Expression of genes encoding for proteins involved in heparan sulphate and chondroitin sulphate chain synthesis and modification in normal and malignant plasma cells.

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Abstract
Summary

Syndecan-1 is a proteoglycan concentrating heparin-binding factors on the surface of multiple myeloma cells, playing likely a major role in multiple myeloma biology. As heparan sulphate and chondroitin sulphate are the bioactive components of syndecan-1, we analyzed the signature of genes encoding 100 proteins involved in synthesis of these chains, i.e. from precursor uptake to post-translational modifications, using Affymetrix microarrays.

Throughout the differentiation of memory B cells into plasmablasts and normal bone marrow plasma cells, we show that expression of enzymes required for heparan sulphate and chondroitin sulphate biosynthesis is increasing, in parallel with syndecan-1 expression. Sixteen genes were significantly different between normal and malignant plasma cells, nine of these genes -EXT2, CHSY3, CSGALNACT1, HS3ST2, HS2ST1, CHST11, CSGALNACT2, HPSE, SULF2- encoding for proteins involved in glycosaminoglycan chain synthesis or modifications. Kaplan-Meier analysis was performed in two independent series of patients: B4GALT7, CSGALNACT1, HS2ST1 were associated with a good prognosis whereas EXT1 was linked to a bad prognosis.

This study provides an overall picture of the major genes encoding for proteins involved in heparan sulphate and chondroitin sulphate synthesis and modifications that can be implicated in normal and malignant plasma cells.

MESH Keywords B-Lymphocytes ; metabolism ; Case-Control Studies ; Cell Line, Tumor ; Chondroitin Sulfates ; biosynthesis ; Gene Expression ; Gene Expression Profiling ; methods ; Heparitin Sulfate ; biosynthesis ; Humans ; Immunologic Memory ; Kaplan-Meiers Estimate ; Multiple Myeloma ; enzymology ; genetics ; immunology ; Oligonucleotide Array Sequence Analysis ; Plasma Cells ; metabolism ; Principal Component Analysis ; Prognosis ; Protein Processing, Post-Translational ; Syndecan-1 ; metabolism

Author Keywords multiple myeloma ; proteoglycans ; heparan sulphate ; chondroitin sulphate ; glycosaminoglycan synthesis and modification pathways

Introduction

Multiple myeloma (MM) is a B cell neoplasia characterized by accumulation of clonal plasma cells, i.e. multiple myeloma cells (MMC), in the bone marrow (BM). A hallmark of plasma cells is the expression of syndecan-1, a proteoglycan containing attachment sites for heparan sulphate (HS) and chondroitin sulphate (CS) chains (Rapraeger, et al 1985), (Kokenyesi and Bernfield 1994). In the BM of healthy individuals, plasma cells are the only cells expressing syndecan-1 (Costes, et al 1999). In patients with MM, all MMC express syndecan-1 with the exception of preapoptotic cells which rapidly lose this proteoglycan expression (Jourdan, et al 1998). In addition, among the major cell-surface heparan sulphate proteoglycans (HSPG), MMC only bear syndecan-1 (Mahtouk, et al 2006). Expression of chondroitin sulphate proteoglycans (CSPG) on MMC has not been fully reported.

HS chains can bind to a large diversity of proteins, including extracellular matrix proteins, growth factors, adhesion molecules, chemokines, interleukins, enzymes or plasma proteins, see (Bishop, et al 2007, Esko and Selleck 2002) for review. In addition, CS chains have been recognized as critical regulators of growth factor- and cytokine signalling (Maeda, et al 1996), (Hirose, et al 2001), (Fthenou, et al 2006).

HS and CS are linear glycosaminoglycans, glucosaminoglycans and galactosaminoglycans respectively, composed of disaccharide units. Both are covalently attached to the syndecan-1 core protein through the linkage region, a common tetrasaccharidic sequence for HS and CS (composed of one xylose unit, two galactose units and one glucuronic acid unit). As summed up in Figure 1, biosynthesis of these

chains mainly takes place in the Golgi apparatus and involves three enzymatic steps, including formation of the linker tetrasaccharide, polymerization of disaccharide units and finally chemical modifications of the sugar backbone at various positions. This ultimate step generates diversity in oligosaccharide sequences, contributing to heterogeneity of binding, in particular through sulphatation pattern, see (Prydz and Dalen 2000) for review. In addition, HS chains can be further modified by exogenous enzymes such as sulphatase 1 and 2 (SULF1 and SULF2), removing selectively 6-O sulphates (Morimoto-Tomita, et al 2002), and heparanase (HPSE), cleaving HS sulphate chains in small bioactive fragments (Vlodavsky, et al 1999). Moreover, the syndecan-1 ectodomain is rapidly shed by several metalloproteinases (MMP) as MMP-7 or MT-MMP-1 (membrane type matrix MMP-1) (Ding, et al 2005), (Kudo, et al 2007). The shedding can be accelerated by activation of thrombin receptor, a G-protein coupled receptor, activation of epidermal growth factor receptor or by others effectors as plasmin(Subramanian, et al 1997), (Fitzgerald, et al 2000).

In patients with MM, high serum levels of soluble syndecan-1 are linked with a poor prognosis, partly as an indicator of the tumour cell mass (Li, et al 2001). Knockdown of syndecan-1 gene expression in MMC injected into severe combined immune deficient mice results in decreased growth (Yang, et al 2007a). In addition, HPSE expression promotes syndecan-1 shedding into its soluble form in association with poor prognosis and tumour growth in vivo (Mahtouk, et al 2007a), (Yang, et al 2007b). Syndecan-1 can regulate activity of heparin-binding growth factors implicated in proliferation and survival of malignant plasma cells by presenting them to their specific receptors (Zimmermann and David 1999). This proteoglycan can thus enhance the binding of heparin-binding epidermal growth factor like growth factor (HB-EGF) or amphiregulin, increasing their biological activities (Mahtouk, et al 2005), (Mahtouk, et al 2006). Syndecan-1 is also critical to capture hepatocyte growth factor (HGF) and to present it to its receptor, cMet (Derksen, et al 2002). Additionally, fibroblast growth factor (FGF), which can induce the PI-3K/AKT pathway involved in myeloma tumour cells survival and proliferation, can bind syndecan-1 (Klein, et al 2003). The functional contribution of the chondroitin sulphate chains still remains to be elucidated. Nevertheless, a report shows the implication of chondroitin synthase 1 (CHSY1), a protein of CS synthesis pathway, in the interaction between MMC and BM microenvironment (Yin 2005).

In this paper, we analysed the expression of genes coding for the major enzymes involved in HS and CS chain synthesis and modifications. We also studied the expression of genes coding for proteins necessary for production and transport of the precursors: sugar nucleotides and 3′-phosphoadenosine 5′-phosphosulphate (PAPS). These building blocks are produced in the cytosol before being translocated to endoplasmic reticulum (ER) and/or Golgi lumens. These enzymatic systems and the translocators are likely to influence availability of substrates and therefore glycosaminoglycan chain synthesis (Toma, et al 1996), (Dick, et al 2008). We determined expression of genes linked with HS and CS chains biosynthesis in memory B cells, plasmablastic cells or normal bone marrow plasma cells using Affymetrix microarrays. These profiles were compared to those of purified MMC of a large panel of newly-diagnosed patients.

Patients, materials and Methods

Cell samples

The 15 XG human myeloma cell lines (HMCL) were obtained in our laboratory (Zhang, et al 1994), (Rebouissou, et al 1998), (Tarte, et al 1999). The other HMCL used - LP1, OPM2, RPMI 8226, SKMM and U266 – were purchased from the ATCC (Rockville, MD, USA). They were maintained in RPMI1640 (Gibco Invitrogen, France), 10% foetal bovine serum (FBS, PAA laboratory GmbH, Austria) and for the IL-6-dependant cell lines, with 2 ng/ml of IL-6 (Abcys SA, Paris, France).

Normal bone marrow plasma cells (BMPC) were obtained from healthy donors after informed consent was given. Plasma cells were purified using an autoMACS with anti-CD138 MACS microbeads (Miltenyi-Biotec, Paris, France) as indicated (Mahtouk, et al 2005). $CD27^{+}$ memory B cells (MBC) and polyclonal plasmablasts (PPC, $CD38^{++}$, $CD20^{-}$) were obtained and generated in vitro from purified $CD19^{+}$ peripheral blood B cells, as previously described (Tarte, et al 2002).

MMC of a total of 171 patients with previously-untreated MM were included in this study after written informed consent was given (123 patients were included at the University hospital of Heidelberg (Germany) and 48 were from Montpellier (France)). These 171 patients were treated with high dose therapy (HDC) and autologous stem cell transplantation (ASCT) (Goldschmidt, et al 2000) and were termed in the following Heidelberg-Montpellier (HM) series. According to the Durie-Salmon classification (Durie and Salmon 1975), 15 patients were of stage IA (who progressed into therapy requiring MM), 24 of stage IIA, 2 of stage IIB, 121 of stage IIIA, and 9 of stage IIIB. According to the International Staging System (ISS), 76 patients were of stage I, 66 of stage II, 29 of stage III. 28 patients had IgAκ MM, 12 IgAλ MM, 64 IgGκ MM, 33 IgGλ MM, 1 IgDλ MM, 18 Bence-Jones κ MM, 11 Bence-Jones λ MM, and 4 non-secreting MM. We also used Affymetrix data of a cohort of 345 purified MMC from previously-untreated patients from the Arkansas Research Group (Little Rock). The patients were treated with total therapy 2 and termed in the following LRTT2 series (Barlogie, et al 2006). These data are publicly available in the NIH Gene Expression Omnibus (GEO) [http://www.ncbi.nlm.nih.gov/geo/] under accession number GSE2658.

Bone marrow stromal cells (MSC) were obtained from the bone marrow of 5 healthy volunteers after informed consent was given and osteoclasts (OST) were obtained in vitro from peripheral blood mononuclear cells as indicated (Corre, et al 2007), (Moreaux, et al 2005). Bone marrow T cells (CD3), monocytes (CD14) and polymorphonuclear neutrophils (PMN) were purified from 5 newly-diagnosed patients after informed consent was given, as indicated (Moreaux, et al 2005).

