PROSTATE.

1986

24. Dagfinn Berntzen: ANALYSIS AND MANAGEMENT OF EXPERIMENTAL AND CLINICAL PAIN.
25. Odd Arnold Kildahl-Andersen: PRODUCTION AND CHARACTERIZATION OF MONOCYTE-DERIVED CYTOTOXIN AND ITS ROLE IN MONOCYTE-MEDIATED CYTOTOXICITY.
26. Ola Dale: VOLATILE ANAESTHETICS.

1987

27. Per Martin Kleveland: STUDIES ON GASTRIN.
28. Audun N. Øksendal: THE CALCIUM PARADOX AND THE HEART.
29. Vilhjalmur R. Finsen: HIP FRACTURES

1988

30. Rigmor Austgulen: TUMOR NECROSIS FACTOR: A MONOCYTE-DERIVED REGULATOR OF CELLULAR GROWTH.
31. Tom-Harald Edna: HEAD INJURIES ADMITTED TO HOSPITAL.
32. Joseph D. Borsi: NEW ASPECTS OF THE CLINICAL PHARMACOKINETICS OF METHOTREXATE.
33. Olav F. M. Sellevold: GLUCOCORTICOIDS IN MYOCARDIAL PROTECTION.
34. Terje Skjærpe: NONINVASIVE QUANTITATION OF GLOBAL PARAMETERS ON LEFT VENTRICULAR FUNCTION: THE SYSTOLIC PULMONARY ARTERY PRESSURE AND CARDIAC OUTPUT.
35. Eyvind Rødahl: STUDIES OF IMMUNE COMPLEXES AND RETROVIRUS-LIKE ANTIGENS IN PATIENTS WITH ANKYLOSING SPONDYLITIS.
36. Ketil Thorstensen: STUDIES ON THE MECHANISMS OF CELLULAR UPTAKE OF IRON FROM TRANSFERRIN.
37. Anna Midelfart: STUDIES OF THE MECHANISMS OF ION AND FLUID TRANSPORT IN THE BOVINE CORNEA.
38. Eirik Helseth: GROWTH AND PLASMINOGEN ACTIVATOR ACTIVITY OF HUMAN GLIOMAS AND BRAIN METASTASES - WITH SPECIAL REFERENCE TO TRANSFORMING GROWTH FACTOR BETA AND THE EPIDERMAL GROWTH FACTOR RECEPTOR.
39. Petter C. Borchgrevink: MAGNESIUM AND THE ISCHEMIC HEART.
40. Kjell-Arne Rein: THE EFFECT OF EXTRACORPOREAL CIRCULATION ON SUBCUTANEOUS TRANSCAPILLARY FLUID BALANCE.
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42. Carl Bredo Dahl: ANIMAL MODELS IN PSYCHIATRY.

1989

43. Torbjørn A. Fredriksen: CERVICOGENIC HEADACHE.
44. Rolf A. Walstad: CEFTAZIDIME.
45. Rolf Salvesen: THE PUPIL IN CLUSTER HEADACHE.
46. Nils Petter Jørgensen: DRUG EXPOSURE IN EARLY PREGNANCY.
47. Johan C. Ræder: PREMEDICATION AND GENERAL ANAESTHESIA IN OUTPATIENT GYNECOLOGICAL SURGERY.
48. M. R. Shalaby: IMMUNOREGULATORY PROPERTIES OF TNF-α AND THE RELATED CYTOKINES.
49. Anders Waage: THE COMPLEX PATTERN OF CYTOKINES IN SEPTIC SHOCK.
50. Bjarne Christian Eriksen: ELECTROSTIMULATION OF THE PELVIC FLOOR IN FEMALE URINARY INCONTINENCE.
51. Tore B. Halvorsen: PROGNOSTIC FACTORS IN COLORECTAL CANCER.

