NOTCH pathway genes' analysis in multiple myeloma remission state

Dubović, Arijana

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INTERNSHIP REPORT

NOTCH pathway genes' analysis in multiple myeloma remission state

By

Arijana Dubović

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Internship institution and address: Department of Molecular Biology at Faculty of Science, University of Zagreb, Horvatovac 102A, 10000 Zagreb
Mentor/Supervisor: Maja Matulić dr. sc., associate professor at Faculty of Science

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Abstract

Multiple myeloma (MM), a second most common haematological malignancy, is a B cells disorder characterized by rapid clonal plasma cells' proliferation and monoclonal gammopathy, leading to severe bone disruptions, hypercalcemia, and abnormal function of the immune system. The origin and the disease developing mechanism are unknown and there is no cure for MM. Existing therapies are sometimes able to halt the disease, but, in most cases, absence of the disease or the remission state is only temporary and MM relapses. NOTCH signalling pathway can be deregulated in the state of the disease. This signalling pathway consists of transmembrane receptors NOTCH which activate by binding to the neighbouring cells' transmembrane ligands DELTA or JAGGED. Ligand binding causes cleavage of the NOTCH intracellular domain, which acts as a part of transcription complex in the nucleus. MM cells either have overexpression of NOTCH receptors or other mechanisms, which lead to the pathway activation. The aim of the thesis was to see if NOTCH pathway deregulation could be detected in cells obtained from patients with multiple myeloma in remission. Thus, we analysed NOTCH pathway genes' expression in peripheral blood samples of MM patients in remission. Peripheral blood samples and a fraction of B cells were analysed for the expression of NOTCH receptors, JAGGED and DELTA ligands, downstream target *HES1*, as well as differentiation factors from IKAROS family. The results showed no uniform pattern which could connect the NOTCH pathway deregulation to the relapse. For the analysis of small cell number samples from selected B cells we optimized a multiplex, semi nested PCR method. This method is valuable as it enables inspection of multiple different genes simultaneously, on a very small cell number, with increased specificity and sensitivity.

Key words: multiple myeloma, B cell, NOTCH signalling pathway, remission, multiplex semi nested PCR

Résumé

Le myélome multiple (MM), la deuxième hémopathie maligne la plus courante, est une maladie des lymphocytes B caractérisé par une surproduction des plasmocytes clonaux et une gammapathie monoclonale, entraînant de graves perturbations osseuses, une hypercalcémie et un fonctionnement anormal du système immunitaire. L'origine et le mécanisme de développement de la maladie sont inconnus et il n'y a pas de remède qui existe jusqu'à présent pour le MM. Les thérapies existantes pour le MM sont parfois capables retenir la progression de la maladie pour un temps, mais dans la plupart des cas une disparition de la maladie ou l'état de rémission n'est que temporaire et le MM rechute. Les voies de signalisation NOTCH preuve être toucher par le MM et être dérégulée. Cette voie de signalisation est constituée de récepteurs transmembranaires de NOTCH qui s'activent en se liant aux ligands transmembranaires DELTA ou JAGGED des cellules voisines. La liaison du ligand provoque le clivage du domaine intracellulaire NOTCH qui fait partie du complexe de transcription dans le noyau cellulaire. Les cellules affectées par le MM ont une surexpression des récepteurs NOTCH ou d'autres mécanismes qui conduisent à l'activation de la voie. L'objectif de la thèse était de voir si la dérégulation de la voie NOTCH peut être détectée dans des cellules obtenues à partir de patients atteints de myélome multiple en rémission. Ainsi, nous avons analysé l'expression des gènes de la voie NOTCH dans des échantillons de sang périphérique de patients atteints de MM en rémission. Les échantillons de sang périphérique et des fractions de lymphocytes B ont été analysés pour détecter l'expression des récepteurs NOTCH, des ligands JAGGED et DELTA, des cibles de signalisation en aval *HES1* et des modificateurs de chromatine du genre IKAROS. Les résultats n'ont montré aucun modèle uniforme qui pourrait relier la dérégulation de la voie NOTCH à la rechute. Pour l'analyse d'échantillons obtenu à partir d'un petit nombre de cellules B sélectionnées nous avons optimisé pour une méthode de PCR multiplex semi-imbriquée. Cette méthode était très importante car elle permettait l'inspection simultanée de plusieurs gènes différents, sur un très petit nombre de cellules, avec une spécificité et une sensibilité élevée.

Mots clés: myélome multiple, voie NOTCH, rémission, rechute, multiplex PCR semi-imbriquée

Presentation of the Laboratory

This master thesis was done as a part of the ongoing project THYMINNOVA, supported by Croatian Scientific Foundation Grant. Project is lead by Professor Mariastefania Antica from the Institute Ruđer Bošković in Zagreb. Project is done in collaboration with Clinical Hospital Merkur, Zagreb and Faculty of Sciences, University of Zagreb. Institute Ruđer Bošković was founded in year 1950 and today is regarded as Croatia's leading scientific institute in the natural and biomedical sciences, as well as marine and environmental research. Multidisciplinary research is organised in 11 divisions and 4 centres gathering a scientific staff of more than 700 researchers and scientists.

Division of the Molecular Biology on Ruđer Bošković Institute investigates molecular basis and functional roles of fundamental biological structures and processes with the aim of general broadening of knowledge of the underlying principles of life. Research is based on the methods of modern molecular biology, biochemistry, cell biology, genetics, biophotonics and bioinformatics.

Experimental work was done with the collaborators at the Department of Molecular Biology at Faculty of Science. The Department belongs to Division of Biology on Faculty of Science, University of Zagreb. This department was established in 1989 with the goal to improve biological research on the molecular level and to start a study program in molecular biology. Molecular biological research is done on plant and animal cell cultures, in the fields of biomedicine, molecular biology of the cell, plant development and bioinformatics.