Preparation of cRNA, microarray hybridization and gene expression profiling analysis

Microarray experiments were performed in the Institute of Research in Biotherapy at the Montpellier University Hospital (France) (http://irb.chumontpellier.fr/en/laboratories_microarray.html). RNA was extracted with the RNeasy Kit (Qiagen, Valencia, CA, USA), the SV-total RNA extraction kit (Promega, Mannheim, Germany) and Trizol (Invitrogen, Karlsruhe, Germany) in accordance to the manufacturer's instructions as indicated (De Vos, et al 2002). RNA was amplified and hybridized (small sample labeling protocol VII, Affymetrix, Santa Clara, CA, USA) to the human genome HG-U133A and HG-U133B or HG-U133 Plus 2.0 GeneChip oligonucleotides arrays, according to the manufacturer's instructions (Affymetrix). Fluorescence intensities were quantified and analyzed using the GCOS (GeneChip Operating Software) software (Affymetrix). Arrays were scaled to an average intensity of 100. A treshold of 1 was assigned to values <1. In the Affymetrix HG-U133 microarrays, a gene is probed by 11 pairs of perfect-match/mismatch oligonucleotides randomly spread over the chip. The signed rank MAS5 algorithm decides after scanning if the corresponding gene can be statistically declared " present" (P call) or "absent" (A call), and delivers a weighted fluorescence signal. Gene expression data were analysed with our bioinformatics platform RAGE [http://rage.montp.inserm.fr/] and Amazonia [http://amazonia.montp.inserm.fr] (Assou, et al 2007). We also used the gene expression profile from 79 human normal tissues samples available from Hogenesch's group on a public data base (Su, et al 2004).

Real-time RT-PCR

RNA was extracted using the RNeasy Kit (Qiagen). We generated cDNA from 100 ng total RNA using Superscript II reverse transcriptase (Invitrogen, Cergy-Pontoise, France). For real-time reverse transcriptase–polymerase chain reaction (RT-PCR), we used Assay-on-Demand primers and probes and the TaqMan Universal Master Mix from Applied Biosystems (Courtaboeuf, France) according to the manufacturer's instructions. Gene expression of SULF2, GPI, CHSY3 and HS3ST2 was measured using the ABI Prism 7000 Sequence Detection System. For each sample, the cycle threshold (Ct) value for the gene of interest was determined and normalized to its respective Ct value for β2-microglobulin (B2M). Then it was compared with a cell type used as a positive control (XG-11 for SULF2, GPI, CHSY3 and one OST sample for HS3ST2 since this gene was not expressed by HMCL) using the following formula: $100/2^{\Delta\Delta Ct}$, in which threshold $\Delta\Delta Ct = (Ct_{unknown} - Ct_{unknown\ B2M}) - (Ct_{positive\ control} - Ct_{B2M\ positive\ control})$. The coefficient of correlation and P values for the correlations between microarrays and real time RT-PCR are indicated on each graph. P values were determined with a Spearman test using SPSS Software. Statistical significance was obtained for P value ≤ 0.05.

Statistical analysis

To cluster the samples according to the similarity of their gene expression patterns, we performed an unsupervised principal component analysis (PCA) with RAGE bioinformatics platform [http://rage.montp.inserm.fr/]. On the graphs related to PCA, the first principal component (pc1) is represented by the X-axis, the second principal component (pc2) by the Y-axis. The percentage indicated on these graphs gives the information of variance for these 2 components.

SAM (significance analysis of microarrays) analysis was applied to our probe sets of interest in the different samples with 1000 permutations, a fold change of 2 and a false discovery rate of 0%.

When PCA and SAM analysis were performed with MMC data, we used data obtained from gene expression analysis of the MMC purified from samples of the 123 patients included in the hospital of Heidelberg.

To determine the link with prognosis for a given probe set, patients were ranked in 4 quartiles according to probe set expression and the event-free (EFS) or overall survivals (OAS) of patients in the lowest quartile were compared to those of patients in the highest quartile. The survival curves were plotted using the Kaplan-Meier method. The statistical significance of differences in overall survival and event-free survival between groups of patients was estimated by the log-rank test (P ≤ 0.05). An event was defined as death. To avoid multiple testing correction, we retained probe sets associated with prognosis in 2 independent series of previously-untreated patients: the HM series of 171 patients and the LRTT2 series of 345 patients.

Results

Affymetrix microarrays to investigate pathways involved in heparan sulphate and chondroitin sulphate chain synthesis and modifications

In Figure 1, proteins involved in synthesis of HS and CS chains, from precursor uptake to post-translational modifications, are listed. These proteins, including their different isoforms, are encoded by 100 genes. Out of these genes, expression of 75 might be evaluated in B and plasma cells using 123 probe sets with Affymetrix U133 Plus 2:0 microarrays (see Table SI). 25 genes could not be studied in reason of a lack of probe set (GLUT7, UGP1) or to defective probe sets having an absent call or a weak Affymetrix signal (below 100) in our samples and a panel of 79 human tissues (Hogenesch data base) (Su, et al 2004).

The first step of HS and CS synthesis corresponds to cytoplasmic synthesis of nucleotide sugars and PAPS, the universal donor of sulphate. We evaluated expression of 16 genes coding for membrane transporters (9 genes for glucose transport and 7 genes for sulphate anion transport), 19 genes coding for enzymes leading to nucleotide sugar synthesis and 2 genes coding for proteins required during sulphate activation in PAPS.

The second step leads to the translocation of these precursors within the lumen of ER and Golgi apparatus where synthesis of HS and CS takes place: 7 genes coding for nucleotide sugar transporters and 2 genes coding for PAPS transporters could be studied with U133 Affymetrix data. In these organelles, a complex set of enzyme reactions encoded by 26 out of the 75 available genes from Affymetrix chips is involved in linkage region synthesis and in HS and CS chain polymerization and modifications. We also analyzed the expression of the 3 genes coding for extracellular enzymes able to modify the pattern of sulphatation (SULF1 and 2) or to produce HS fragments of 10 to 20 sugar units long (HPSE).

Gene expression profiles associated with normal B cell and plasma cell samples

To determine whether the normal populations of memory B cells (MBC), plasmablasts (PPC) and bone marrow plasma cells (BMPC) present a specific expression profile for HS and CS synthesis and modifications pathways, an unsupervised principal component analysis (PCA) using the 123 probe sets was performed. PCA clearly delineated three distinct clusters: MBC, PPC and BMPC (Fig 2A). The first principal component accounted for 39% of variance and separated MBC from PPC and BMPC. The second principal component accounted for 24% of variance separating PPC and BMPC. We then identified the genes separating MBC from PPC + BMPC along the first PCA axis and PPC from BMPC along the second axis. We used a supervised analysis with the SAM (significance analysis of microarrays) software (1000 permutations, 2-fold change in expression with a 0% false discovery rate (FDR)) in order to identify probe sets linked to each type of cells. Results in Table I underline the weak level of expression of genes involved in each step of synthesis and modifications of HS and CS chains in MBC compared to plasma cells. PPC and BMPC cluster was associated with 33 overexpressed probe sets interrogating 25 genes and MBC cluster by 8 overexpressed probe sets interrogating 4 genes only. Out of the 25 plasma cell genes, 1 encoded for glucose transporter (SLC2A5), 2 for a sulphate anion transporter (SLC26A2, SLC26A6), 1 for an activator of sulphate in PAPS (PAPSS1), 1 for a PAPS transporter (SLC35B3), 11 for nucleotide sugar synthesis enzymes (GALK1, GALK2, PGM1, PGM2, PGM3, GFPT1, GNPNAT1, UAP1, UGDH, UGP2 and GNPDA1), 2 for a nucleotide sugar transporter (SLC35B1, SLC35A4), 2 for enzymes implicated in HS synthesis and modification (EXT1, HS2ST1) and 5 for enzymes involved in CS synthesis and modification (CHPF, CHST11, CHST12, GALNAC4S-6ST and CSGlcA-T).

Regarding genes dissociating PPC and BMPC along the 2nd PCA axis, 10 genes were increased in PPC compared to BMPC - SLC2A3, HK2, GPI, PGM2, GFPT1, GALK2, PAPSS1, SLC35D1, SLC35B4, and HS2ST1 - and 12 genes were overexpressed in BMPC compared to PPC - SULF2, PAPSS2, UAP1, GALK1, CHST12, CSGlcA-T, CSGALNACT1, HS3ST2, HS6ST1, SLC2A10, SLC35D2, and SLC26A11 (Table II). These data emphasize an increased expression of genes coding for proteins involved in synthesis of HS and CS chains throughout B cell differentiation, in PPC and mainly in BMPC. Thus, out of the 123 probe sets initially included, a total of 63 probe sets discriminates the 3 populations (33 + 8 probe sets discriminating B cells from plasma cells and 10 + 12 probe sets separating plasmablasts from mature plasma cells).

Gene expression profiles linked with multiple myeloma cells

Unsupervised PCA clustering of MMC together with normal MBC, PPC and BMPC delineated two groups according to first PCA component accounting for 18% of variance: a group of MMC only (principal component 1>0) and a group of normal cells and MMC (principal component 1<0) (Fig 2B). Similar clustering was obtained with the limited list of 63 probe sets reported above. Running only normal plasmablasts and mature plasma cells together with MMC yielded the same clustering. In order to identify probe sets overexpressed in MMC compared to normal plasma cells (PPC + BMPC), normal PPC or normal BMPC, a supervised analysis was realized with SAM software (1000 permutations, fold-change ≥ 2, FDR = 0%). Expression of 16/75 genes was significantly different between BMPC and MMC. Results are shown in Table III. Twelve probe sets interrogating 11 genes were up-regulated in MMC in comparison with BMPC: HK2, GPI, GNPDA2, PAPSS1, PAPSS2, EXT2, HS2ST1, CHST11, CHSY3, HPSE and SULF2. CHSY3 gene presented the highest variance among all the 75 genes studied. CHSY3 was only expressed by MMC, unlike normal BMPC, PPC or MBC (Fig 3A) and had a present call in MMC of 105 out of 123 patients included in the Heidelberg patient cohort. Two other genes are remarkable because the encoded proteins have already documented functions in MM (SULF2) (Dai, et al 2005) or other cancers (GPI) (Watanabe, et al 1996), (Tsutsumi, et al 2003b). SULF2 gene was overexpressed 3.7 fold in MMC in comparison with BMPC (Fig 3B). GPI, whose expression was significantly enhanced in PPC, had a 2.08-fold change in expression between MMC and BMPC (Fig 3C).