1990

52. Asbjørn Nordby: CELLULAR TOXICITY OF ROENTGEN CONTRAST MEDIA.
53. Kåre E. Tvedt: X-RAY MICROANALYSIS OF BIOLOGICAL MATERIAL.
54. Tore C. Stiles: COGNITIVE VULNERABILITY FACTORS IN THE DEVELOPMENT AND MAINTENANCE OF DEPRESSION.
55. Eva Hofsli: TUMOR NECROSIS FACTOR AND MULTIDRUG RESISTANCE.
56. Helge S. Haarstad: TROPHIC EFFECTS OF CHOLECYSTOKININ AND SECRETIN ON THE RAT PANCREAS.
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58. Tarjei Rygnestad: DELIBERATE SELF-POISONING IN TRONDHEIM.
59. Arne Z. Henriksen: STUDIES ON CONSERVED ANTIGENIC DOMAINS ON MAJOR OUTER MEMBRANE PROTEINS FROM ENTEROBACTERIA.
60. Steinar Westin: UNEMPLOYMENT AND HEALTH: Medical and social consequences of a factory closure in a ten-year controlled follow-up study.
61. Ylva Sahlin: INJURY REGISTRATION, a tool for accident preventive work.
62. Helge Bjørnstad Pettersen: BIOSYNTHESIS OF COMPLEMENT BY HUMAN ALVEOLAR MACROPHAGES WITH SPECIAL REFERENCE TO SARCOIDOSIS.
63. Berit Schei: TRAPPED IN PAINFUL LOVE.
64. Lars J. Vatten: PROSPECTIVE STUDIES OF THE RISK OF BREAST CANCER IN A COHORT OF NORWEGIAN WOMAN.

1991

65. Kåre Bergh: APPLICATIONS OF ANTI-C5a SPECIFIC MONOCLONAL ANTIBODIES FOR THE ASSESSMENT OF COMPLEMENT ACTIVATION.
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68. Trond Sand: THE EFFECTS OF CLICK POLARITY ON BRAINSTEM AUDITORY EVOKED POTENTIALS AMPLITUDE, DISPERSION, AND LATENCY VARIABLES.
69. Kjetil B. Åsbakk: STUDIES OF A PROTEIN FROM PSORIATIC SCALE, PSO P27, WITH RESPECT TO ITS POTENTIAL ROLE IN IMMUNE REACTIONS IN PSORIASIS.
70. Arnulf Hestnes: STUDIES ON DOWN´S SYNDROME.
71. Randi Nygaard: LONG-TERM SURVIVAL IN CHILDHOOD LEUKEMIA.
72. Bjørn Hagen: THIO-TEPA.
73. Svein Anda: EVALUATION OF THE HIP JOINT BY COMPUTED TOMOGRAMPHY AND ULTRASONOGRAPHY.

1992

74. Martin Svartberg: AN INVESTIGATION OF PROCESS AND OUTCOME OF SHORT-TERM PSYCHODYNAMIC PSYCHOTHERAPY.
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79. Turid Nilsen: PYROPHOSPHATE IN HEPATOCYTE IRON METABOLISM.
80. Olav Haraldseth: NMR SPECTROSCOPY OF CEREBRAL ISCHEMIA AND REPERFUSION IN RAT.
81. Eiliv Brenna: REGULATION OF FUNCTION AND GROWTH OF THE OXYNTIC MUCOSA.

1993

82. Gunnar Bovim: CERVICOGENIC HEADACHE.
83. Jarl Arne Kahn: ASSISTED PROCREATION.
84. Bjørn Naume: IMMUNOREGULATORY EFFECTS OF CYTOKINES ON NK CELLS.
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86. Jie Ming Shen: BLOOD FLOW VELOCITY AND RESPIRATORY STUDIES.
87. Piotr Kruszewski: SUNCT SYNDROME WITH SPECIAL REFERENCE TO THE AUTONOMIC NERVOUS SYSTEM.
88. Mette Haase Moen: ENDOMETRIOSIS.
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91. Kjell Å. Salvesen: ROUTINE ULTRASONOGRAPHY IN UTERO AND DEVELOPMENT IN CHILDHOOD.

1994

92. Nina-Beate Liabakk: DEVELOPMENT OF IMMUNOASSAYS FOR TNF AND ITS SOLUBLE RECEPTORS.
93. Sverre Helge Torp: *erb*B ONCOGENES IN HUMAN GLIOMAS AND MENINGIOMAS.
94. Olav M. Linaker: MENTAL RETARDATION AND PSYCHIATRY. Past and present.
95. Per Oscar Feet: INCREASED ANTIDEPRESSANT AND ANTIPANIC EFFECT IN COMBINED TREATMENT WITH DIXYRAZINE AND TRICYCLIC ANTIDEPRESSANTS.
96. Stein Olav Samstad: CROSS SECTIONAL FLOW VELOCITY PROFILES FROM TWO-DIMENSIONAL DOPPLER ULTRASOUND: Studies on early mitral blood flow.
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103. Unni Syversen: CHROMOGRANIN A. Phsysiological and Clinical Role.