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1. Introduction

Blood is an essential body fluid which circulates through vasculatory system providing the organism with oxygen, nutrition, waste removal and defence against pathogens. It's composed of blood cells, dissolved nutrients and various proteins suspended in plasma. Blood cells include red blood cells which carry oxygen, platelets which help blood clotting in injuries and white blood cells which, as a part of humane immune system, fight infections (Junqueira *et al*., 1999). As there is an immense number of various infections and different pathogens, no wonder that white blood cells are the most versatile blood cell group, involved in both, innate and adaptive immunity. B cells take part in the adaptive immunity. They produce antibodies which recognize pathogen's antigen and neutralize pathogen by mechanisms of agglutination, opsonisation, complement activation etc. Antibody consists of one pair of heavy and one pair of light immunoglobulin chains, which are characterized by great variability. Great variability arises from a number of gene recombinations during B cell differentiation (Junqueira *et al*., 1999). All blood cells differentiate from the same hematopoietic stem cells (HSCs) which make haematopoiesis one of the most complex processes in the organism (Murphy *et al*., 2016). From HSCs, cells differentiate into two subgroups: myeloid and lymphoid lineages. B cells belong to the lymphoid lineage. Differentiation begins in bone marrow where HSCs reside together with all supporting cells such as fibroblasts, osteoblasts, endothelial cells, adipocytes (Travlos, 2006). HSCs have the ability to selfrenew, but also to produce multipotent progenitor cells. These cells further develop into common myeloid (CMP) or common lymphoid progenitor (CLP) from which myeloid or lymphoid cells develop, respectively. CLP further produces lymphoblast cells which differentiate into B cells, T cells and natural killer cells. In B cell lineage, differentiation has two stages: primary and secondary. Primary is restricted to the bone marrow where HSC is first transformed to the CLP and CLP gradually into immature B cell. It is characterized by production and functional rearrangements of immunoglobulin (Ig) gene segments coding for heavy (H) and light (L) chains. According to the level of differentiation, three phenotypically different B cells types are observed: pre-B cells, pro-B cells and immature B cells. Immature B cells expressing IgM immunoglobulin are the final step of the primary differentiation and cells are thereby checked for autoreactivity. If cell is autoreactive, it will be either destroyed or sent for secondary L gene rearrangements (Pieper *et al.*,

2013; Eibel *et al.*, 2014). Once immature B cell is ready, it leaves bone marrow and circulates through blood system. When in spleen or lymph node, during organism infection, pathogen antigens are presented to the immature B cells, the secondary differentiation starts. Cells undergo somatic hypermutations and fast proliferation producing either memory B cells or plasma cells able to fight specific pathogen by production of specific antibodies. Before their final release, cells are checked for antigen specificity. Cells with low specificity undergo apoptosis, while cells with strong affinity toward antigen undergo final differentiation changes. Memory B cells circulate through blood system, being on guard for secondary infections arising from the same pathogen. Some plasma cells stay in spleen or lymph node and some return to the bone marrow. From there, plasma cells secrete antibodies which circulate through blood system, also being on guard for secondary infections (Pieper, et al. 2013; Eibel *et al.*, 2014).

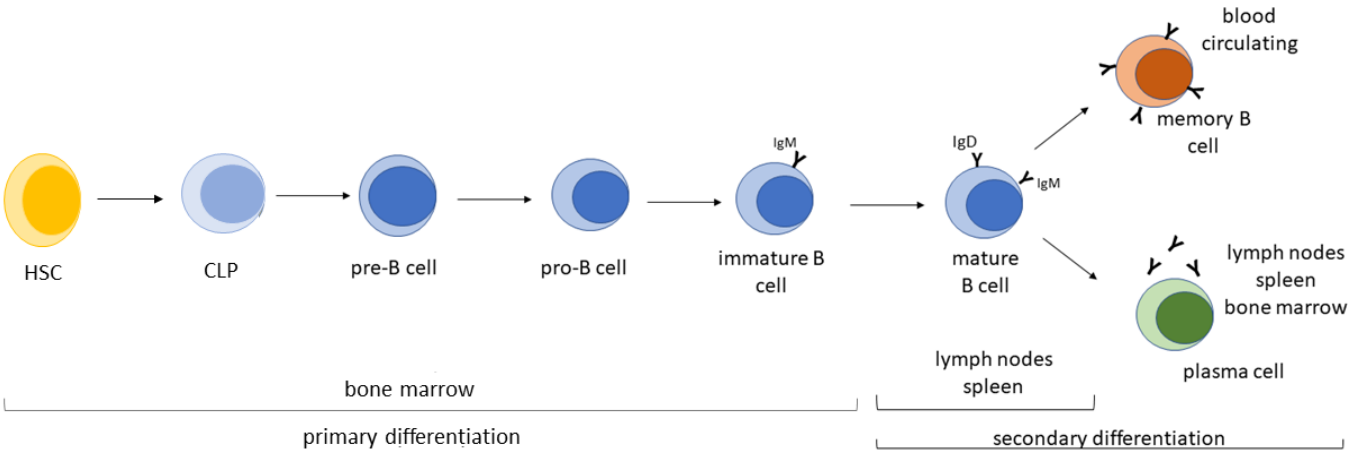

Figure 1 B cell differentiation scheme. B cell differentiation is divided into two stages: primary and secondary differentiation. Primary is restricted to the bone marrow and starts from hematopoietic stem cell (HSC) differentiating into common lymphoid progenitor (CLP). It is characterised by functional immunoglobulin gene segments rearrangements. There are three stages of early developing B cells: pre-B cell, pro-B cell and immature B cell. Secondary differentiation is initiated by antigen presentation to the immature B cell which undergoes intense proliferation and somatic hypermutation producing memory B cell or plasma cell at the end. Secondary differentiation happens in secondary lymphoid organs such as lymph nodes or spleen (Pieper *et al*., 2013; Eibel *et al*., 2014). (created in Microsoft PowerPoint)

As stated earlier, this process is one of the most complex processes in the body and is therefore led by both, intrinsic and extrinsic factors. Intrinsic factors include transcription factors which regulate cell's fate. In the context of B cells, there are 5 master transcription factors which are essential for B cell committed lineage: IKAROS, PAX5, PU.1, E2A and EBF (Glimcher & Singh,

1999). Extrinsic factors include cytokines, growth factors and adhesion molecules produced by supporting microenvironment cells and other hematopoietic cells (Porwit *et al.*, 2011).