Moreover, 7 probe sets interrogating 5 genes were under expressed in MMC compared to BMPC: SLC2A3, GALK1, HS3ST2, CSGALNACT2 and CSGALNACT1. A loss of HS3ST2 expression has been documented in some epithelial cancers (Tsutsumi, et al 2003b), and this is also the case in 39 out of the 123 MMC purified from patients of the Heidelberg group (Fig 3D).

Microarray data for these four genes of interest (CHSY3, SULF2, GPI, and HS3ST2) were validated (Fig 4) by real-time RT-PCR ($P \leq 0.05$) using RNA of 7 human myeloma cell lines (HMCL) or 7 osteoclast (OST) samples (HS3ST2 probe set is not expressed in HMCL). Supplementary information regarding genes differentially expressed between MMC and PPC or MMC and PPC+BMPC is given in Table SII.

Gene expression profiles associated with human myeloma cell lines

Unsupervised PCA clustering of primary MMC and HMCL using the 123 probe sets defined previously yielded to 2 well identified clusters, a MMC and a HMCL cluster. The first PCA component accounted for 24% and the second one for 8% (data not shown). Using SAM analysis (1000 permutations, fold change ≥ 2, FDR = 0%), 47 of the 123 probe sets were differentially expressed between MMC and HMCL: 30 being overexpressed in MMC and the 17 others in HMCL (Table IV). The 30 MMC probe sets interrogated 4 genes implicated in glucose transport (SLC2A1, SLC2A9, SLC2A10, SLC2A13), 1 gene involved in sulphate anion transport (SLC26A1), 1 gene coding for PAPSS2, 1 gene coding for SLC35B3, 2 genes implicated in sugar metabolism (GALK1, PGM3), 1 gene coding for the nucleotide sugar transporter SLC35D2, 5 genes implicated in HS synthesis and modifications (EXT1, HS2ST1, HS3ST2, HS3ST4 and NDST1), 1 gene coding for SULF2, and 5 genes involved in CS chain polymerization and modifications: CSGALNACT1, GalNAc4S-6ST, CHST11, CHSY3 and CHPF. This last observation underlines an increased expression of enzymes implicated in CS chain polymerization in MMC. Among the 17 probe sets up-regulated in HMCL, 9 probe sets are linked with genes encoding for enzymes involved in sugar metabolism: PGM2, GALE, GALK2, GNPDA1, HK2, GPI, GFPT1, GNPNAT1, and GALM. This result indicates an enhanced sugar metabolism capacity in cell lines. Other genes encoded for the SLC35B4 nucleotide sugar transporter, for B3GAT3, for HS3ST3B1, for SULF1 and for HPSE.

Expression of genes coding for extracellular enzymes involved in HS modifications

The function of HSPG, and thus of syndecan-1, can be regulated by extracellular enzymes able to modulate the structure of HS chains. Among these enzymes, 2 sulphatases (SULF1 and SULF2) are known to possess endoglucosamine-6 sulphatase activity. Another enzyme, HPSE, acts as an endoglucuronidase that can cleave HS chains, producing fragments more biologically active than native chains. Given the major role of the BM microenvironment in the pathogenesis of myeloma, see (Mitsiades, et al 2006) for review, we examined gene expression level of these 3 enzymes in bone marrow T cells (CD3), monocytes (CD14) and polymorphonuclear neutrophils (PMN) purified from 5 patients with newly diagnosed myeloma. In addition, bone marrow stromal cells (MSC) (n= 5) and OST (n=7) were generated in vitro from cells of 5 healthy donors and 7 patients with myeloma, respectively. As it can be seen in Figure 5, SULF1 which was not expressed by MMC (data not shown) was exclusively expressed by MSC. SULF2 was expressed by all types of cells studied, except for CD3 cells. Besides, an over expression of this enzyme was observed in CD14 cells (median, 1678) and PMN (median, 724) in comparison with MSC (median, 267) and OST (median, 67). Moreover, HPSE was mainly expressed by OST and CD14 cells as previously published (Mahtouk, et al 2007b). Taken together, these data suggest that the BM environment could be involved in the regulation of the structure of HS chains and thus in their function.

Probe sets linked to prognosis

Out of the 123 probe sets, 4 were associated with prognosis in the 2 independent series of previously-untreated patients, the HM series of 171 patients and the LR-TT2 series of 345 patients treated with TT2 therapy. Overall survival (OAS) and event-free survival (EFS) data are shown in Figure 6 and are summed up in Table V. B4GALT7, CSGALNACT1 and HS2ST1 expressions were correlated with a good prognosis and EXT1 expression was associated with a bad prognosis. Expression of B4GALT7, CSGALNACT1, HS2ST1 and EXT1 was evaluated in the 8 MM subtypes defined by Zhan et al. (Table VI) (Zhan, et al 2006). The poor prognosis of EXT1 is related to its higher expression in the poor prognosis group of patients with t(4;14) and spiked MMSET (MS). On the other hand, the frequency of the good prognosis genes is decreased in the Proliferation (PR) and MMSET (MS) bad prognosis groups.

Discussion

In the present study, we have first analysed the change in expression profiles of 75 genes that could be interrogated with Affymetrix microarrays and encoding for HS and CS chains biosynthesis and modifications pathways, throughout B cell to plasma cell differentiation. These chains are the major bioactive components of syndecan-1 that is a hallmark of plasma cell differentiation. Our work shows that, in the differentiation from MBC to PPC and BMPC, gene expression of enzymes required for HS and CS biosynthesis is increasing, in parallel with syndecan-1 expression in the cells. The expression of these 75 genes makes it possible to classify MBC, PPC and BMPC into 3 distinct and homogeneous clusters, characterized by a set of overexpressed genes. Only 4/75 genes were demonstrated to have an increased expression in MBC compared to other types of cells. These genes encode for proteins involved in precursor transport and

synthesis, which are probably necessary for other metabolic processes. The 22 genes defining the PPC cluster indicate that these cells are, above all, able to enhance their production of PAPS and of nucleotide sugars, and then to promote their transport within ER and Golgi apparatus. Gene expression profile of PPC also indicates that these cells possess the majority of the different enzymes required for linkage region synthesis, polymerization and modifications of HS and CS chains, even if syndecan-1 expression in PPC is low (data not shown). Thus, PPC have the machinery to enhance their metabolic potential, with a special effect on sugar metabolism, and are then prompt to increase the synthesis of syndecan-1 HS and CS chains when differentiating into mature plasma cells (PC). The BMPC cluster is characterized by the high expression of the overall enzymes required for HS and CS synthesis and modifications in accordance with the high levels of syndecan-1 HS and CS chains in these cells. Of note, mature PC have to continuously synthesize a lot of these chains since membrane syndecan-1 has a 2-hour turnover, being rapidly cleaved by MMP into soluble syndecan-1. Among the genes that were highly overexpressed in BMPC in comparison to PPC, HS3ST2 and SULF2 encode for a sulphotransferase and for a sulphatase, respectively. These enzymes are involved in the late modifications of heparan sulphate chains. They are responsible of their structural diversity by modulating sulphatation, which governs interactions between HSPG and a broad range of proteins.

Regarding MMC, the unsupervised PCA analysis indicates that MMC are closed to BMPC, MBC and PPC being in distant clusters. Sixteen genes are however differently expressed between MMC and BMPC with a fold change ranging from 10.33 (CHSY3) to 2 fold (GNPDA2) with a 0% FDR.

Among these genes, one is involved in initial steps of precursor synthesis: GPI. This gene encodes for an enzyme responsible for isomerization of glucose-6-phosphate and fructose-6-phosphate. In our study, it was associated with OAS in Kaplan–Meier analysis realized from data of the LR-TT2 cohort but was not significant in HM cohort (data not shown). In LR-TT2, upper levels of expression appeared to be associated with shorter OAS, suggesting that this parameter could be linked to a bad prognosis. GPI is an ubiquitous cytosolic enzyme that plays a critical role in sugar metabolism. Interestingly, this protein can be secreted and molecular cloning and sequencing studies have ascribed multiple identities to the secreted form: autocrine motility factor, neuroleukin and maturation factor (Watanabe, et al 1996). GPI is thus considered as being a multifunctional protein and is involved in different malignant processes as proliferation, survival or angiogenesis (Funasaka, et al 2001), (Tsutsumi, et al 2003b), (Tsutsumi, et al 2003a). Its contribution to MM is not known but could be of particular interest.

Of note, among the genes differently expressed between MMC and BMPC, 9 genes – EXT2, CHSY3, CSGALNACT1, HS2ST1, HS3ST1, CHST11, CSGALNACT2, HPSE, SULF2- encode for proteins involved in HS or CS chain synthesis or modifications.