1995

104. Odd Gunnar Brakstad: THERMOSTABLE NUCLEASE AND THE *nuc* GENE IN THE DIAGNOSIS OF *Staphylococcus aureus* INFECTIONS.
105. Terje Engan: NUCLEAR MAGNETIC RESONANCE (NMR) SPECTROSCOPY OF PLASMA IN MALIGNANT DISEASE.
106. Kirsten Rasmussen: VIOLENCE IN THE MENTALLY DISORDERED.
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109. Arild Faxvaag: STUDIES OF IMMUNE CELL FUNCTION *in mice infected with* MURINE RETROVIRUS.

1996

110. Svend Aakhus: NONINVASIVE COMPUTERIZED ASSESSMENT OF LEFT VENTRICULAR FUNCTION AND SYSTEMIC ARTERIAL PROPERTIES. Methodology and some clinical applications.
111. Klaus-Dieter Bolz: INTRAVASCULAR ULTRASONOGRAPHY.
112. Petter Aadahl: CARDIOVASCULAR EFFECTS OF THORACIC AORTIC CROSS-CLAMPING.
113. Sigurd Steinshamn: CYTOKINE MEDIATORS DURING GRANULOCYTOPENIC INFECTIONS.
114. Hans Stifoss-Hanssen: SEEKING MEANING OR HAPPINESS?
115. Anne Kvikstad: LIFE CHANGE EVENTS AND MARITAL STATUS IN RELATION TO RISK AND PROGNOSIS OF CANCER.
116. Torbjørn Grøntvedt: TREATMENT OF ACUTE AND CHRONIC ANTERIOR CRUCIATE LIGAMENT INJURIES. A clinical and biomechanical study.
117. Sigrid Hørven Wigers: CLINICAL STUDIES OF FIBROMYALGIA WITH FOCUS ON ETIOLOGY, TREATMENT AND OUTCOME.
118. Jan Schjøtt: MYOCARDIAL PROTECTION: Functional and Metabolic Characteristics of Two Endogenous Protective Principles.
119. Marit Martinussen: STUDIES OF INTESTINAL BLOOD FLOW AND ITS RELATION TO TRANSITIONAL CIRCULATORY ADAPATION IN NEWBORN INFANTS.
120. Tomm B. Müller: MAGNETIC RESONANCE IMAGING IN FOCAL CEREBRAL ISCHEMIA.
121. Rune Haaverstad: OEDEMA FORMATION OF THE LOWER EXTREMITIES.
122. Magne Børset: THE ROLE OF CYTOKINES IN MULTIPLE MYELOMA, WITH SPECIAL REFERENCE TO HEPATOCYTE GROWTH FACTOR.
123. Geir Smedslund: A THEORETICAL AND EMPIRICAL INVESTIGATION OF SMOKING, STRESS AND DISEASE: RESULTS FROM A POPULATION SURVEY.

1997

124. Torstein Vik: GROWTH, MORBIDITY, AND PSYCHOMOTOR DEVELOPMENT IN INFANTS WHO WERE GROWTH RETARDED *IN UTERO*.
125. Siri Forsmo: ASPECTS AND CONSEQUENCES OF OPPORTUNISTIC SCREENING FOR CERVICAL CANCER. Results based on data from three Norwegian counties.
126. Jon S. Skranes: CEREBRAL MRI AND NEURODEVELOPMENTAL OUTCOME IN VERY LOW BIRTH WEIGHT (VLBW) CHILDREN. A follow-up study of a geographically based year cohort of VLBW children at ages one and six years.
127. Knut Bjørnstad: COMPUTERIZED ECHOCARDIOGRAPHY FOR EVALUTION OF CORONARY ARTERY DISEASE.
128. Grethe Elisabeth Borchgrevink: DIAGNOSIS AND TREATMENT OF WHIPLASH/NECK SPRAIN INJURIES CAUSED BY CAR ACCIDENTS.
129. Tor Elsås: NEUROPEPTIDES AND NITRIC OXIDE SYNTHASE IN OCULAR AUTONOMIC AND SENSORY NERVES.
130. Rolf W. Gråwe: EPIDEMIOLOGICAL AND NEUROPSYCHOLOGICAL PERSPECTIVES ON SCHIZOPHRENIA.
131. Tonje Strømholm: CEREBRAL HAEMODYNAMICS DURING THORACIC AORTIC CROSSCLAMPING. An experimental study in pigs.

1998

132. Martinus Bråten: STUDIES ON SOME PROBLEMS REALTED TO INTRAMEDULLARY NAILING OF FEMORAL FRACTURES.
133. Ståle Nordgård: PROLIFERATIVE ACTIVITY AND DNA CONTENT AS PROGNOSTIC INDICATORS IN ADENOID CYSTIC CARCINOMA OF THE HEAD AND NECK.