This immense system, controlled by so many overlapping processes on a number of differentiation stages, unfortunately gives out many threatening possibilities depending on the deregulated segment. Today, many immunodeficiencies, autoimmune disorders and B cell hematopoietic malignancies are connected to versatile B cells dysfunctions (Pieper *et al.*, 2013). Multiple myeloma (MM) is a second most common haematological malignancy (Colombo et al., 2013). It is characterized by rapid clonal plasma cells' proliferation which takes up most of the bone marrow space leading to severe, painful bone destructions and hypercalcemia. Clonal plasma cells produce identical antibodies unable to fight different pathogens which makes ones' immunity extremely vulnerable (Gao et al., 2016; S. Xu et al., 2018). Exact origin or mechanism of clonal plasma cells' development is unknown. There are some disease hallmarks which enable, more or less specific, targeting clonal plasma cells for therapy. Unfortunately, due to insufficient knowledge of the disease, recovered state or remission is often temporary and MM returns after a certain period of time. Disease return is called relapse (Sabol & Delgado-Calle, 2021). Deregulated NOTCH signalling pathway is one of the hallmarks of the disease and much effort is put to demystify its role in this disease. This survey is concentrated on possible NOTCH dysregulations in remission state, which may, as we believe, be connected to the relapse.

1.2. NOTCH signalling pathway

NOTCH signalling pathway is evolutionary highly conserved and has a role in many different cell functions (Kopan & Ilagan, 2009). It was discovered nearly 100 years ago in *Drosophila melanogaster* by observing a notched wings' phenotype in the *notch* mutated flies (Shellenbarger *et al.*, 1975). Since then, many NOTCH dependent functions, such as cellular differentiation, proliferation, apoptosis, morphogenesis and embryonic development have been discovered (Canalis, 2018; Mirandola et al., 2011). Signalling by NOTCH is initiated by ligand binding to the cell membrane NOTCH receptor. There are 4 NOTCH receptors, single-pass transmembrane molecules. They bind to their ligands, also transmembrane proteins on neighbouring cells. There are two classes of NOTCH ligands, JAGGED and DELTA-LIKE. JAGGED family has two members, JAGGED 1 and 2, and DELTA-LIKE three: DLL-1, DLL-3, and DLL-4. NOTCH

receptor goes through complex process of modifications and cleavages. Final cleavage is performed after binding the ligand when GAMMA-SECRETASE cleaves receptor and releases NOTCH intracellular domain or NICD intracellularly. NOTCH intracellular domain (NCID) travels to the nucleus. Together with DNA binding protein, CSL, a transcriptional complex is assembled on the promoters of target genes and transcription is enabled (Figure 1) (Capaccione & Pine, 2013; Colombo et al., 2015; Gragnani et al., 2021).

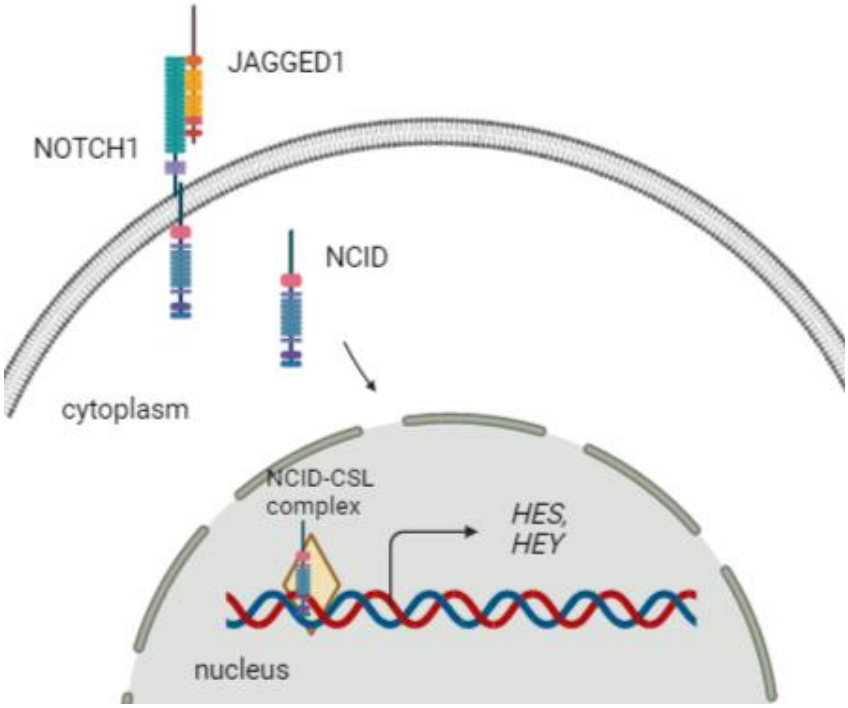

Figure 2 Canonical NOTCH pathway. Neighbouring cell ligand JAGGED1 attaches to NOTCH1 receptor, NOTCH intracellular domain (NCID) is released and travels to the nucleus. In the nucleus, together with DNA binding protein CSL, NCID forms NCID-CSL complex which regulates expression of downstream genes (Colombo et al., 2015). (created in Biorender.com)

Described signal transduction is used in most NOTCH dependent processes and is known as the "canonical pathway". However, NOTCH activity is further regulated at various points in the pathway depending on the intensity, signal duration and cellular context (Kopan & Ilagan, 2009). NOTCH complex controls a large number of genes depending on the cell type. Some of the most common downstream targets are HES and HEY families, repressors of genes included in lineage commitment decisions (Capaccione & Pine, 2013). As NOTCH pathway is involved in many

fundamental processes over a wide range of tissues, aberrant gain or loss of NOTCH signalling components may lead to multiple human disorders. As already mentioned, multiple myeloma is one of the disorders where NOTCH pathway is believed to be a hallmark. Almost all of the NOTCH pathway components, from receptors and ligands to regulated genes, were found to be overexpressed in MM patients (Colombo et al., 2016; Meurette & Mehlen, 2018; Palano et al., 2020). However, exact role of NOTCH pathway in MM cells still needs to be explained.

2.1. Materials and methods

2.1.1. Cell samples

MM patients were diagnosed and treated in accordance with Informed consent and Ethics Review Board approval by Ethics Committee of the University of Zagreb Medical School and the experiments were performed according to the Declaration of Helsinki. Blood samples from MM patients in remission were collected in sterile sacks by leucapheresis. Nine samples were from patients in MM state of remission and two samples from healthy individuals. Cell line 697 – B-cell precursor acute lymphoblastic leukaemia was acquired from Leibniz Institue DSMZ, Germany. Cell line was grown in RPMI medium (Sigma Aldrich, USA) with the addition of 10% foetal bovine serum (Sigma Aldrich) and incubated in humid conditions at 37 ^{o}C with 5% CO2.