EXT2 gene encodes for exostose multiple 2, a protein involved in the first step of HS chain polymerization. Of note, EXT2 is one of the three genetic loci, with EXT1 and EXT3, mutated in hereditary multiple exostoses, an autosomal dominant disease characterized by the formation of osteochondromas in relation with a disorganization of chondrocytes, leading to random ossification (Zak, et al 2002). The protein EXT2 combines with EXT1 to form an hetero-oligomeric complex that catalyzes the HS chain elongation (Busse, et al 2007). These enzymes do not appear to play a redundant role in HS polymerization (Zak, et al 2002). In our study, we observed that EXT1 expression in MMC is associated with an adverse prognosis in the 2 independent patient series. This result is not in accordance with previous reports suggesting that EXT1 functions as a tumour suppressor gene in patients with hereditary multiple exostoses (Hecht, et al 1995, Piao, et al 1997). In multiple myeloma, apart from its role in HS chain polymerization, another function of the protein EXT1 should be investigated. HS chains bind to a plethora of proteins, including different growth factors involved in myeloma biology. Sulphatation pattern of glycosaminoglycan chains is a critical element responsible for diversity in oligosaccharide sequences and, as a consequence, for heterogeneity of binding. HS2ST1 encodes for a 2-O sulphotransferase (heparan sulphate 2-O-sulphotransferase 1). This enzyme transfers sulphate to the C-2 position of the iduronic acid residues of HS chains. In our study, we observed an overexpression of this gene in MMC. Besides, the gene encoding for this enzyme was correlated to a good prognosis. Additionally, HS3ST2, an other enzyme implicated in HS chain modifications, is of interest. This protein exhibits heparan sulphate glucosaminyl 3-O-sulphotransferase activity and is thus able to modulate sulphatation pattern in C-3 position of the sugar backbone. Interestingly, a loss of HS3ST2 expression has been documented in some epithelial cancers (Tsutsumi, et al 2003b) and a similar result was obtained in our study when comparing BMPC and MMC, a decreased expression being obtained in this last category of cells. These observations are consistent with a major role of HS sulphatation in myeloma biology, most likely through the modulation of growth factor binding.

Notably, our study is the first one to focus on several genes involved in CS chain synthesis and modification in myeloma. CSGALNACT1, also known as chondroitin sulphate N-acetylgalactosaminyltransferase 1, is involved in the initiation of CS chain synthesis (Sato, et al 2003). Our results show that overexpression of this gene is associated with a good prognosis in HM and LR-TT2 groups. CHSY3 gene encodes for a glycosyltransferase implicated in CS synthesis (Yada, et al 2003). Our data show that CHSY3 is not expressed by normal BMPC but by MMC with a high level of expression. Moreover, CHSY3 correlated with a good prognosis in Kaplan-Meier analysis realized from EFS data of the HM cohort and from OAS data of the LR-TT2 cohort (data not shown). Recently, it was shown that co-expression of CHSY3 with CHPF (CHPF gene is expressed by MMC, data not shown) confers a CS polymerization activity (Izumikawa, et al 2007). Another enzyme involved in CS sulphatation, CHST11, was overexpressed in MMC too. We can speculate that these enzymes could extend and modify CS chains in MMC conferring on them more controlled growth rate and better

prognosis. They could be involved in MM biology and clinical response through various proteoglycans. This remains to be elucidated. To examine expression of proteoglycans with CS chains in our samples, a supervised analysis was realized with SAM software as described previously (see Table SIII, Table SIV and Table SV). We first compared MBC samples to the group of PPC+BMPC. The MBC population was characterized by an overexpression of SPOCK2 (a gene encoding for testican 2) whereas the genes APLP2 and SRGN (a gene encoding for serglycin) were overexpressed in the cluster PPC+BMPC. Of note, the secretion of serglycin by HMCL and MMC has already been described (Theocharis, et al 2006). When we compared BMPC and MMC, we found that VCAN and COL9A2 genes were overexpressed in normal plasma cells whereas none of the genes encoding for CSPG was overexpressed in MMC.

Two MMC genes encode for enzymes involved in HS chain modifications: SULF2 and HPSE. SULF2 gene is already overexpressed in BMPC compared to PPC and its expression increases in MMC. Recent data have shown that SULF2, and the first member of the corresponding endosulphatase family SULF1, act as strong inhibitors of myeloma tumour growth in vivo (Dai, et al 2005). In our study, SULF1 was not expressed by normal plasma cells or MMC but by MSC, which further underlines the role of the myeloma microenvironment. Of note, SULF1 is expressed by some HMCL emphasizing that these HMCL can express environment genes, making it possible to escape from environment dependence. This is the case for IL-6 (Jernberg-Wiklund, et al 1992), HB-EGF (De Vos, et al 2001), (Wang, et al 2002) or for heparanase (Mahtouk, et al 2007a). This last enzyme cleaves HS chain into bioactive 10–20 saccharide fragments. Transduction of HPSE gene in HMCL confers a growth advantage in vivo (Yang, et al 2005). One mechanism is that heparanase can control syndecan-1 gene expression and syndecan-1 shedding into a soluble form that also confers MMC growth advantage (Yang, et al 2007b).

Among the genes whose expression is associated to prognosis in our study, B4GALT7 encodes for a glycosyltransferase (beta 1,4-galactosyltransferase I) involved in HS chain polymerization in the trans-Golgi apparatus. In addition, this enzyme can be located at the cell membrane and functions as a receptor for extracellular glycoside ligands by binding terminal N-acetylglucosamine residues (Rodeheffer and Shur 2002). Mutations of this gene resulting in absent or defective enzyme activity have been associated with Ehlers-Danlos syndrome (progeroid variant) (Furukawa and Okajima 2002). Additionally, several studies have suggested a contribution of B4GALT7 to cancer development and metastasis. Expression of this enzyme was increased in astrocytomas in comparison with normal brain tissue (Xu, et al 2001). An overexpression of this enzyme has been observed in hepatoma in comparison with normal liver tissues, the gene B4GALT7 being under the control of the viral regulatory protein Hbx. Besides, in this model, an up-regulation of B4GALT7 was associated with hepatoma cell growth in vivo and in vitro (Wei, et al 2008). In other studies, the surface activity of the enzyme was a determining factor for invasive behaviour of murine melanoma cell lines whereas activity of the Golgi enzyme remained unchanged in cells with different metastatic and invasive capacities (Johnson and Shur 1999, Passaniti and Hart 1990). The same observation was made for metastatic lung cancer cells, an up-regulation of B4GALT7 being under the control of the transcription factor E1AF (Zhu, et al 2005). In the current study, the result related to B4GALT7 challenges these data, this gene being linked to good prognosis. B4GALT7 is involved in the initial step of linkage region synthesis in Golgi apparatus. As this step is shared by HS and CS chains biosynthesis, one hypothesis is that B4GALT7 overexpression promotes production of proteoglycans other than syndecan-1 and is able to control myeloma growth. The cellular location of B4GALT7 has not been described in myeloma cells and could be studied to understand the role of this enzyme in myeloma biology.

In conclusion, this study shows an increased expression of enzymes required for HS and CS biosynthesis in parallel with syndecan-1 expression through B cell differentiation from MBC to BMPC. Of interest, some of these genes correlate with prognosis, which suggests their important function in MM biology. Additionally, this study underlines enzymes implicated in CS synthesis and modification. Their implication in MM could be linked to CS proteoglycans expressed by plasma cells.

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Footnotes:

Authors' information: CB designed research, performed the experiments and wrote the paper. DH, MH, JFS, PQ, JFR and HG collected bone marrow samples and clinical data. TR, ACS and KM participated in the bioinformatics analysis of the data. DH and HG participated in the writing of the paper. BK is the senior investigator who designed research and wrote the paper.

List of abbreviations

Acetyl-CoA: acetyl-Coenzyme A
ADP: adenosine di-phosphate
ASCT: autologous stem cell transplantation
ATP: adenosine tri-phosphate

B2M: β2-microglobulin
BM: bone marrow
BMPC: bone marrow plasma cell
CD14: monocyte
CD3: bone marrow T cell
CHSY1: chondroitin synthase 1
CS: chondroitin sulphate
CSPG: chondroitin sulphate proteoglycan
Ct: cycle threshold
EFS: event-free survival
ER: endoplasmic reticulum
FDR: false discovery rate
FGF: fibroblast growth factor
Fru: fructose
Gal: galactose
GalNAc: N-acetyl galactosamine
GEP: gene expression profile
GlcN: glucosamine
GlcNAc: N-acetylglucosamine
GCOS: GeneChip Operating Software (Affymetrix)
Glc: glucose
HBEGF: heparin-binding epidermal growth factor like growth factor
HDC: high dose therapy
HGF: hepatocyte growth factor
HM: Heidelberg-Montpellier series of patients
HS: heparan sulphate
HSPG: heparan-sulphate proteoglycan
LR-TT2: Little Rock series of patients treated with total therapy 2
MBC: memory B cell
MM: multiple myeloma
MMC: multiple myeloma cell
MMP: metalloproteinase
MS: MMSET (multiple myeloma SET domain)
MSC: bone marrow stromal cell
NAD: nicotinamide adenine dinucleotide
OAS: overall survival
OST: osteoclast
PAPS: 3′-phosphoadenosine 5′-phosphosulphate
PC: plasma cell
pc1: first principal component
pc2: second principal component
PCA: principal component analysis
PI3K: phosphatidylinositol 3-kinase
PMN: polymorphonuclear neutrophil
PPC: plasmablast
PPi: inorganic phosphate
PR: proliferation
SAM: significance analysis of microarrays
UDP: uridine di-phosphate
UTP: uridine tri-phosphate
Xyl: xylose

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Fig 1

Pathways for HS and CS chains biosynthesis: from precursor up-take to extracellular modifications

The first step corresponds to monosaccharide and sulphate uptake through specialized transport systems (GLUT, SLC26 and SLC13 families). These precursors are activated in UDP-sugars and PAPS in the cytosol via a set of enzymes (GALM, GALK, GALT, GALE, PGM1, PGM2, UGP2, UGDH, UXS1, GFPT, GNPDA, GNPNAT1, PGM3 and UAP1 for nucleotide sugars synthesis and PAPSS1 and 2 for sulphate activation). UDP-sugars and PAPS are actively transported into the Golgi lumen and/or RE (SLC35 family). Thereafter, glycosaminoglycan synthesis is initiated. A linkage region is produced: a xylose, two galactose and one glucuronic acid residues are added on an attachment site of the proteoglycan core (XYLT, B4GALT7, B3GALT6 and B3GAT3). Then, the glycosaminoglycan lineage depends on the addition of the fifth nucleotide sugar. Addition of a glucosaminyl residue by EXTL2 generates glycosaminoglycans (HS chains), whereas a galactosaminyl residue added by CSGALNACT1 creates galactosaminoglycans (CS chains). For these two types of chains, enzymes catalyse elongation and polymerization (EXT1/EXT2 for HS chains, CSGlcA-T, CSGALNACT2, CHSY1, CHSY3 and CHPF for CS chains). As the chains grow, they are modified at various positions: deacetylation/N-sulphatation of glucosamine by NDST, epimerisation of glucuronic acid to iduronic acid by GLCE and O-sulphatation in various positions by HS2ST, HS6ST or HS3ST in HS chains and sulphatation by CHST11, 12, 13, CHST3, GalNac4S-6ST and UST in various sites in CS chains. Finally, proteoglycans bearing HS and CS chains can be transported to plasma membrane. Extracellular enzymes, as SULF1 and 2 which exhibit sulphatase activity on HS chains removing sulphate from the 6-position of glucosamine and HPSE able to cleave HS in little fragments of 10 to 20 saccharidic units, are responsible of the latest modifications. When gene expression is associated to prognosis in our data, proteins are colour-coded in green for good genes and in red for bad genes.

