

Genomic Studies of Multiple Myeloma Reveal an Association Between X Chromosome Alterations and Genomic Profile Complexity

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The genomic profile of multiple myeloma (MM) has prognostic value by dividing patients into a good prognosis hyperdiploid group and a bad prognosis nonhyperdiploid group with a higher incidence of IGH translocations. This classification, however, is inadequate and many other parameters like mutations, epigenetic modifications, and genomic heterogeneity may influence the prognosis. We performed a genomic study by array-based comparative genomic hybridization on a cohort of 162 patients to evaluate the frequency of genomic gains and losses. We identified a high frequency of X chromosome alterations leading to partial Xq duplication, often associated with inactive X (Xi) deletion in female patients. This partial X duplication could be a cytogenetic marker of aneuploidy as it is correlated with a high number of chromosomal breakages. Patient with high level of chromosomal breakage had reduced survival regardless the region implicated. A higher transcriptional level was shown for genes with potential implication in cancer and located in this altered region. Among these genes, *IKBKG* and *IRAK1* are members of the NFκB pathway which plays an important role in MM and is a target for specific treatments. © 2016 Wiley Periodicals, Inc.

INTRODUCTION

Multiple myeloma (MM) is a hematological disorder characterized by an uncontrolled accumulation of clonal plasma cells in the bone marrow. The prognosis is partially determined by genomic alterations revealed by array-based comparative genomic hybridization (aCGH) and/or fluorescence in situ hybridization (FISH). In contrast to myeloid and lymphoid leukemias which display mainly whole chromosome alterations due to mitotic errors, MM often harbor partial genomic alterations and translocations (Munshi and Avet-Loiseau, 2011). This difference may be explained by the high transcriptomic and splicing activity of the type of cell implicated in the disease. Trisomies of odd chromosomes and hyperdiploidy (HD) are considered to be associated with a good prognosis while *del(1p)*, *dup(1q)*, *del(13q)*, *del(17p)*, and *t(4;14)*, associated with nonhyperdiploidy (NHD), are considered as poor prognosis markers. However, many MM show a heterogeneous genomic profile and alterations with an unknown prognostic impact. In these cases, the

genetic markers provide limited prognostic information reliability. A clinical impact of the chromosomal breakage level was proposed in some recent studies, which were based on quantifying genomic alterations, particularly those with chromosome breaks. Indeed, structural chromosomal alterations seem to have a poor prognostic impact and to reflect deficiencies in genomic repair systems (Chung et al., 2013; Przybytkowski et al., 2014).

High throughput sequencing data of MM samples show many mutations in genes known to be

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implicated in cancerogenesis but also in genes with a role in RNA processing, protein folding or coding for cancer testis antigens (Chapman et al., 2011). However, the low frequency of these mutations suggests that they may be passengers. Indeed, most mutations involve weakly expressed genes (Rashid et al., 2014). Other types of genomic alteration like loss of heterozygosity (LOH) have been linked to adverse outcome in some cancers but their prognostic significance in MM remains unknown (Walker and Morgan, 2006; Gronseth et al., 2015).

The X chromosome is often altered in cancers and carries more point mutations than other chromosomes. Genetic studies of medulloblastoma have shown a twofold increase in X gonosome mutations compared to autosomes (Jäger et al., 2013). Cancer-testis antigen genes on X are often mutated in MM and their expression seems to be related to disease progression (de Carvalho et al., 2012). The impact of X chromosome alterations in cancer is unknown but a correlation was shown between the development of particular forms of breast cancer (sporadic basal-like cancer) and loss of inactive X (Xi) markers, sometimes with duplication of the remaining chromosome leading to a copy number neutral loss of heterozygosity (CNN-LOH). These alterations may result in a localized over-expression of X chromosome genes (Richardson et al., 2006; Sun et al., 2015).

In this study, we used the aCGH technology on a cohort of 162 MM patients to detect copy number variations (CNV). We focused on genomic X profiles and evaluated the impact of X alterations on transcriptomic profiles using RNA-seq and qRT-PCR.

MATERIALS AND METHODS

Ethics

Approval was obtained from the Institutional Review Board (Ethical Committee of the Faculty of Medicine of the University of Liège) in compliance with the Declaration of Helsinki. This work consisted of a prospective study and did not lead to any changes in the treatment of the enrolled patients.