2.1.2. Leucapheresis – peripheral blood collecting

Leucapheresis is a blood cells' harvest procedure where patient's or donor's blood cells are collected in a continuous-flow centrifuge, while the rest of the blood is returned to the patient. Collected blood samples include lymphocytes, monocytes, NK cells and additionaly hematopoietic stem cells (HSCs) (Chen et al., 2019). Leucapheresis was performed at Clinical Hospital Merkur in Zagreb.

2.1.3. Flow cytometry

Peripheral blood cells were separated on a density gradient and analysed and sorted by flow cytometry (SONYSH800). Flow cytometer is a sophisticated instrument which sheds laser light on each cell and detects forward and light scattered from the cell. Detectors provide information on physical cell characteristics such as size and granularity. Cells can be labelled with fluorescent dye labelled antibody where detectors collect the information about the change in wavelength caused by fluorescence. According

to their fluorescence, cells can also be physically sorted. Using flow cytometry one can count cells, sort them, analyse cell proliferation, viability and expression of different molecules bound to specific labelled antibodies (Adan et al., 2017; McKinnon, 2018). For this survey, flow cytometry was used for sorting and counting $CD19^+$ and CD34+ blood cells. Flow cytometry was performed at the Laboratory of Cellular and Molecular Immunology at Ruđer Bošković Institute in Zagreb.

2.1.4. In silico primer design

To analyse gene expression, one needs to design effective, specific primers which will amplify target genes. Representative target gene sequence should be found by searching the NCBI *Nucleotide Base* and selecting the gene reference sequence. Sets of primers are then found with the help of NCBI *Primer Designing Tool* program by inserting targeting gene sequence. For seminested PCR three primers are required: forward1 (F1) and reverse (R) for PCR I reaction and forward2 (F2) and reverse for PCRII. In that way, reaction sensitivity and specificity is increased. Desired amplicon in PCR II reaction should be around 100-200 bp long or 200-400 bp for PCR I reaction. To choose the most suitable set of primers one should follow some recommendations. Each primer should be around 20 nt long, GC content should be around 40-60%, 3' end should be G/C nucleotide, repetitive sequences should be avoided and primers should have similar Tm temperatures, around 60 °C. It's necessary for primers to be on different exons. Possible off targets should be checked using NCBI *Primer Blast* program, as well as possible secondary structures, which could decrease efficiency of PCR reaction. Possible secondary structures include binding of two identical primers together (*primer self dimer*), binding of two different primers *forward-reverse* (*primer cross dimer*) and formation of *hairpin structure*. For seminested PCR, compatibility of all primers R and F1, as they are present together in the same reaction tube, should be examined using *ThermoFisher Scientific Multiple Primer Analyzer*.

Table 1. Table of primers used in experiments

GENE	PRIMER SEQUENCE	GENE	PRIMER SEQUENCE
AIOLOS F1	GGCAGCGACATGGAAGATATAC	*HPRT R*	TCCCCTGTTGACTGGTCATT
AIOLOS F2	GTCTGTGCCCGCAGAAAGTG	*DELTEX1 F*	CCCTCGCCACTGCTATCTAC
AIOLOS R	ATCTTCATTGGCTGGGCCTT	*DELTEX1 R*	GGTCTTGTGGTGGATCTCGT
DLL-1 F1	CTCAGTCAGCATCATCGGGG	*IKAROS F*	CACTCCGTTGGTAAACCTC
DLL-1 F2	ACAGCGCCGACAAGAATGG	*IKAROS R*	CCTATCTTGCACAGGTCTTC
DLL-1 R	TCAGATGCTTCTCCACCCCT	*JAGGED1 F1*	CCAGTCTTCACCTTGTGCCT
DLL-4 F2	AGTTCCCCACAGTGACAAGA	*JAGGED1 F2*	AATGGCTACCGGTGTGTCTG
DLL-4 R	GCCTTATACCTCCGTGGCAA	*JAGGED1 R*	ACACCAGACCTTTGAGCAGG
GUSB F1	ACTTCTCTGACAACCGACGC	*JAGGED2 F*	AGCTGGACGTCAACGACTGT
GUSB F2	GTACGAACGGGAGGTGATCC	*JAGGED2 R*	GGAGCAGTTCTTGCCACCAA
GUSB R	CATTCACCCACACGATGGCA	*NOTCH1 F1*	GATGCCAGGACCCCAACC
HES1 F1	AGCACAGAAAGTCATCAAAGCC	*NOTCH1 F2*	CGTGGTGGACCGCAGAG
HES1 F2	GAAAGATAGCTCGCGGCATT	*NOTCH1 R*	TGGCACGATTTCCCTGACCA
HES1 R	GGTACTTCCCCAGCACACTT	*NOTCH2 F*	AAGCAGAGTCCCAGTGCCTA
HES1 F	AACACGACACCGGATAAACC	*NOTCH2 R*	GGCACTCATCCACTTCATAC
HES1 R	CCGCGAGCTATCTTTCTTCA	*PAX5 F*	AACACGACACCGGATAAACC
HPRT F	TGCTCGAGATGTGATGAAGG	*PAX5 R*	CCGCGAGCTATCTTTCTTCA

2.1.5. RNA isolation

Cells, previously pelleted, are lysed in TRI Reagent (Sigma-Aldrich) by repeated pipetting. One mL of the Reagent is sufficient to lyse 5-10*10^6 cells. By allowing samples to stand for 5 minutes at room temperature, complete dissociation of nucleoprotein complexes is ensured. Afterwards, for phase separation, 0.2 mL of chloroform per mL of TRI Reagent is used. Sample is then shaken vigorously for 15 s and allowed to stand 2-15 min at room temperature. Samples are centrifuged at 12 000*g for 15 min at 2-8 °C. After centrifugation, mixture is separated into 3 phases: red organic phase (proteins), interphase (DNA) and colourless upper phase (RNA). RNA phase is transferred to a clean tube and 0.5 mL of 2-propanol per mL of TRI Reagent is added. Sample is allowed to stand for 5-10 min and then centrifuged at 12 000*g for 15 min at 2-8 °C to precipitate RNA. Supernatant is removed and RNA pellet is washed by adding 1 mL 75% ethanol per 1 mL TRI Reagent. Sample is centrifuged at 12000*g for 5 min at 2-8 °C. RNA pellet is then air dried for 5-10 min. Pellet shouldn't be dried completely as it would become less soluble. To dissolve RNA pellet appropriate volume of nuclease-free water is added, concentration is measured spectroscopically using NanoVue and quality by agarose gel electrophoresis. Samples are stored at -70 °C.