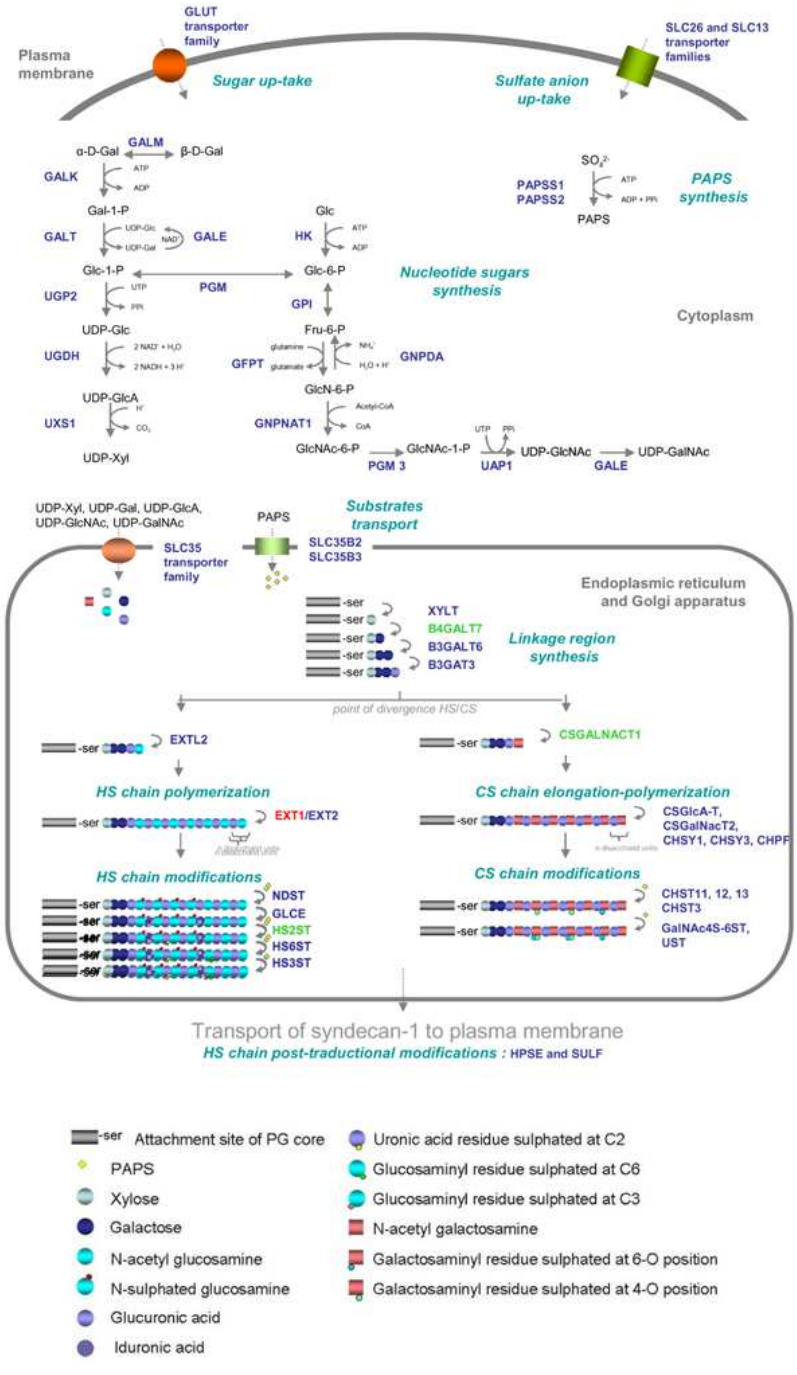


Fig 2

A global view of variation of gene expression in MBC, PPC, BMPC and MMC using PCA

Unsupervised PCA using the list of 75 genes involved in HS and CS biosynthesis pathway was performed to obtain a global view of the variation in gene expression among MBC, PPC, BPMC and MMC. (A). MBC, PPC and BMPC were separated in 3 distinct clusters by a PCA. The first principal component is represented by the X-axis (pc1, variance: 39%), the second principal component by the Y-axis (pc2, variance: 24%). According to pc1, PPC and BMPC clustered together on the left side and MBC clustered separately on the right side. The second component makes it possible to separate BMPC from PPC. (B) PCA clustering of MMC together with normal MBC, PPC and BMPC delineated two groups according to first PCA component (pc1, variance: 18%): a group of MMC only with a pc1 value ≥ 0 and a group of normal cells and MMC with a pc1 value < 0.

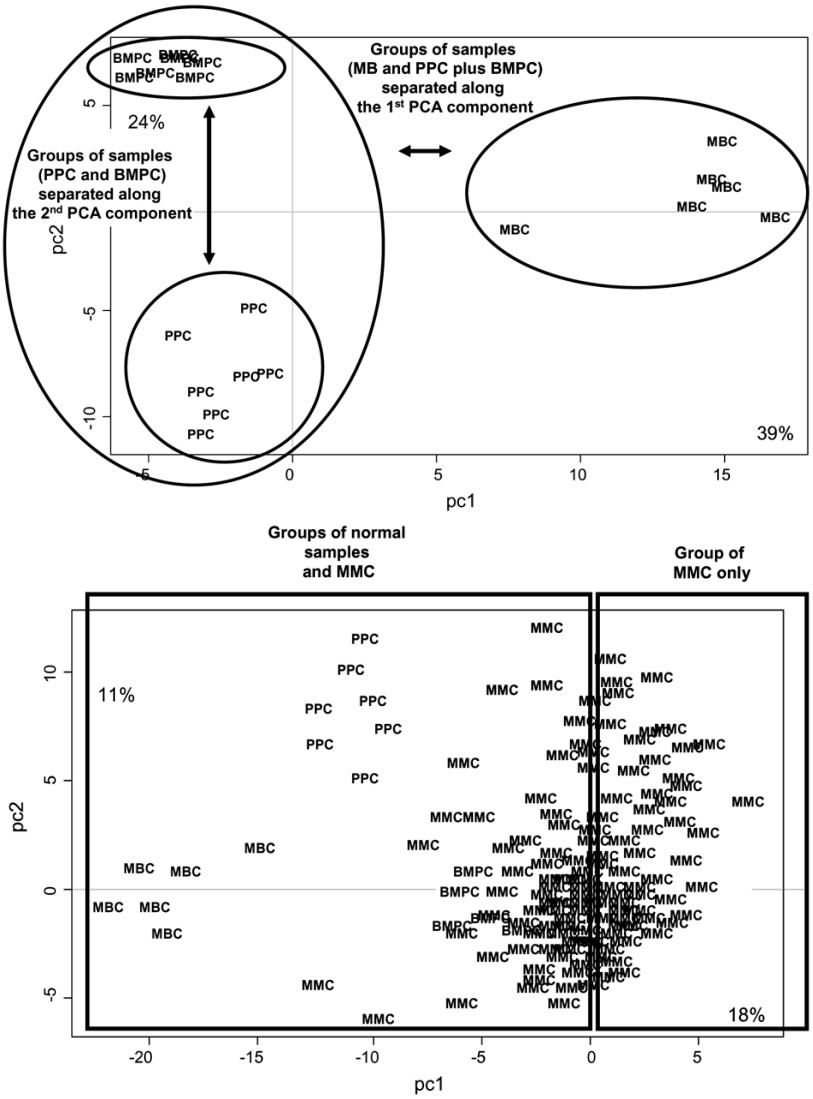


Fig 3

Expression data of CHSY3, SULF2, GPI and HS3ST2 in MBC, PPC, BMPC, MMC and HMCL

Histograms show the probe set signal corresponding to CHSY3 (3A), SULF2 (3B), GPI (3C) and HS3ST2 (3D) gene expression on the Y-axis as arbitrary units determined by the Affymetrix software from 6 MBC, 7 PPC, 7 BMPC, MMC of 123 patients of the Heidelberg series and 20 HMCL. Filled histograms indicate a “present” Affymetrix call and white ones an “absent” call in the sample. When several probe sets were available for a same gene, we used the probe set with the highest variance.