Patients and Sample Preparation

Bone marrow samples and clinical data were obtained after informed consent from patients followed at the CHU of Liège and other regional medical centers (Table 1). Only patients with

medullar plasmocytosis over 10% based on bone marrow smear examination were included in the study. EasySep Human CD138 Positive Selection Kit (Stemcells Technologies, Grenoble, France) was used to enrich plasma cells populations. Plasma cell purity evaluation was performed by cytometry with CD138 antibody or morphologically by examining Giemsa stained slides. Samples with plasma cells purity over 80% were selected for genomic DNA (gDNA) and RNA extraction using AllPrep DNA/RNA extraction kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's instructions.

aCGH, CNV, and SNP Analysis

The plasma cells were analyzed with the SurePrint G3 Human CGH Microarray Kit 8x60K (Agilent Technologies, Santa Clara, CA) according to the manufacturer's instructions, and the results were interpreted using the Cytogenomics software (Agilent Technologies). The arrays were scanned with a G2565CA microarray scanner (Agilent Technologies) and the images were extracted and analyzed with the CytoGenomics software v2.0 (Agilent Technologies). An ADM-2 algorithm (cutoff 6.0), followed by a filter to select regions with three or more adjacent probes and a minimum average log₂ ratio of ± 0.25 , was used to detect copy number changes. The quality of each experiment was assessed by the measurement of the derivative log ratio spread with the CytoGenomics software v2.0. Genomic positions were based on the UCSC human reference sequence (hg19) (NCBI build 37 reference sequence assembly).

Genotyping of 25 patients from our cohort was also performed with Genome-Wide Human SNP array 6.0 and analyzed with the Genotyping Console 4.0 (Affymetrix, Santa Clara, CA). Unpaired analyses were performed using 270 Hapmap files as references. The regional CG correction parameter was selected and the UCSC human reference sequence (hg19) (NCBI build 37 reference sequence assembly) was chosen as annotation files. The thresholds for minimum number of markers per segment and the minimal genomic size of a segment were 5 and 5 Mb, respectively, in the SNP analysis.

Gene Transcription Profiling

RNA quality control and quantification were done with spectrophotometer ND-1000 and

TABLE I. Patients' Characteristics

	F	64	39%
Sex	M	98	61%
Durie & Salmon	1	29	18%
	2	19	12%
	3	81	50%
	Unknown	33	20%
ISS	1	38	23%
	2	24	15%
	3	34	21%
	Unknown	66	41%
Heavy chain	IgA	28	17%
	IgD	1	0,6%
	IgG	70	43%
	Light chains	32	20%
	Nonsecretory	1	0,6%
	Unknown	30	18%
Light chain	Kappa	72	44%
	Lambda	42	26%
	Nonsecretory	1	0,6%
	Unknown	47	29%
Status	Diagnosis	78	48%
	Follow-up	60	37%
	Unknown	24	15%
Age (years); range		75	(36–92)
Survival (months); range		29	(1–157)
Plasmocytosis (%); range		37.2	(10–94)

F, female; M, male.