2.1.6. Reverse transcription

By reverse transcription, isolated mRNA is converted to cDNA which later enables expressed genes amplification and detection by PCR. The reaction mixture contains 100 ng – 2 µg RNA, 1 µL dNTP (10 µM, Sigma-Aldrich), 125 ng random hexamers (Carl Roth GmbH, Germany) and nuclease free water up to the volume of 20 µL. The mixture is heated for 5 min at 65 °C and cooled down on 4 °C. After cooling, 4 µL 5x buffer MMLV (250 mM Tris-HCl (pH 8.3), 375 mM KCl, 15 mM $MgCl_2$ (Applied Biosystems, USA), 2 µL DTT (0.1 M, Applied Biosystems), and 1 µL RNase inhibitor (40 U, Applied Biosystems) are added. Mixture is incubated for 10 min at 25 °C. Finally, 1 µL of MMLV Superscript reverse transcriptase (200 U⁄µl, Invitrogen, USA) is added. Final reaction volume is 20 µL and reaction conditions are: 50 min at 42 °C and 15 min 72 °C. After RT, samples can be stored on -20°C or -80°C.

2.1.7. Polymerase chain reaction

To check whether potential genes are expressed in cell samples, polymerase chain reaction (PCR) with specific targeting primers F2 or F1 and R is done. Reaction mixture (15 µL) is prepared from 7.5 µL GreenDream mix (composition: DreamTaq DNA Polymerase, 2X DreamTaq Green buffer, dNTPs and 4

mM $MgCl_2$) (Thermo Fisher Scientific, USA), 0.6 µL forward primer (5 mM, Metabion International AG, Germany), 0.6 µL reverse primer (5 mM, Metabion International AG), 1 µL cDNA template and 5.3 µL water. Reaction conditions are as follows: 94 °C 5 min, 40 cycles (94 °C 30 s, 56 °C 30 s, 72 °C 30 s), 72 °C 7 min.

2.1.8. Gel electrophoresis

Amplified DNA from PCR reaction is analysed by horizontal agarose gel electrophoresis. Gel is prepared as 1.3% or 1.5 % agarose in 1x TAE buffer (50x TAE buffer composition: EDTA disodium salt 50 mM, Tris-HCl 2 M, acetic acid 1 M) with the addition of ethidium bromide solution (10 µL/mL). Electrophoresis is carried out at 80 V for approximately 20 minutes and the samples are visualised under UV light (Sambrook *et al*., 2006).

2.1.9. Densitometry

Images acquired by photographing agarose gels after electrophoresis are analysed by densitometry to enable result comparison. Densitometry is done according to ImageJ Gel Analysis protocol using ImageJ program (Schneider et al., 2012).

2.1.10. Multiplex seminested PCR

Multiplex seminested polymerase chain reaction (multiplex) is a novel method used when one wants to amplify multiple genes from a small number of cells with increased sensitivity and specificity compared to regular PCR. Multiplex reaction is performed in three separate stages: reverse transcription (RT), polymerase chain reaction I (PCR I) and polymerase chain reaction II (PCR II). Reverse transcription is done directly on lysed cell samples, without previous RNA isolation. Number of cells should be 500-2500. RT reaction mixture is prepared by heating 5 µL of cell sample with 1 µL RNA inhibitor (40 U, Invitrogen) for 2 min at 65 °C , and then cooling down on 4 °C. 0.2 µL of each 10 µM reverse primer, which are previously tested to be compatible using *ThermoFisher Scientific Multiple Primer Analyzer* program, 1 µL dNTP (10 µM, Sigma-Aldrich,) and nuclease-free water up to 13 µL are added. Mixture is then heated for 5 min at 65 °C. Afterwards, 4 µL 5x RT buffer (composition: 250 mM Tris-HCl, 375 mM KCl, 15 mM $MgCl_2$, pH=8.3, Invitrogen) and 2 µL of DTT (0.1 M , Invitrogen) are added to the mixture which is then heated for 2 min at 42 °C. Finally, 1 µL MMLV reverse transcriptase (200 U/µL, Invitrogen) is added and reaction is set up for 50 min 42 °C and 15 min 70 °C. The second stage in multiplex is PCR I. It's performed with F1 and R primers, from all target genes, present in a single tube. During PCR I, 200-400 bp long

amplicons are generated. Reaction mixture (25 µL) is prepared by mixing 2.5 µL 10x buffer (composition: 500 mM KCl, 100 mM Tris-HCl, pH 8.3; 15 mM $MgCl_2$ and 0.01% (w/v) gelatine, Applied Biosystems), 2 µL dNTP (2.5 µM, Sigma-Aldrich), 2 µL $MgCl_2$ (25 mM, Applied Biosystems), 2.5 µL GC enhancer (Applied Biosystems), primer mix (created by adding 0.375 µL 1 µM F1 and 0.375 µL 1 µM R, for every primer pair), 2.5 µL cDNA, water and 0.125 µL AmpliTaq (5 U/µL, Applied Biosystems). Reaction conditions are as follows: 95 °C 3 min, 15 cycles (94 °C 30 s, 56 °C 30 s, 72 °C 30 s), 72 °C 5 min. The third stage of multiplex is PCR II where PCR I generated amplicons are further amplified using F2 and R primers, each pair in separate tube. PCR II generates 100-250 bp long amplicons. As mentioned before, by performing two separate PCR reactions (seminested PCR) sensitivity and specificity are increased. Increased sensitivity arises from the high total cycle number – PCR I and PCR II, while increased specificity arises from the binding of two separate primer sets to the same target sequence – F1 and R and, in the second reaction, F2 and R (Green & Sambrook, 2019). Reaction mixture for PCR II (15 µL) is prepared by mixing 7.5 µL GreenDream mix (Thermo Fisher Scientific), 0.3 µL of primers R and F2, 10 µM, 1 µL PCR I reaction and 5.3 µL water. Reaction conditions are as follows: 94 °C 5 min, 42 cycles (94 °C 30 s, 58 °C 30 s, 72 °C 20 s), 72 °C 7 min. Amplified DNA is analysed by 1.5% agarose gel electrophoresis.

3. Results

Aim was to analyse NOTCH pathway genes' expression of white blood cells in multiple myeloma remission. Thus, blood samples of multiple myeloma patients in remission were separated on a density gradient and analysed. All samples were collected by leucapheresis.