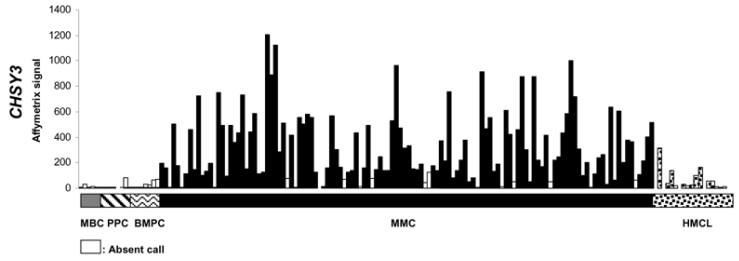


Figure 3A

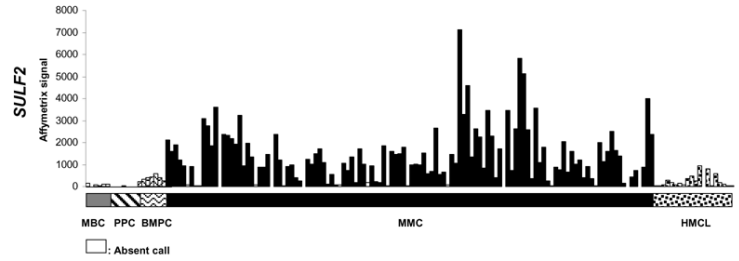


Figure 3B

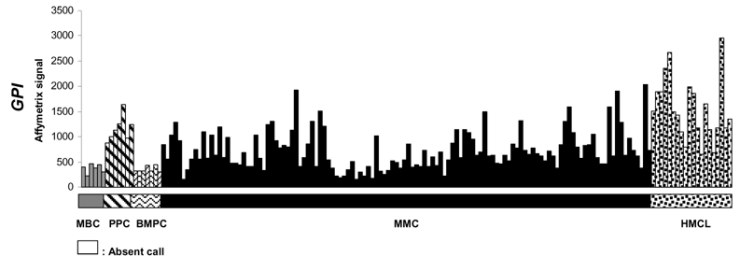


Figure 3C

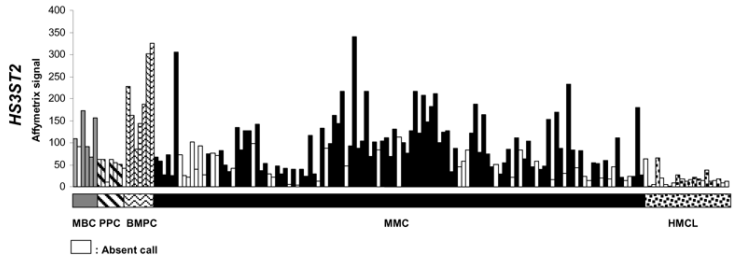


Figure 3D

Fig 4

Validation of Affymetrix microarray data of CHSY3, GPI, SULF2 and HS3ST2 genes

Gene expression of CHSY3 (4A), SULF2 (4B) and GPI (4C) were assayed with real-time RT-PCR in 7 HMCL samples and normalized with B2M. XG-11 was used as a positive control. Gene expression of HS3ST2 (4D) was studied in 7 OST and normalized with B2M. One OST sample was used as a positive control. The P values of the correlation between microarrays and real time RT-PCR data were determined with a Spearman test and are indicated in the figure.

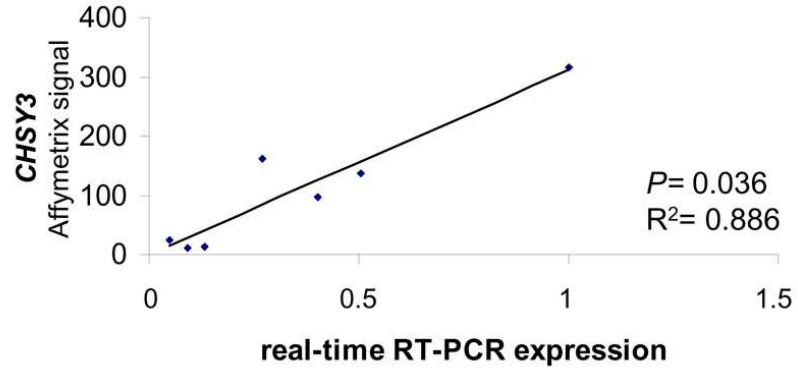


Figure 4A

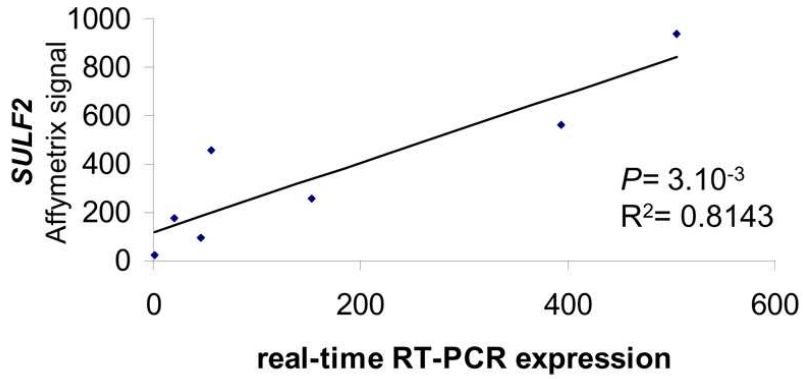


Figure 4B

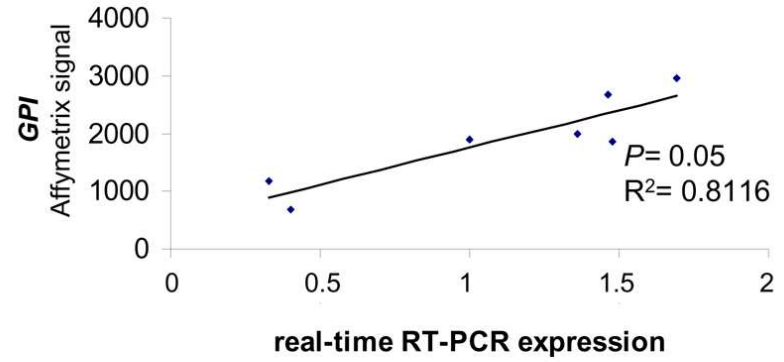


Figure 4C

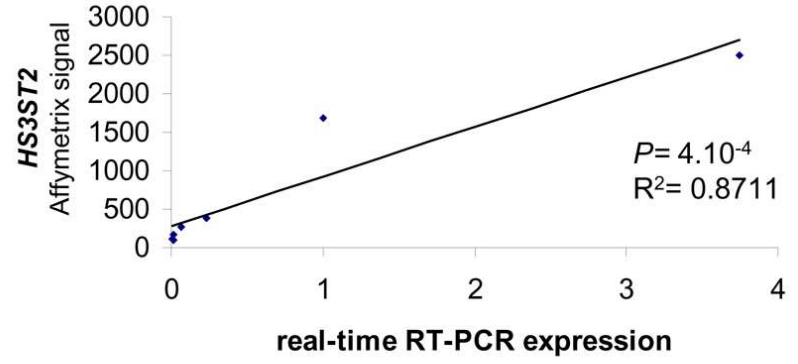


Figure 4D

Fig 5

Expression of extracellular enzymes implicated in modifications of HS chains in BM environment

The histograms show the Affymetrix signals of 3 probe sets interrogating SULF1 (5A), SULF2 (5B) and HPSE (5C) expression in 5 cell populations of the BM environment: MSC, OST, CD14, PMN and CD3 cells. Filled histograms indicate a "present" Affymetrix call and white ones an "absent" call in the sample. When several probe sets were available for a same gene, we used the probe set with the highest variance.

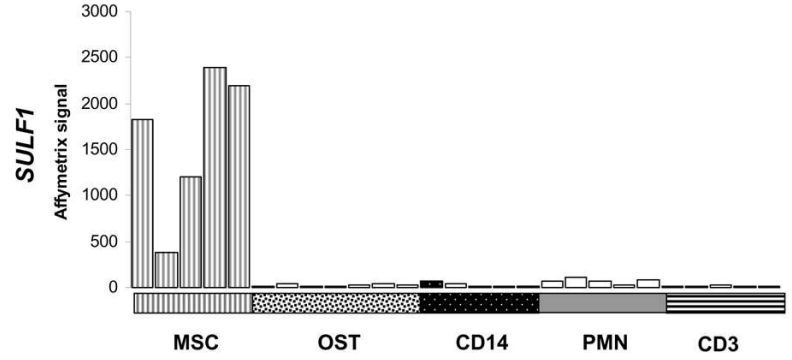


Figure 5A

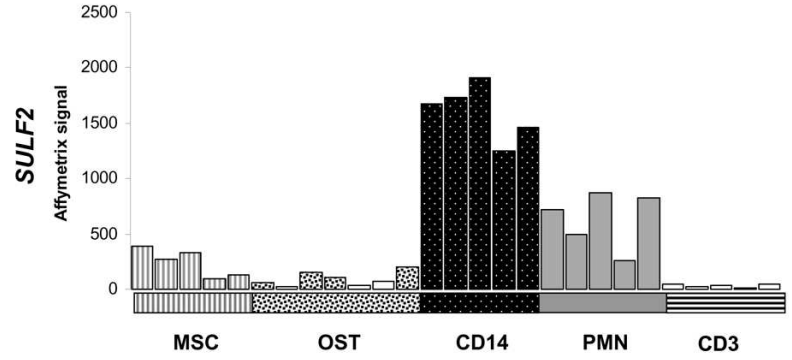


Figure 5B

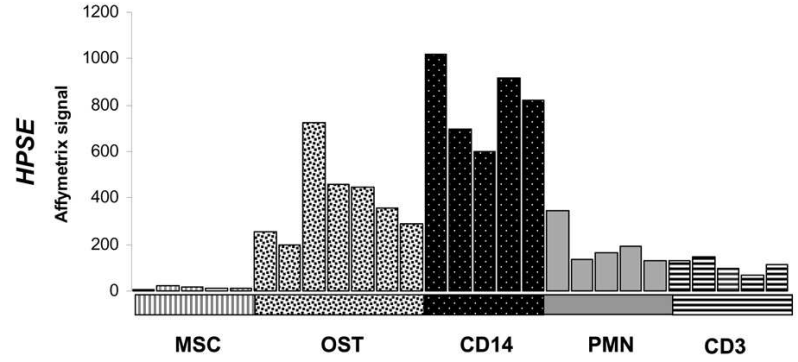
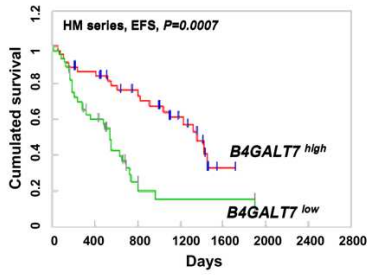


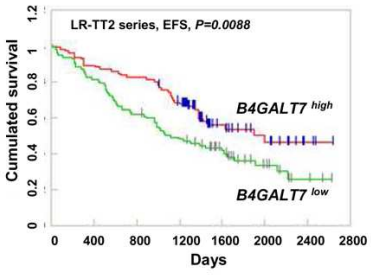
Figure 5C

Fig 6

Overall survival and event-free survival related to 4 genes in 2 independent patient series

Data are Kaplan-Meier survival curves and/or event-free survival curves of patients with the highest or lowest expression (quartile) of B4GALT7 (6A), CSGALNACT1 (6B), HS2ST1 (6C) and EXT1 (6D). The HM series comprises 171 patients and the LR-TT2 series 345 patients. P values are indicated in each panel.