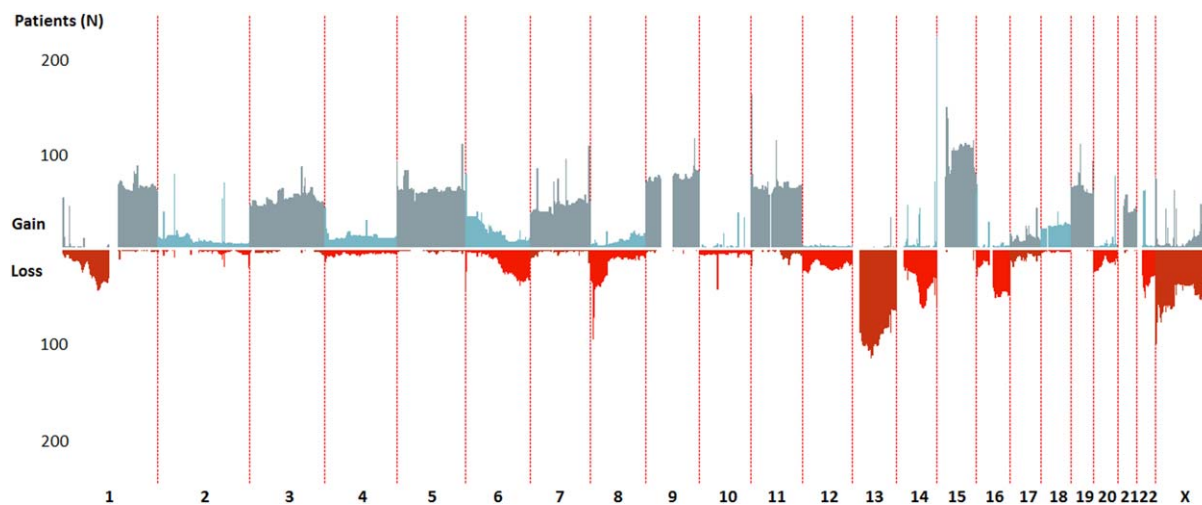


Figure 1. Prevalence of genomic aberration. Bars above the x axis indicates gains and below the x axis losses. Gains of whole chromosome most frequently involved 3, 5, 7, 9, 11, 19, and 21. Losses most frequently involved 13, 1p, 6q, 8p, 14, and 16q. [Color figure can be viewed at wileyonlinelibrary.com]

Agilent 2100 bioanalyser using RNA 6000 Nano Kit (Agilent). Libraries were prepared using TruSeq Stranded Total RNA Sample Prep Kit

(Illumina, Essex, UK) from 400 ng of total RNA and following the manufacturer's instructions. Briefly, rRNA was depleted before purification,

TABLE 2. Sequences of Primers Used for Real-Time Polymerase Chain Reaction

Gene name	Forward primer sequence	Reverse primer sequence
IRAK1	caa cgt cct tct gga tga gag gct	atg ctg ctc tgg ctg ggg ct
ENOX2	gcc aga gac acc ttg gta gtg ccc a	gcc tca gga cat tcg ccc caa acc
BCAP31	tgg ctt ctg agg ata ctg cgt cta	gcc cgc aag agc gac tcc ta
SASH3	ccc tga gaa gat ggc gct ggc ctt	ggc tgc aga gct cac tgc ctg t
RBMX	ctg agc tgc tag gaa gcc cct a	tga caa tgg gtt caa gct cca acg
IKBKG	tcc cac agc tat gac acc gga agc	gcg gac tgt gaa cgc tgg tag g
BCORL1	gga ttc gca tgt gtg gca tc	cgt gag ctc cac ctt gga aa
MTMR1	tgt gga ctg gat gat gcc ttc gac	tct agg gtc ctg ctg aca gag c
NSDHL	cca cat ccc cta ctg ggt ggc cta	tga agg tgg gct gca gct gga tga c
RBMX2	cac tgg ccc taa gaa gca cag cag c	gga ttt ggg gag ctt ctg ccc ctc
XIST	tcc tta gta gtc atg tct cct tag	aac aac aag cct att ctt ctg ag

TABLE 3. Most Frequent Minimal Common Altered Regions

Genomic alteration	Frequency (%)
del(X)	47 (only women)
dup(X)(q27q28)	21
del(X)(p22.3q21)	25 (only women)
dup(1q)	40
del(1)(p31p32)	13
del(1)(p11p13)	16
dup(3)	30
dup(5)	38
dup(7)	27
del(8p)	13
dup(9)	43
dup(11)	34
del(12)(p12p13)	17
del(13)	46
del(14)(q24q31)	14
dup(15)	46
del(16q)	18
dup(18)	13
dup(19)	39
dup(21)	23
del(22)	14

fragmentation and cDNA double strand synthesis. After 3' adenylation and ligation of adapters, DNA fragments were enriched. The size of the library was evaluated on an Agilent Technologies 2100 Bioanalyzer using a DNA 1000 chip (Agilent). A size of approximately 300 bp was expected. Libraries were run on HiSeq2000 sequencer (Illumina). All DNA libraries were sequenced using an Illumina HiSeq2000, producing 2 × 100 bp paired-end reads with multiplexing. Fastq data were analyzed using the TopHat Alignment software from Basespace (Illumina).

Reverse-Transcription and Real-Time PCR

Reverse transcription was performed on 50 ng RNA using RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo scientific, Erembodegem,

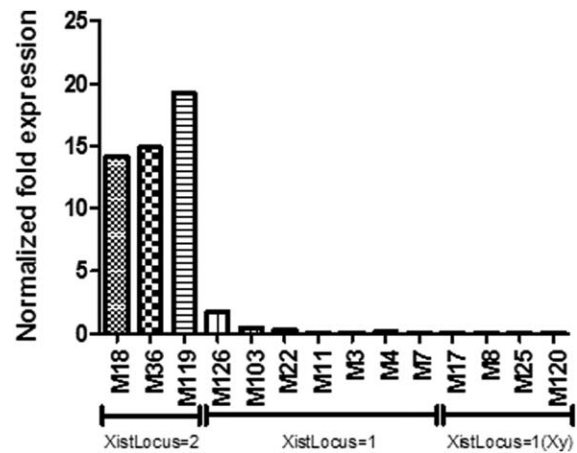


Figure 2. Xist expression levels in relation to X chromosome profiles. The expression levels of Xist were compared between female patients with partial X deletion including the Xist locus (M126, M103, M22, M11, M3, M4, and M7) and female patients without deletion including this locus (M18, M36, and M119). Patients with a copy number of 1 for the Xist locus show a drastic decrease in its expression. Four males were tested as negative controls. The β2-microglobuline gene was used for normalization.