3.1 NOTCH pathway genes' expression analysis in white blood cells from multiple myeloma patients in state of remission

NOTCH pathway genes' expression was analysed in white blood cells from three multiple myeloma patients in the state of remission and from one healthy individual. RNA was isolated from cells and reversely transcribed to cDNA. cDNA was used for gene expression analysis by PCR, using specific gene targeting primers. Ten genes linked to the NOTCH pathway were analysed: receptors *NOTCH1* and *NOTCH2*, ligands *JAGGED1* and *2*, *DLL-1* and *4*, genes under NOTCH control *HES1* and *DELTEX1* and B cells transcription factors, possibly interfering with NOTCH signalling: *AIOLOS* and *IKAROS*. Being important for B cell differentiation, transcription factor *PAX5* was also included. Figure 3 presents the results, amplified DNA run on agarose gel.

Images from electrophoresis were analysed by densitometry using ImageJ program (Supplement 1).

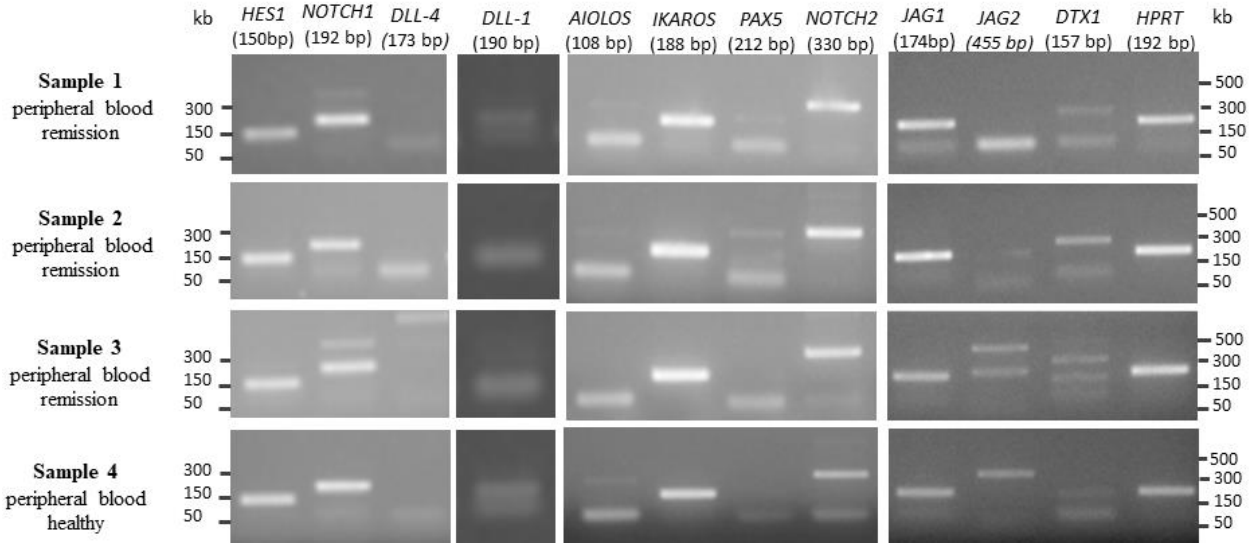

Figure 3 NOTCH pathway genes' expression in white blood cells from patients in multiple myeloma remission. White blood cells were extracted from peripheral blood by separation on a density gradient. RNA was isolated from blood cells of patients in MM remission and from one healthy individual, reversely transcribed to cDNA and amplified by PCR, using specific gene targeting primers. *JAG1:JAGGED1, JAG2:JAGGED2, DTX1:DELTEX1.*

Results in Figure 3 show that all blood samples from MM patients in remission expressed control gene *HPRT*, confirming positive PCR. Expression of *HES1*, *NOTCH1*, *AIOLOS*, *IKAROS*, *NOTCH2* and *JAGGED1* is present in all four blood samples. Expression of *JAGGED2* and *DELTEX1* is present only in samples 3 and 4. *DELTEX1* is weakly expressed in all the samples. Weak expression of *DLL-1* is observed in samples 1 and 4 and weak *PAX5* expression in sample 2. *DLL-4* expression is not detected in any of 4 blood samples (Figure 3).

3.2 NOTCH pathway genes' expression in sorted samples of multiple myeloma patients in state of remission

Seven white blood samples from multiple myeloma patients in state of remission and one from healthy individual were collected for NOTCH pathway genes' expression analysis of sorted

samples. Blood cells, previously incubated with labelled antibodies targeting $CD19^+$ and $CD34^+$ antigens, were sorted according to the fluorescence intensity by flow cytometry. $CD19^+$ is characteristic for B cells, and $CD34^+$ for hematopoietic stem cells. Six samples of MM patients in remission and one sample from healthy individual contained B cells, while one sample contained HSC cells. Sorted samples contained 500-2500 cells and were snap-frozen in small aliquots. Prepared aliquots were analysed by multiplex, seminested PCR. Cells were lysed and mRNA was reversely transcribed to cDNA, without previous RNA isolation, using a mixture of specific primers targeting *AIOLOS, DLL-1, HES1, JAGGED1, NOTCH1* and *GUSB*. cDNA is then amplified using seminested PCR in reactions PCR I and PCR II. Small aliquot of B-cell precursor acute lymphoblastic leukaemia cell line 697 was also reversely transcribed and amplified using the same method, as a positive control. Figure 4 presents the results from control 697 cells. Figure 5 presents the results of cDNA amplification of small aliquots of B cells obtained from blood samples, run on agarose gel. Images from electrophoresis were analysed by densitometry using ImageJ program (Supplement 1).

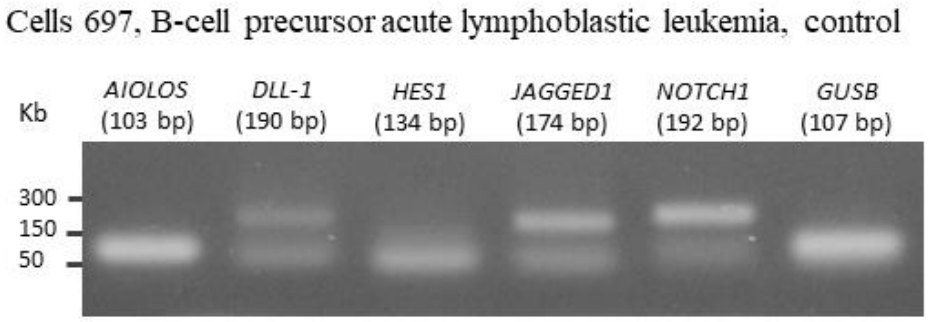

Figure 4 NOTCH pathway genes' expression in small cell sample of 697 cell line, cells of B-cell precursor acute lymphoblastic leukaemia. Cells were lysed and mRNA was directly transcribed to cDNA. Gene expression was analysed by multiplex, seminested PCR. Sample contained around 1700 cells. cDNA was further amplified with specific gene targeting primers. Amplicons were separated on agarose gel.