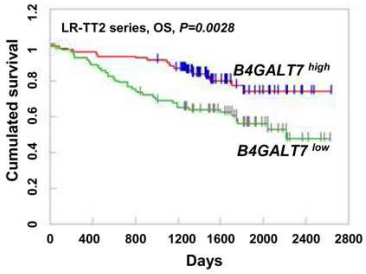
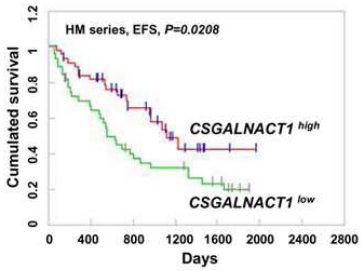
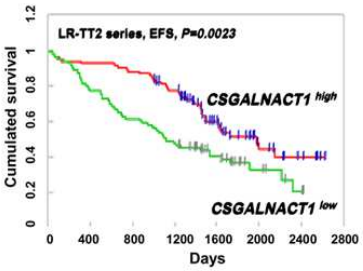
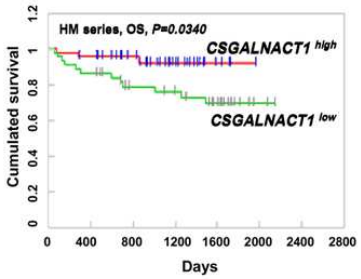


Figure 6A







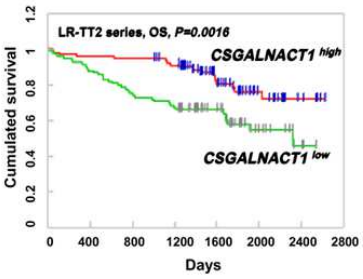
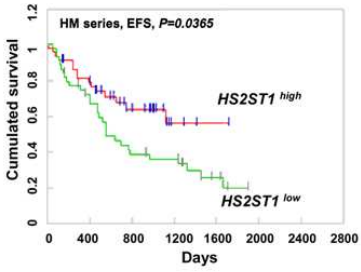


Figure 6B



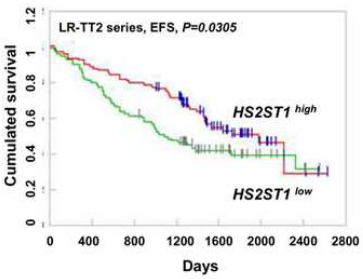


Figure 6C

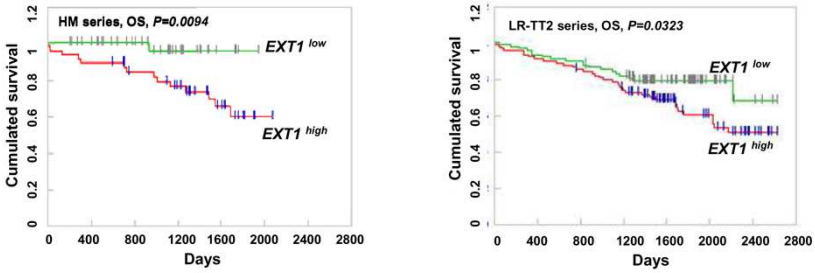

HM series, OS, P=0.0094
EXT1 low
EXT1 high
Cumulated survival
Days
LR-TT2 series, OS, P=0.0323
EXT1 low
EXT1 high
Cumulated survival
Days

Figure 6D

Table I

SAM-defined overexpressed genes in MBC and in PPC plus BMPC

		Gene name	Function	Probe set	Fold-change	Median in MBC	Median in PPC	Median in BMPC
Comparison MBC versus PPC plus BMPC	**SAM-defined genes overexpressed in MBC**	SLC2A3	Glucose transport	202499_s_at	7.24	2738	474	146
		SLC2A3///SLC2A14	Glucose transport	222088_s_at	5.46	567	154	50
		SLC2A3	Glucose transport	202497_x_at	5.29	863	357	28
		SLC2A3	Glucose transport	23657l_at	4.40	304	63	82
		SLC35D2	Nucleotide sugar transport	213082_s_at	3.80	132	6	50
		SLC2A3///SLC2A14	Glucose transport	216236_s_at	2.86	536.5	182	175
		UST	CS chain modification	205139_s_at	2.78	74.5	20	33
		SLC2A1	Glucose transport	201250_s_at	2.42	482	212	191
	SAM-defined genes overexpressed in PPC plus BMPC	PGM3	Sugar metabolism	210041_s_at	28.25	8.5	231	191
		CHST12	CS chain modification	218927_s_at	17.17	33.5	405	1025
		CHST11	CS chain modification	226372_at	15.25	12.5	350	207
		SLC35B3	PAPS transport	222691-at	11.16	32.5	737	823
		GALK2	Sugar metabolism	205219_s_at	6.48	8.5	162	53
		HS2ST1	HS chain modification	203284_s_at	6.26	60	487	374
		UGDH	Sugar metabolism	203343_at	4.96	71.5	592	413
		UAP1	Sugar metabolism	209340_at	4.94	270.5	797	1855
		GNPDA1	Sugar metabolism	202382_s_at	4.69	21.5	133	182
		PGM3	Sugar metabolism	221788_at	4.49	34.5	106	206
		GALNAC4S-6ST	CS chain modification	203066_at	4.31	292.5	1329	1499
		GNPNAT1	Sugar metabolism	225853_at	4.27	78.5	480	261
		SLC2A5	Glucose transport	204430_s_at	4.11	68	373	202
		SLC26A2	Sulphate anion transport	224959_at	4.08	65	266	357
		CSGlcA-T	CS chain polymerization	55093_at	3.98	77.5	214	539
		SLC35B1	Nucleotide sugar transport	202433_at	3.84	195.5	944	773
		GFPT1	Sugar metabolism	202722_s_at	3.82	199	811	634
		SLC26A6	Sulphate anion transport	221572_s_at	3.69	25	80	114
		SLC35A4	Nucleotide sugar transport	224626_at	3.35	77	259	265
		SLC26A2	Sulphate anion transport	205097_at	3.23	43.5	97	185
		HS2ST1	HS chain modification	203283_s_at	3.16	54.5	236	110
		GALK1	Sugar metabolism	229458_s_at	3.06	26.5	35	158
		CHPF	CS chain modification	202175_at	3.02	69.5	136	265
		CSGlcA-T	CS chain	221799_at	2.91	79.5	214	273

	polymerization					
UGP2	Sugar metabolism	205480_s_at	2.86	444.5	1506	1426
PGM2	Sugar metabolism	225366_at	2.71	48.5	154	100
GFPT1	Sugar metabolism	20272l_s_at	2.69	94.5	381	118
PAPSS1	PAPS synthesis	209043_at	2.56	316	1321	470
HS2ST1	HS chain modification	203285_s_at	2.49	72	206	153
PGM1	Sugar metabolism	201968_s_at	2.46	260	687	652
EXT1	HS chain polymerization	201995_at	2.20	82	152	217
PGM2	Sugar metabolism	223738_s_at	2.17	84	199	127
CHST12	CS chain modification	222786_at	2.00	113	203	248

A supervised analysis was performed with the SAM software (1000 permutations) in order to compare the gene expression profile of the MBC population and of the group PPC plus BMPC (these two populations were grouped according to PCA result shown in Figure 2A). This analysis identified probe sets related to each type of cells. Only probe sets with a 2-fold change in expression at least and a 0 % false discovery rate were retained. Genes were ranked according to the fold-change.

Table II

SAM-defined overexpressed genes in PPC and BMPC

		Gene name	Function	Probe set	Fold-change	Median in MBC	Median in PPC	Median in BMPC
comparison PPC versus BMPC	**SAM-defined genes overexpressed in PPC**	SLC2A3	Glucose transport	202497_x_at	11.79	863	357	28
		SLC35B4	Nucleotide sugar transport	238418_at	10.67	3	30	3
		HK2	Sugar metabolism	202934_at	8.73	335.5	716	66
		PGM2	Sugar metabolism	225367_at	3.73	350.5	1293	311
		SLC2A3	Glucose transport	202498_s_at	3.47	147.5	106	34
		GPI	Sugar metabolism	208308_s_at	3.31	393	1131	314
		GFPT1	Sugar metabolism	20272l_s_at	3.19	94.5	381	118
		SLC2A3///SLC2A14	Glucose transport	222088_s_at	3.00	567	154	50
		PAPSS1	PAPS synthesis	209043_at	2.68	316	1321	470
		SLC35B4	Nucleotide sugar transport	22588l_at	2.65	137	376	142
		SLC2A3	Glucose transport	202499_s_at	2.58	2738	474	146
		SLC35D1	Nucleotide sugar transport	209711_at	2.50	184	277	132
		GALK2	Sugar metabolism	205219_s_at	2.47	8.5	162	53
		HS2ST1	HS chain modification	203283_s_at	2.02	54.5	236	110
	SAM-defined genes overexpressed in BMPC	SULF2	HS chain modification	224724_at	37.00	81	6	383
		SLC2A10	Glucose transport	221024_s_at	22.15	3	3	89
		PAPSS2	PAPS synthesis	203060_s_at	7.03	22.5	18	94
		SULF2	HS chain modification	233555_s_at	6.34	22.5	18	94
		SLC26A11	Sulphate anion transport	226679_at	4.32	211.5	61	252
		HS3ST2	HS chain modification	219697_at	4.19	100	54	188
		CSGALNACT1	CS chain polymerization	219049_at	3.50	727	418	1469
		GALK1	Sugar metabolism	229458_s_at	3.43	26.5	35	158
		SLC35D2	Nucleotide sugar transport	213083_at	3.21	321.5	71	227
		HS6ST1	HS chain modification	225263_at	2.74	146	101	275
		CHST12	CS chain modification	218927_s_at	2.34	33.5	405	1025
		CSGlcA-T	CS chain polymerization	55093_at	2.29	77.5	214	539
		UAP1	Sugar metabolism	209340_at	2.22	270.5	797	1855

A supervised analysis was performed with the SAM software in order to compare the gene expression profile of PPC and BMPC as indicated in Table I legend.