134. Egil Lien: SOLUBLE RECEPTORS FOR **TNF** AND **LPS**: RELEASE PATTERN AND POSSIBLE SIGNIFICANCE IN DISEASE.
135. Marit Bjørgaas: HYPOGLYCAEMIA IN CHILDREN WITH DIABETES MELLITUS
136. Frank Skorpen: GENETIC AND FUNCTIONAL ANALYSES OF DNA REPAIR IN HUMAN CELLS.
137. Juan A. Pareja: SUNCT SYNDROME. ON THE CLINICAL PICTURE. ITS DISTINCTION FROM OTHER, SIMILAR HEADACHES.
138. Anders Angelsen: NEUROENDOCRINE CELLS IN HUMAN PROSTATIC CARCINOMAS AND THE PROSTATIC COMPLEX OF RAT, GUINEA PIG, CAT AND DOG.
139. Fabio Antonaci: CHRONIC PAROXYSMAL HEMICRANIA AND HEMICRANIA CONTINUA: TWO DIFFERENT ENTITIES?
140. Sven M. Carlsen: ENDOCRINE AND METABOLIC EFFECTS OF METFORMIN WITH SPECIAL EMPHASIS ON CARDIOVASCULAR RISK FACTORES.

1999

141. Terje A. Murberg: DEPRESSIVE SYMPTOMS AND COPING AMONG PATIENTS WITH CONGESTIVE HEART FAILURE.
142. Harm-Gerd Karl Blaas: THE EMBRYONIC EXAMINATION. Ultrasound studies on the development of the human embryo.
143. Noèmi Becser Andersen:THE CEPHALIC SENSORY NERVES IN UNILATERAL HEADACHES. Anatomical background and neurophysiological evaluation.
144. Eli-Janne Fiskerstrand: LASER TREATMENT OF PORT WINE STAINS. A study of the efficacy and limitations of the pulsed dye laser. Clinical and morfological analyses aimed at improving the therapeutic outcome.
145. Bård Kulseng: A STUDY OF ALGINATE CAPSULE PROPERTIES AND CYTOKINES IN RELATION TO INSULIN DEPENDENT DIABETES MELLITUS.
146. Terje Haug: STRUCTURE AND REGULATION OF THE HUMAN UNG GENE ENCODING URACIL-DNA GLYCOSYLASE.
147. Heidi Brurok: MANGANESE AND THE HEART. A Magic Metal with Diagnostic and Therapeutic Possibilites.
148. Agnes Kathrine Lie: DIAGNOSIS AND PREVALENCE OF HUMAN PAPILLOMAVIRUS INFECTION IN CERVICAL INTRAEPITELIAL NEOPLASIA. Relationship to Cell Cycle Regulatory Proteins and HLA DQBI Genes.
149. Ronald Mårvik: PHARMACOLOGICAL, PHYSIOLOGICAL AND PATHOPHYSIOLOGICAL STUDIES ON ISOLATED STOMACS.
150. Ketil Jarl Holen: THE ROLE OF ULTRASONOGRAPHY IN THE DIAGNOSIS AND TREATMENT OF HIP DYSPLASIA IN NEWBORNS.
151. Irene Hetlevik: THE ROLE OF CLINICAL GUIDELINES IN CARDIOVASCULAR RISK INTERVENTION IN GENERAL PRACTICE.
152. Katarina Tunòn: ULTRASOUND AND PREDICTION OF GESTATIONAL AGE.
153. Johannes Soma: INTERACTION BETWEEN THE LEFT VENTRICLE AND THE SYSTEMIC ARTERIES.
154. Arild Aamodt: DEVELOPMENT AND PRE-CLINICAL EVALUATION OF A CUSTOM-MADE FEMORAL STEM.
155. Agnar Tegnander: DIAGNOSIS AND FOLLOW-UP OF CHILDREN WITH SUSPECTED OR KNOWN HIP DYSPLASIA.
156. Bent Indredavik: STROKE UNIT TREATMENT: SHORT AND LONG-TERM EFFECTS
157. Jolanta Vanagaite Vingen: PHOTOPHOBIA AND PHONOPHOBIA IN PRIMARY HEADACHES