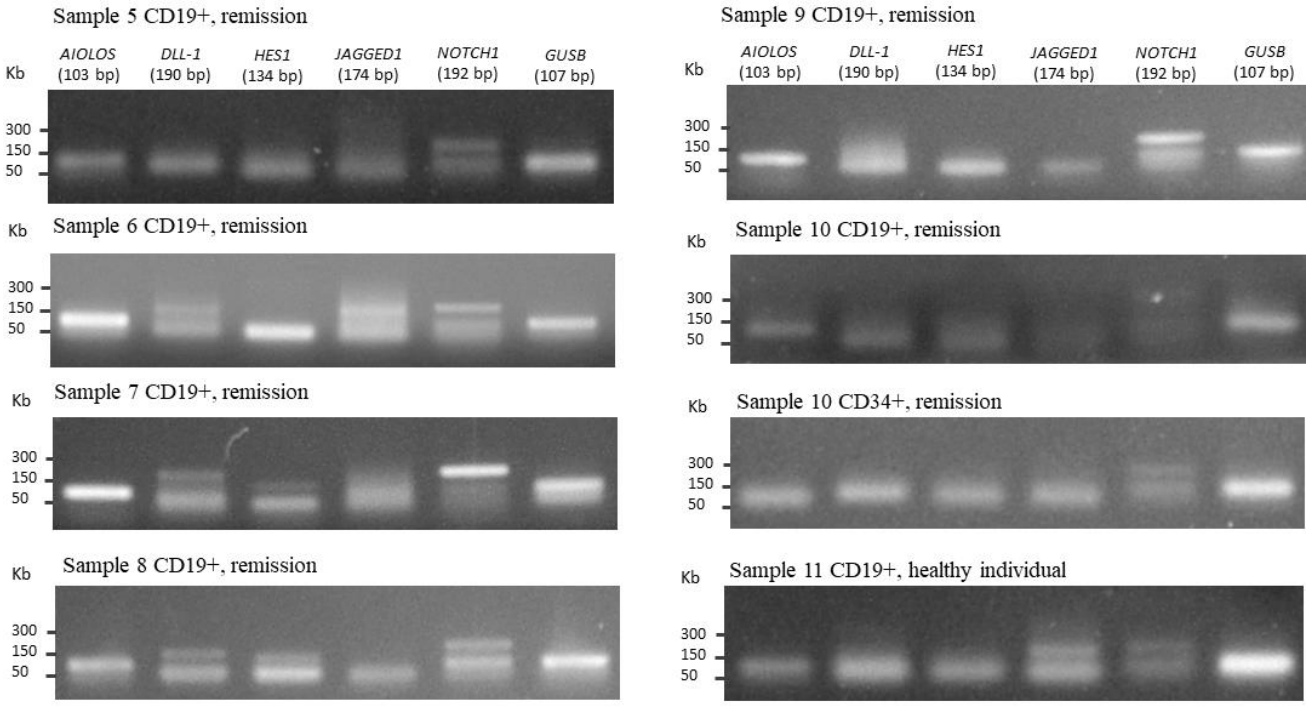

Figure 5 NOTCH pathway genes' expression in small cell samples of multiple myeloma patients in remission. White blood cells were extracted from peripheral blood samples on a density gradient. Blood cells were separated by flow cytometry depending on the presence of CD19 or CD34 antigens. 6 samples contained CD19+ and one CD34+ cells from multiple myeloma patients in remission and one contained CD19+ cells from a healthy individual. Cells were lysed and mRNA was directly transcribed to cDNA. Gene expression was analysed by multiplex, seminested PCR. Samples contained 500-2500 cells. cDNA was further amplified with specific gene targeting primers. Amplicons were run on agarose gel.

Results in Figure 4 show that cells 697 expressed *AIOLOS, DLL-1, JAGGED1, NOTCH1* and *GUSB*, but not *HES1*. Results in Figure 5 show that all small cell samples from MM patients in remission expressed control gene, *GUSB*, confirming positive reaction of RT and PCR. *NOTCH1* is present in all samples, but noticeably weaker in samples 5, 10 $CD34^+$, 11 and 10 $CD19^+$. *AIOLOS* is also expressed in all cell samples, but in samples 5, 10 and 11 it is less expressed than in samples 6, 7, 8 and 9. Cell samples 6, 7 and 8 expressed *DLL-1*, while cell samples 5, 9, 10 and 11 didn't. *JAGGED1* was expressed only in cell samples 6 and 11 and *HES* only in samples 7 and 8.

4. Discussion

NOTCH signalling pathway regulates cell differentiation, apoptosis and proliferation, as well as many other different processes such as angiogenesis, haematopoiesis and development of different types of tissues (Mirandola *et al.*, 2011; Canalis, 2018). All these processes are dependent on the cellular context or cell type and synchronized with other signalling pathways. Deregulation of NOTCH signalling can lead to different diseases and is linked to development of several types of tumours. There is a growing number of evidences indicating that NOTCH pathway plays a critical role in MM progression. Many researches showed overexpression of almost all NOTCH pathway linked genes. Jundt (Jundt *et al.*, 2004) demonstrated overexpression of receptors *NOTCH1* and *2* and ligand *JAGGED1* in cultured and primary MM cells. Houde (Houde *et al.*, 2004) showed overexpression of *JAGGED2* in malignant plasma cells from MM patients and cell lines. Škrtić (Škrtić *et al.*, 2010) showed by immunohistochemical analysis overexpression of *NOTCH1* and *2*, as well as *JAGGED1* and *2* in tumour plasma cells. Furthermore, Colombo (Colombo *et al.*, 2016) observed that high expression of *JAGGED1, NOTCH1* and downstream NOTCH targets *HES5* and *HES6* correlated with malignant progression and high-risk disease. It was found that chromosomes duplicated in one type of multiple myeloma often code for molecules participating in the NOTCH pathway (Zhan et al., 2006). Activation of this pathway in stromal cells leads to release of cytokines and growth factors which support myeloma cell proliferation, drug resistance and angiogenesis (Colombo et al., 2016; Davies et al., 2001; Liu et al., 2014). Xu (Xu *et al.*, 2012) showed how DLL-1/NOTCH interaction promotes MM cell proliferation, predominantly in $CD138^+$ MM cells, by reducing the expression of p21 and p27, thus accelerating MM cell cycling. Mirandola (Mirandola *et al.*, 2013) showed how activity of NOTCH pathway can increase plasma cell migration, growth and survival through expression of CXCR4/SDF1alpha chemokine and by increasing the bone marrow levels of interleukin-6 (IL-6) (Colombo et al., 2016). Among the most prominent effects of MM is its influence on the osteoclasts and osteoblasts, leading to bone resorption. This is a consequence of the release of the osteoclastogenic soluble factor RANKL, which can be released by bone marrow stromal cells under the NOTCH signalling (Colombo et al., 2014).