Table III

SAM-defined overexpressed genes in BMPC and in MMC

		Gene name	Function	Probe set	Fold-change	Median in PPC	Median in BMPC	Median in MMC
Comparison MMC versus BMPC	**SAM-defined genes overexpressed in MMC**	CHSY3	CS chain polymerization	242100_at	10.33	5	26	218
		SULF2	HS chain modification	224724_at	3.67	6	383	1042
		SULF2	HS chain modification	233555_s_at	3.17	5	69	207
		HK2	Sugar metabolism	202934_at	2.84	716	66	174
		CHST11	CS chain modification	226368_at	2.62	189	121	273
		PAPSS2	PAPS synthesis	203058_s_at	2.32	3	51	87
		HS2ST1	HS chain modification	230465_at	2.32	53 (range: 8–64)	39 (range: 31–58)	68 (range: 1–596)
		PAPSS1	PAPS synthesis	209043_at	2.16	1321	470	1006
		GPI	Sugar metabolism	208308_s_at	2.08	1131	314	633
		HPSE	HS chain modification	219403_s_at	2.07	57 (range: 11 – 85)	40 (range: 12–81)	57 (range: 1 – 895)
		EXT2	HS chain polymerization	202012_s_at	2.06	188	193	374
		GNPDA2	Sugar metabolism	227022_at	2.01	103	72	125
	SAM-defined genes overexpressed in BMPC	GALK1	Sugar metabolism	229458_s_at	4.23	35	158	26
		SLC2A3	Glucose transport	202499_s_at	3.54	474	146	36
		SLC2A3///SLC2A14	Glucose transport	216236_s_at	2.93	182	175	50
		HS3ST2	HS chain modification	219697_at	2.43	54	188	74
		CSGALNACT2	CS chain polymerization	218871_x_at	2.39	753	963	374
		CSGALNACT2	CS chain polymerization	222235_s_at	2.38	410	558	228
		CSGALNACT1	CS chain polymerization	219049_at	2.25	418	1469	530

A supervised analysis was performed with the SAM software in order to compare the gene expression profile of the BMPC and MMC populations and to underline probe sets related to each type of cells as indicated in Table I.

Table IV

SAM-defined overexpressed genes in MMC and HMCL

Gene name	Function	Probe set	Fold-change	Median in MMC	Median in HMCL
SULF1	HS chain modification	212353_at	15.10	8	18 (range: 1 – 1303)
HS3ST3B1	HS chain modification	22736l_at	8.46	4	156
SULF1	HS chain modification	212354_at	6.18	14	15 (range: 1–693)
SLC35B4	Nucleotide sugar transport	238418_at	5.88	7	92
HK2	Sugar metabolism	202934_at	4.68	174	827.5
HPSE	HS chain modification	22288l_at	3.89	13	25.5 (range: 1–445)
GNPDA1	Sugar metabolism	202382_s_at	3.22	169	475
GALK2	Sugar metabolism	205219_s_at	2.81	84	211

comparison MMC versus HMCL	**SAM-defined genes overexpressed in HMCL**	GALE	Sugar metabolism	202528_at	2.78	54	129.5
		GALM	Sugar metabolism	235256_s_at	2.70	65	206.5
		HPSE	HS chain modification	219403_s_at	2.55	57	127.5
		GNPNAT1	Sugar metabolism	225853_at	2.48	242	577
		GFPT1	Sugar metabolism	20272l_s_at	2.42	167	399.5
		SLC35B4	Nucleotide sugar transport	22588l_at	2.42	219	581.5
		B3GAT3	Synthesis of linkage region	203452_at	2.18	88	172.5
		GPI	Sugar metabolism	208308_s_at	2.12	633	1465
		PGM2	Sugar metabolism	225367_at	2.07	482	1140
	SAM-defined genes overexpressed in MMC	SLC35D2	Nucleotide sugar transport	213083_at	27.43	176.5	3
		SLC35D2	Nucleotide sugar transport	213082_s_at	21.87	53.5 (range: 2 – 693)	3
		CSGALNACT1	CS chain polymerization	219049_at	11.73	531	30
		GALK1	Sugar metabolism	229458_s_at	6.44	26.5 (range: 1–181)	3
		CHSY3	CS chain polymerization	242100_at	6.37	214.5	25
		SULF2	HS chain modification	233555_s_at	6.07	205.5	30
		SLC2A10	Glucose transport	221024_s_at	5.95	45.5 (range: 1 – 686)	3
		SULF2	HS chain modification	224724_at	5.88	1034	146
		HS3ST2	HS chain modification	219697_at	4.90	74.5	17
		SLC2A9	Glucose transport	219991_at	4.61	101	23
		EXT1	HS chain polymerization	215206_at	4.54	69.5	15
		CHST11	CS chain modification	226368_at	3.93	270	86
		HS3ST4	HS chain modification	228206_at	3.87	79.5	19
		EXT1	HS chain polymerization	239227_at	3.60	63.5	16
		NDST1	HS chain modification	202607_at	3.52	49.5 (range: 6 – 252)	18
		PAPSS2	PAPS synthesis	203060_s_at	3.36	105.5	14
		EXT1	HS chain polymerization	237310_at	3.35	46.5 (range: 7 – 222)	15
		HS2ST1	HS chain modification	230465_at	3.25	64.5	25
		PAPSS2	PAPS synthesis	203058_s_at	3.21	89	12
		EXT1	HS chain polymerization	232174_at	3.13	110	35
		PAPSS2	PAPS synthesis	203059_s_at	2.92	63.5	19
		SLC35D2	Nucleotide sugar transport	231437_at	2.55	41.5 (range: 4– 121)	15
		SLC26A1	Sulphate anion transport	205058_at	2.36	35 (range: 4–104)	15
		CHST11	CS chain modification	219634_at	2.33	163	84
		SLC2A1	Glucose transport	201250_s_at	2.33	189	83
		SLC2A13///C120RF40	Glucose transport	227176_at	2.29	102.5	54
		PGM3	Sugar metabolism	221788_at	2.18	182.5	60
		CHPF	CS chain polymerization	202175_at	2.10	181.5	102
		SLC35B3	PAPS transport	231003_at	2.05	70	32
		GALNAC4S-6ST	CS chain modification	244874_at	2.01	68	30

A supervised analysis was performed with the SAM software in order to compare the gene expression profile of the MMC and the HMCL and to determine probe sets linked to each type of cell as indicated in Table I.

Table V

Correlation of B4GALT7, CSGALNACT1, HS2ST1 and EXT1 gene expression with prognosis in HM and LR-TT2 series of patients.

Gene Probe set Good/bad gene		B4GALT7 53076_at good	CSGALNACT1 219049_at good	HS2ST1 203285_s_at good	EXT1 239227_at bad
Heidelberg-Montpellier series (n=171)	**EFS (P)**	0.00070	0.02080	0.03650	NS
	OAS (P)	NS	0.03400	NS	0.00940
Little Rock series (n=345)	**EFS (P)**	0.00888	0.00230	0.03050	NS
	OAS (P)	0.00280	0.00160	NS	0.03230

Data are the P values of the 4 probe sets - B4GALT7, CSGALNACT1, HS2ST1 and EXT1 -having a significant EPS and/or OAS in both HM and LR-TT2 series. When different probe sets interrogating the same gene were associated to prognosis, the probe set with the highest variance was selected. "NS" (not significant") indicates that Kaplan-Meier analysis is not significant.

Table VI

B4GALT7, CSGALNACT1, HS2ST1 and EXT1 expression in MMC according to the molecular classification of multiple myeloma

		Patients subgroup								
		All patients	**PR**	**LB**	**MS**	**HY**	**CD1**	**CD2**	**MF**	**MY**
Probe set		**100%**	**8.4%**	**9.0%**	**12.1%**	**18.8 %**	**6.3%**	**11.9%**	**6.0%**	**27.5 %**
53076_at	**frequency of patients with B4GALT7** ^{high}	25%	**3.4 % (P = 10 $^{-5}$)**	9.7%	**7.1 % (P = 5.10 $^{-4}$)**	32.3 %	**45.5 % (P = 2.10 $^{-3}$)**	36.6%	15.0%	31.6 %
219049_at	**frequency of patients with CSGALNACT1** ^{high}	25%	**3.4 % (P = 10 $^{-5}$)**	**45.2 % (P = 2.10 $^{-3}$)**	19.0%	12.3 %	**45.5 % (P = 2.10 $^{-3}$)**	**65.9% (P = 6.10 $^{-9}$)**	30.0%	12.6 %
203085_s_at	**frequency of patients with HS2ST1** ^{high}	25%	**3.4 % (P = 10 $^{-5}$)**	32.3%	**7.1 % (P = 5.10 $^{-4}$)**	27.7 %	31.8%	**78.0 % (P = 6.10 $^{-4}$)**	**0.0 % (P = 9.10 $^{-8}$)**	15.8 %
239227_at	**frequency of patients with EXT1** ^{high}	25%	27.6%	22.6%	**45.2% (P = 2.10 $^{-3}$)**	15.4 %	27.3%	22.0%	15.0%	23.2 %

For each probe set, patients were classified in 4 quartiles according to probe set signal and we examined the frequency of patients belonging to the highest quartile in the 8 molecular subgroups defined by Zhan et al ([Zhan, et al 2006]): PR: proliferation, LB: low bone disease, MS: MMSET, HY: hyperdiploid, CD1 and CD2: CCND1/CCND3, MF: MAF/MAFB and MY: myeloid group. Data are shown in bold and italic when the percentage of patients with B4GALT7 ^{high} , CSGALNACT1 ^{high} , HS2ST1 ^{high} or EXT1 ^{high} MMC in a given group was significantly different from that in all patients with a Chisquare test ($P \leq 0.05$).