Belgium), following the manufacturer's instructions. Real time PCR was performed using Kapa Sybr Fast qPCR LightCycler 480 readymix kit (Sopachem, Eke, Belgium). Real-time PCR reactions were carried out on LightCycler 480 Real-Time PCR system using specific primers (Table 2). The relative expression quantification was calculated using β-2-microglobuline as a reference gene.

Conventional and Molecular Cytogenetics

Screening for immunoglobulin heavy chain gene (IGH) rearrangements (t(4;14)(p16;q32)) with the dual-fusion translocation probes (Abbott Molecular, Wavre, Belgium) and 17p deletions (LSI p53, 17p13.1) (Abbott Molecular) were performed on purified plasma cells. Cell suspensions from 120h-cultured bone marrow cells were used retrospectively and prospectively. A minimum of

20 Giemsa-banded metaphases were karyotyped by conventional cytogenetic analyses using standard procedures. FISH using whole painting chromosome probes for X (Kreatech, Diegem, Belgium) was performed on fixed slides with 120 h-cultured bone marrow cells and selected plasma cells.

RESULTS

Frequent X Chromosome Alterations

In our patient population, we confirmed the earlier observations that trisomies chromosomes 3, 5, 7, 9, 11, 19, and 21 were the most frequent whole chromosome gains (Smadja, 2001; Kumar et al., 2012). Most monosomies involved chromosomes X (female

patients), 13, and 22. Minimal recurrent altered regions, in decreasing order of frequency, were: dup(1q), dup(X)(q27q28), del(16q), del(1)(p31p32), del(1)(p11p13), del(8p), del(14)(q24q31), del(12)(p12p13), and del(X)(p22.3q21) (Fig. 1). Complete and partial X deletions were observed in 47% ($N = 31$) and 17% ($N = 10$) of the female patients respectively. Partial X duplication was seen in 21% ($N = 34$) of the patients regardless of sex (Tables 3). Transcriptomic evaluation of Xist in female patients with partial X deletion showed a drastic decrease in this noncoding gene leading to the conclusion that the deleted chromosome is the inactive one (Fig. 2). X deletions were not present in male tumors, probably because X nullisomy does not allow cell survival.

Genotyping of 25 patients revealed CNN-LOH of several genomic regions but the incidence of such alterations was quite low in our cohort and did not involve any specific regions. CNN-LOH of Xq25q28 was observed in three patients (two females and one male) and was associated with the loss of Xpter to Xq25 in the two female patients (Table 4). Therefore, the tumors from these two female patients lacked the Xi chromosome and partially duplicated the active chromosome. This kind of alteration could modify the gene expression profile of this region.

X Chromosome Transcription Profile

Transcriptomic screening by RNA-seq was performed on two female cases with partial X deletion and compared to three cases without any abnormality of the X chromosome. Although we could

TABLE 4. Regions Involved in CNN-LOH Detected in 25 Patients

LOH	Number of patient
UPD(1)(pterp22)	1
UPD(3)(p21p14)	2
UPD(4)(q27q28)	1
UPD(4)(q32q35)	1
UPD(5p)	1
UPD(5q)	1
UPD(9)(p21qter)	1
UPD(10)	1
UPD(13)	1
UPD(14)(q23q24)	2
UPD(14)	1
UPD(17)(q22q23)	1
UPD(20)(q11qter)	1
UPD(X)(q25qter)	3