2000

158. Ola Dalsegg Sæther: PATHOPHYSIOLOGY DURING PROXIMAL AORTIC CROSS-CLAMPING CLINICAL AND EXPERIMENTAL STUDIES
159. xxxxxxxxx (blind number)
160. Christina Vogt Isaksen: PRENATAL ULTRASOUND AND POSTMORTEM FINDINGS – A TEN YEAR CORRELATIVE STUDY OF FETUSES AND INFANTS WITH DEVELOPMENTAL ANOMALIES.
161. Holger Seidel: HIGH-DOSE METHOTREXATE THERAPY IN CHILDREN WITH ACUTE LYMPHOCYTIC LEUKEMIA: DOSE, CONCENTRATION, AND EFFECT CONSIDERATIONS.
162. Stein Hallan: IMPLEMENTATION OF MODERN MEDICAL DECISION ANALYSIS INTO CLINICAL DIAGNOSIS AND TREATMENT.

163. Malcolm Sue-Chu: INVASIVE AND NON-INVASIVE STUDIES IN CROSS-COUNTRY SKIERS WITH ASTHMA-LIKE SYMPTOMS.
164. Ole-Lars Brekke: EFFECTS OF ANTIOXIDANTS AND FATTY ACIDS ON TUMOR NECROSIS FACTOR-INDUCED CYTOTOXICITY.
165. Jan Lundbom: AORTOCORONARY BYPASS SURGERY: CLINICAL ASPECTS, COST CONSIDERATIONS AND WORKING ABILITY.
166. John-Anker Zwart: LUMBAR NERVE ROOT COMPRESSION, BIOCHEMICAL AND NEUROPHYSIOLOGICAL ASPECTS.
167. Geir Falck: HYPEROSMOLALITY AND THE HEART.
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169. Dalius Bansevicius: SHOULDER-NECK REGION IN CERTAIN HEADACHES AND CHRONIC PAIN SYNDROMES.
170. Bettina Kinge: REFRACTIVE ERRORS AND BIOMETRIC CHANGES AMONG UNIVERSITY STUDENTS IN NORWAY.
171. Gunnar Qvigstad: CONSEQUENCES OF HYPERGASTRINEMIA IN MAN
172. Hanne Ellekjær: EPIDEMIOLOGICAL STUDIES OF STROKE IN A NORWEGIAN POPULATION. INCIDENCE, RISK FACTORS AND PROGNOSIS
173. Hilde Grimstad: VIOLENCE AGAINST WOMEN AND PREGNANCY OUTCOME.
174. Astrid Hjelde: SURFACE TENSION AND COMPLEMENT ACTIVATION: Factors influencing bubble formation and bubble effects after decompression.
175. Kjell A. Kvistad: MR IN BREAST CANCER – A CLINICAL STUDY.
176. Ivar Rossvoll: ELECTIVE ORTHOPAEDIC SURGERY IN A DEFINED POPULATION. Studies on demand, waiting time for treatment and incapacity for work.
177. Carina Seidel: PROGNOSTIC VALUE AND BIOLOGICAL EFFECTS OF HEPATOCYTE GROWTH FACTOR AND SYNDECAN-1 IN MULTIPLE MYELOMA.

2001

178. Alexander Wahba: THE INFLUENCE OF CARDIOPULMONARY BYPASS ON PLATELET FUNCTION AND BLOOD COAGULATION – DETERMINANTS AND CLINICAL CONSEQUENSES
179. Marcus Schmitt-Egenolf: THE RELEVANCE OF THE MAJOR hISTOCOMPATIBILITY COMPLEX FOR THE GENETICS OF PSORIASIS
180. Odrun Arna Gederaas: BIOLOGICAL MECHANISMS INVOLVED IN 5-AMINOLEVULINIC ACID BASED PHOTODYNAMIC THERAPY
181. Pål Richard Romundstad: CANCER INCIDENCE AMONG NORWEGIAN ALUMINIUM WORKERS
182. Henrik Hjorth-Hansen: NOVEL CYTOKINES IN GROWTH CONTROL AND BONE DISEASE OF MULTIPLE MYELOMA
183. Gunnar Morken: SEASONAL VARIATION OF HUMAN MOOD AND BEHAVIOUR
184. Bjørn Olav Haugen: MEASUREMENT OF CARDIAC OUTPUT AND STUDIES OF VELOCITY PROFILES IN AORTIC AND MITRAL FLOW USING TWO- AND THREE-DIMENSIONAL COLOUR FLOW IMAGING
185. Geir Bråthen: THE CLASSIFICATION AND CLINICAL DIAGNOSIS OF ALCOHOL-RELATED SEIZURES
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187. Trude Helen Flo: RESEPTORS INVOLVED IN CELL ACTIVATION BY DEFINED URONIC ACID POLYMERS AND BACTERIAL COMPONENTS
188. Bodil Kavli: HUMAN URACIL-DNA GLYCOSYLASES FROM THE UNG GENE: STRUCTRUAL BASIS FOR SUBSTRATE SPECIFICITY AND REPAIR
189. Liv Thommesen: MOLECULAR MECHANISMS INVOLVED IN TNF- AND GASTRIN-MEDIATED GENE REGULATION
190. Turid Lingaas Holmen: SMOKING AND HEALTH IN ADOLESCENCE; THE NORD-TRØNDELAG HEALTH STUDY, 1995-97
191. Øyvind Hjertner: MULTIPLE MYELOMA: INTERACTIONS BETWEEN MALIGNANT PLASMA CELLS AND THE BONE MICROENVIRONMENT
192. Asbjørn Støylen: STRAIN RATE IMAGING OF THE LEFT VENTRICLE BY ULTRASOUND. FEASIBILITY, CLINICAL VALIDATION AND PHYSIOLOGICAL ASPECTS