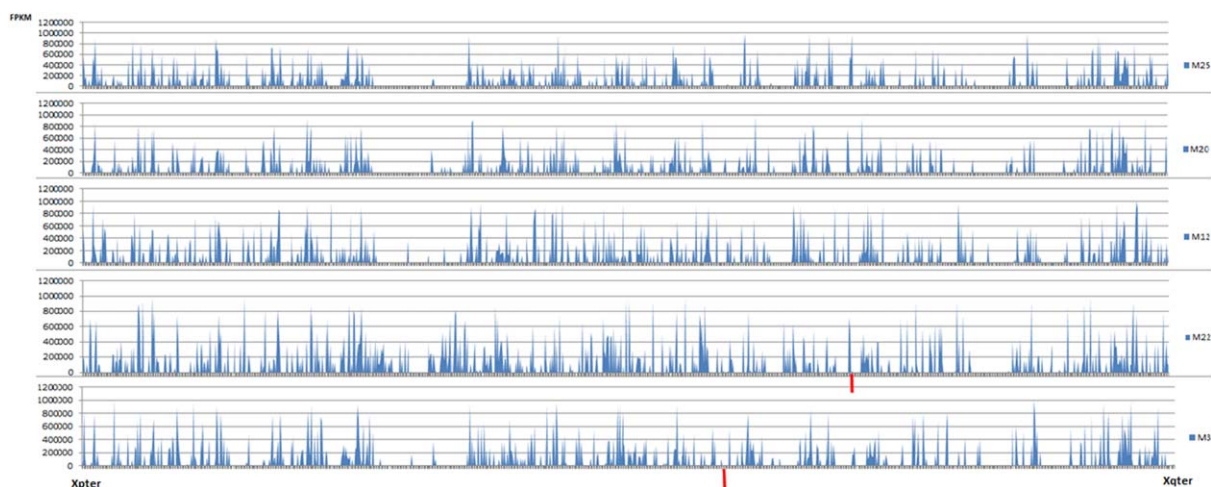


Figure 3. Expression profiles along the X chromosome. RNA-seq performed on patients with abnormal X profile (M12, M20, and M25) did not show any different profiles between these and those with partial X alterations (region located to the right of the red bar) (M3 and M22). [Color figure can be viewed at wileyonlinelibrary.com]

not detect any significant overall difference between the groups (Fig. 3), several genes were selected to confirm their high transcription level on a larger number of patients. We analyzed 10 of

them (*IKBK*G, *IRAK1*, *RBMX*, *NSDHL*, *RBMX2*, *MTMR1*, *ENOX2*, *BCORL1*, *SASH3*, and *BCAP31*) by RT-qPCR and detected that all of them showed a higher transcriptional level in patients with partial X alterations (Fig. 4). To confirm this observation on a larger cohort we performed a comparative analysis based on GEO-website available GEP (GSE 26760) and aCGH (GSE 26863) data. In this cohort, *IKBK*G and *IRAK1*, both involved in the NFKB pathway, were also over-expressed in tumors with partial X alterations. A similar observation was made for *NSDHL*, *BCAP31*, and *RBMX2*, three genes for which a clear link with cancer and MM has not yet been established (Fig. 5).

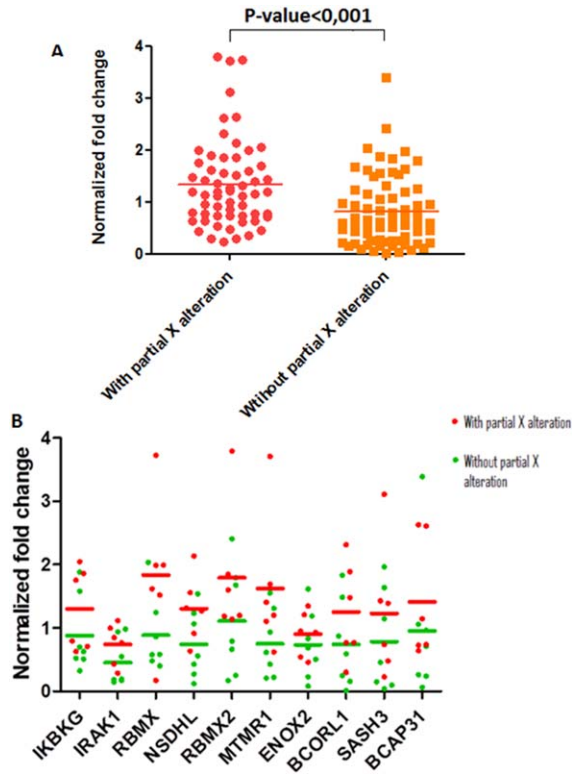


Figure 4. Expression levels of 10 Xq-linked genes analyzed together (A) and independently (B). The average expression level in the Xq region is higher for patients with partial X alterations ($N = 6$) compared to those without partial X alterations ($N = 7$) (A). No significant difference was detected when we tested each gene independently (B). The β 2-microglobuline gene was used for normalization. [Color figure can be viewed at wileyonlinelibrary.com]

Genomic X Profile and Aneuploidy

Currently, the most reliable genomic marker to evaluate the prognosis in MM remains the detection of CNVs. We tried to establish whether the number of alterations could be linked to a specific X chromosome profile. Poor prognosis alterations including *del(1p)*, *dup(1q)*, *del(13q)*, *del(16q)*, *del(17p)* and *t(4;14)* were not correlated with the X profiles. However, the total number of alterations and alterations with an unknown prognostic impact were significantly more frequent in patients with partial alterations of the X chromosome compared to patients with X monosomy or normal X profile (Fig. 6A). Therefore, patients with partial X alterations have a higher incidence of structural chromosomal alterations, while gains and losses of whole chromosomes were not significantly different between the three groups (Fig.

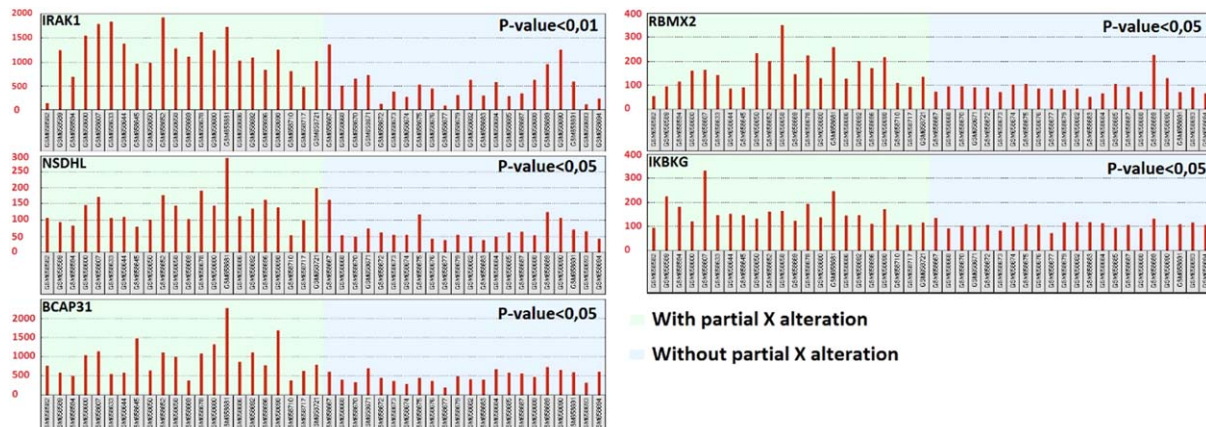


Figure 5. Expression profiles of the X-linked genes *IKBK*G, *IRAK1*, *RBMX*, *NSDHL*, and *RBMX2* obtained from the GEO dataset GSE 26760. Groups were built based on X genomic profile (GSE 26863). Patients with partial X alterations (green color) overexpressed these X-linked genes compared to those without partial X alterations (blue color). [Color figure can be viewed at wileyonlinelibrary.com]

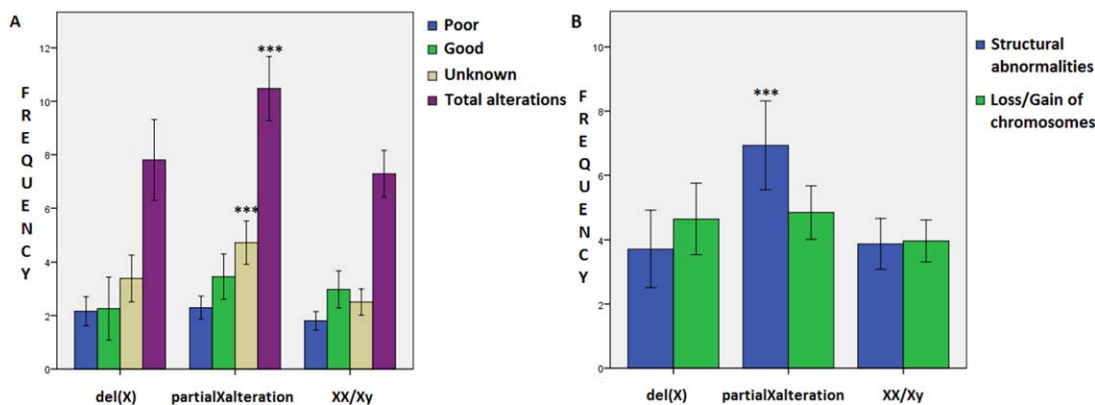


Figure 6. (A) Association between X chromosome profiles and prognostic groups, and (B) between whole chromosome gain/loss and structural abnormalities. There were three different groups based on the types of alterations: poor (negative impact on prognosis which include del(1p32), del(1p22), dup(1q21), del(8p), del(12p12p13), del(13), del(16q), del(17p), and dup(20q)), good (trisomy of uneven-numbered chromosomes) and unknown (unknown impact on prognosis). There was a significant association between partial X alterations