193. Kristian Midthjell: DIABETES IN ADULTS IN NORD-TRØNDELAG. PUBLIC HEALTH ASPECTS OF DIABETES MELLITUS IN A LARGE, NON-SELECTED NORWEGIAN POPULATION.
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195. Ulrik Wisløff: CARDIAC EFFECTS OF AEROBIC ENDURANCE TRAINING: HYPERTROPHY, CONTRACTILITY AND CALCUIM HANDLING IN NORMAL AND FAILING HEART
196. Øyvind Halaas: MECHANISMS OF IMMUNOMODULATION AND CELL-MEDIATED CYTOTOXICITY INDUCED BY BACTERIAL PRODUCTS
197. Tore Amundsen: PERFUSION MR IMAGING IN THE DIAGNOSIS OF PULMONARY EMBOLISM
198. Nanna Kurtze: THE SIGNIFICANCE OF ANXIETY AND DEPRESSION IN FATIQUE AND PATTERNS OF PAIN AMONG INDIVIDUALS DIAGNOSED WITH FIBROMYALGIA: RELATIONS WITH QUALITY OF LIFE, FUNCTIONAL DISABILITY, LIFESTYLE, EMPLOYMENT STATUS, CO-MORBIDITY AND GENDER
199. Tom Ivar Lund Nilsen: PROSPECTIVE STUDIES OF CANCER RISK IN NORD-TRØNDELAG: THE HUNT STUDY. Associations with anthropometric, socioeconomic, and lifestyle risk factors
200. Asta Kristine Håberg: A NEW APPROACH TO THE STUDY OF MIDDLE CEREBRAL ARTERY OCCLUSION IN THE RAT USING MAGNETIC RESONANCE TECHNIQUES

2002

201. Knut Jørgen Arntzen: PREGNANCY AND CYTOKINES
202. Henrik Døllner: INFLAMMATORY MEDIATORS IN PERINATAL INFECTIONS
203. Asta Bye: LOW FAT, LOW LACTOSE DIET USED AS PROPHYLACTIC TREATMENT OF ACUTE INTESTINAL REACTIONS DURING PELVIC RADIOTHERAPY. A PROSPECTIVE RANDOMISED STUDY.
204. Sylvester Moyo: STUDIES ON STREPTOCOCCUS AGALACTIAE (GROUP B STREPTOCOCCUS) SURFACE-ANCHORED MARKERS WITH EMPHASIS ON STRAINS AND HUMAN SERA FROM ZIMBABWE.
205. Knut Hagen: HEAD-HUNT: THE EPIDEMIOLOGY OF HEADACHE IN NORD-TRØNDELAG
206. Li Lixin: ON THE REGULATION AND ROLE OF UNCOUPLING PROTEIN-2 IN INSULIN PRODUCING ß-CELLS
207. Anne Hildur Henriksen: SYMPTOMS OF ALLERGY AND ASTHMA VERSUS MARKERS OF LOWER AIRWAY INFLAMMATION AMONG ADOLESCENTS
208. Egil Andreas Fors: NON-MALIGNANT PAIN IN RELATION TO PSYCHOLOGICAL AND ENVIRONTENTAL FACTORS. EXPERIENTAL AND CLINICAL STUDES OF PAIN WITH FOCUS ON FIBROMYALGIA
209. Pål Klepstad: MORPHINE FOR CANCER PAIN
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