and the incidence of unknown prognosis alterations. Generally these patients seem to have more genomic alterations (A). Patients with partial X alterations have more structural abnormalities than those with complete X deletion or normal X profiles. Furthermore, the incidence of whole chromosome gains/losses seems to be approximately the same between the different groups (B). [Color figure can be viewed at wileyonlinelibrary.com]

6B). We did not find any direct association between the X chromosome profiles and overall patient survival; however, our data indicated a tendency to a worse outcome for patients with a partial X alteration, compared to normal X profiles or complete X losses (Fig. 7A). However, we observed that a high level of chromosomal breakage (more than 3 breaks) was associated with a reduced overall survival (Fig. 7B).

DISCUSSION

MM is characterized by numerous chromosomal rearrangements, possibly indicating the presence of chromosomal instability. Indeed, it has been demonstrated that DNA double strand break repair mechanisms are impaired in tumors leading to multiple rearrangements (Cagnetta et al., 2015; Herrero et al., 2015). aCGH and sequencing data have confirmed a genetic evolution of MM during different phases of the disease, with the diagnostic and relapse aCGH profiles being different (Bolli et al., 2014; Keats et al., 2012).

We performed a genomic study of MM, after positive selection of tumor cells, in order to detect chromosomal rearrangements and to study their clinical and biological implications. We observed frequent rearrangements on the X chromosome. Tumors in female MM patients frequently showed monosomy X or a copy number of 1 from pter to the Xq region (47 and 12% of the female patients, respectively). The exact position on Xq differed but was located between Xq21 and Xq25. Genotyping of two female patients with such a

copy number profile showed a CNN-LOH of the Xq region. In male patients, a partial duplication, involving the same area, was observed in 28% of patients. These alterations are too frequent and too similar to represent passenger events and should thus contribute to the development or progression of the MM. In our study, we found that this recurrent X chromosome alteration was a cytogenetic marker associated with numerous genomic breakpoints, maybe reflecting chromosomal instability. Interestingly, a complete duplication of the active X has been described in basal-like breast cancers as well as in other tumors (Sirchia et al., 2005; Kang et al., 2015) and one study found a globally increased expression of X-linked genes in breast cancers with a duplication of the active X chromosome (Richardson et al., 2006) while other reports showed a partial reactivation of gene transcription from the inactive X in some tumors (Lose et al., 2008; Chaligné et al., 2015). The partial duplication of the X long arm seems to be very rare in other cancers and may be a characteristic of MM.

Many chromosomal alterations have a limited impact on gene transcription. For example, tumors with trisomy of odd chromosomes do not show specific transcriptomic profiles, in contrast, translocations involving IGH, are linked to a specific profile (Broyl et al., 2010). Indeed, processes by which chromosome number aberrations occur are different than those leading to structural aberrations. The first type of alteration implicate deregulation of protein involved in centrosome biogenesis leading to centrosome dysfunction or

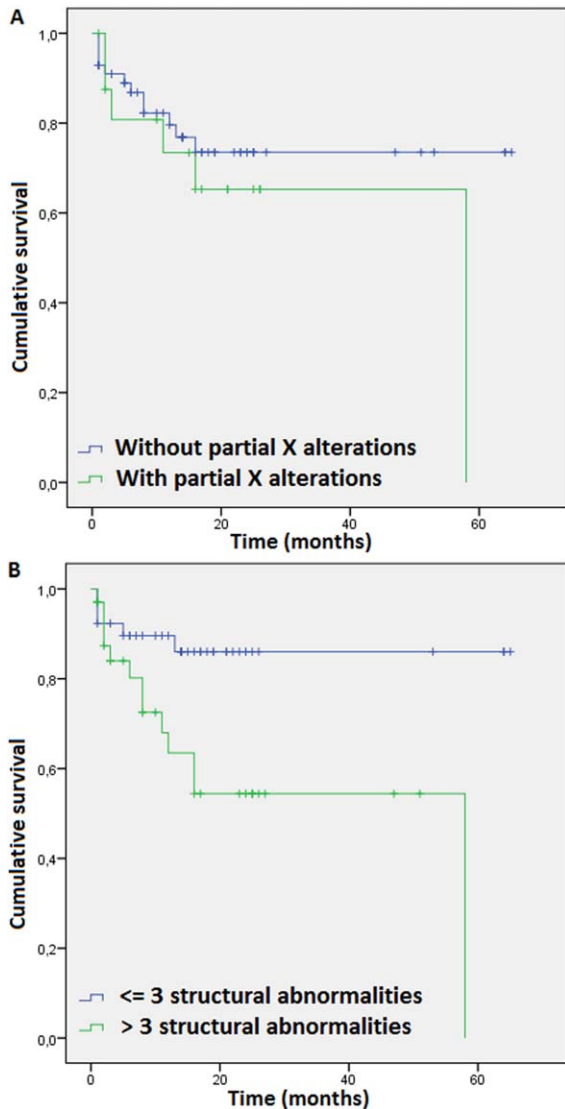


Figure 7. Overall survival in relation to presence of partial X alterations (A) and the number of structural aberrations (B). We performed aCGH at diagnosis of 70 patients. Based on X chromosome profiles, there was no statistical difference in OS between the groups with or without partial X alterations (A). Patients with more than three structural abnormalities had reduced overall survival compared to these with three or less structural abnormalities. *P* value (log rank) = 0.008 (B). [Color figure can be viewed at wileyonlinelibrary.com]

abnormal centrosome number as responsible for segregation errors. The origin of the second type of alteration is more complex, it may be partially explained by the presence of fragile sites and defect in double-strand break (DSB) repair (Vitre and Cleveland, 2012). Homologous recombination (HR) and nonhomologous end-joining (NHEJ) are important pathways in the DSB repair system. Deregulation of genes involved in these pathways such as *ATM*, *NBN*, *XRCC4-6*, *PRKDC*, *DCLRE1C*, and *LIG4* leads to a higher level of chromosomal breakage with partial losses or gains

of genomic material (Krem et al., 2015). These chromosomal fragments could be translocated and submitted to other gene expression regulation processes, resulting in a more important impact on the phenotype and prognosis of partial X alteration as compared to complete X monosomy. Based on transcriptomic data, we observed that partial duplication of the active X chromosome does not have any significant impact on the transcription of the complete gene set from this region. However, a more specific data analysis based on our cohort and on a GEP online-available data set indicated a higher transcriptomic level of some Xq genes that are known to be involved in pathways activated in MM. Among these genes, *IKBKKG* and *IRAK1* belong to the NFKB pathway, which plays an important role in MM progression, treatment resistance and is targeted by proteasome inhibitors. Interestingly, a global study of genomic rearrangements in human cancers identified NFKB pathway genes, including *IRAK1* and *IKBKKG*, as frequently involved in amplified regions (Beroukhim et al., 2010). Therefore, these two genes are likely to be very relevant for MM progression and treatment response, as demonstrated in other cancers (Aigelsreiter et al., 2012; Wee et al., 2015). Other genes that are located in this region are also biologically relevant. Indeed, *ENOX2* has been associated with tumor cell migration (Wang et al., 2011; Zeng et al., 2012), *BCAP31* with anti-cancer immune response (Sankaranarayanan et al., 2015) and *RBMX/RBMX2* code for RNA-binding proteins with possible roles in gene expression and DNA repair (Adamson et al., 2012). These data therefore indicate that the simultaneously increased expression of several genes on Xq probably plays a role in signaling pathway activation and in the biology of MM cells, most probably in advanced disease with high chromosomal instability.

X chromosome alterations might thus constitute a predictive marker for response to some targeted drug. However, this hypothesis needs to be evaluated in a large prospective study. An aCGH analysis or FISH study with probes targeting the Xp and Xq telomeric regions could easily detect the partial duplication of the active X chromosome. Conventional karyotype would be less accurate due to the difficulty in recognizing the break-points. Indeed, there was no abnormality identifiable as a partial X chromosome when we analyzed the karyotypes of two patients. Thus, the distal Xq seems to be translocated to an unidentified autosome (Supporting Information Fig. 1).

Moreover, low proliferation rates often lead to non-informative karyotypes.

Even if we did not find any outcome association with X chromosome changes, partial X alterations were associated with a higher number of chromosomal breakages. We demonstrated that patients with more than three chromosomal breaks, regardless of the region where they occurred, had a decreased overall survival. These results show the importance of whole genome screening and the benefit of aCGH for patients with multiple myeloma.

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