There are some available chemotherapeutics, such as thalidomide, lenalidomide, bortezomib, which are sometimes able to halt the disease (Gao et al., 2016). However, absence of the disease

or the remission state is often temporary. MM relapses and there is no clear explanation for this reoccurrence. For this reason, we inspected the expression of NOTCH pathway linked genes in MM patients in the state of remission. We examined NOTCH pathway expression in the white blood cells samples and in B cells fraction only. Our aim was to check whether there is a possibility of a NOTCH pathway characteristic expression pattern in the state of remission and to see whether these genes still retain their activity. Results showed no uniform pattern, characteristic for the MM remission state and different from healthy individuals. However, our survey was only small scaled, so further more extensive surveys are needed to confirm our findings. Expression of all inspected genes was generally stronger in white blood cells samples than in B cells only. We believe difference is due to NOTCH pathway activity in many different processes across many different cell types. Compared to B cells fractions only, white blood cells samples contain many different cell types such as T cells, NK cells, monocytes etc. In white blood cells samples, when compared with control sample, we detected expression of *NOTCH1* and *2* receptors and *HES1* indicating activity of the pathway, as well as *JAGGED1*. *JAGGED2* was expressed in 1/3 samples, as well as *AIOLOS*, both present in control sample. *DLL1*, together with *DELTEX1* and *PAX5*, were expressed in 1 of three MM samples, and *IKAROS* was present in all samples. Some of these genes were investigated in a B cell fraction of MM patients in remission. *AIOLOS* and *NOTCH1* were present in all samples, as well as in a control healthy sample. *HES1*, indicating activity of NOTCH pathway, was not expressed in control, and was only expressed in two of six samples. *JAGGED1* was detected in control sample, but only in one MM sample. *DLL-1*, not expressed in control, was present in 3/7 of MM samples. One blood sample was fractionated on both, CD 19+ B cells and CD34+ progenitor cells. The expression pattern of NOTCH molecules was not the same in these two cell types. Three samples were previously analysed by immunohistochemistry (data not shown, personal observation Škrtić). Sample 10, which showed low expression of the most NOTCH signalling molecules, also had low expression of NOTCH1 and JAGGED1, and no expression of DLL-1 when analysed immunocytochemically. Data from samples 6 and 9 obtained by immunostaining also corresponded to expression found in multiplex PCR for NOTCH1 expression. Analysis of other proteins showed that JAGGED1 was expressed in both samples, as well DLL1, although at low level. We detected *DLL1, NOTCH1* and *JAGGED1* in sample 6, while in sample 9 only *NOTCH1*.

In our experiments we used a novel PCR from a small cell number or multiplex seminested PCR method introduced by Peixoto and collaborators (Peixoto *et al.*, 2004.) We believe that this method proves great for medicinal diagnostic as it is fast and simple, requiring no special equipment and highly trained staff. Method enables to inspect multiple disease characteristic gene expressions simultaneously. Additionally, only small sample is needed, so patient biopsy could be less invasive.

5. Conclusion

Due to increasing evidences of NOTCH signalling pathway importance in MM disease, as well as frequent disease reoccurrences after various treatments, we wanted to inspect whether there is a possible connection between NOTCH signalling pathway dysregulation and MM reoccurrence. Accordingly, we analysed NOTCH pathway genes' expression in nine blood samples from MM patients in state of remission and compared it to expression in samples of two healthy individuals. Our results indicate that there is no clear NOTCH pathway dysregulation pattern connected to MM in the state of remission. However, we tested small number of samples, so more extensive surveys are necessary. In accordance with NOTCH pathway involvement in many fundamental processes across different cell types, expression of NOTCH pathway linked genes was higher in white blood cells samples compared to B cell fractions only. Furthermore, we optimized small cells' sample PCR for analysis of NOTCH pathway connected genes expression.

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7. Supplement

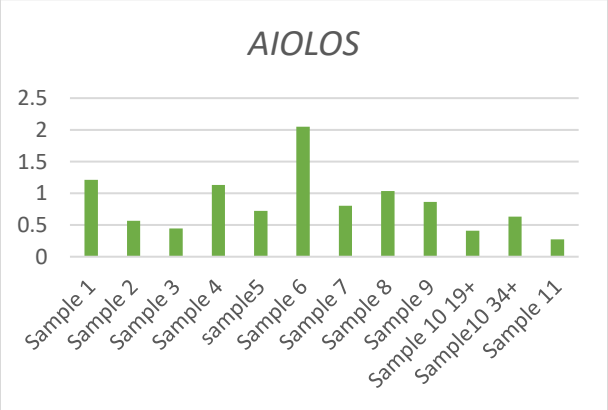

Figure *1* *AIOLOS* expression in blood cell samples from patients in MM remission, obtained by PCR. Samples 1-4 included patients in MM remission and one healthy individual. White blood cells were separated from peripheral blood on a density gradient. RNA was isolated from white blood cells, reversely transcribed to cDNA and amplified by PCR, using specific gene targeting primers For samples 5-11, white blood cells were separated from peripheral blood on a density gradient. Blood cells were separated by flow cytometry depending on the presence of CD19 or CD34 antigens. 6 samples contained CD19+ and one, sample 10, CD34+ cells from multiple myeloma patients in remission, one contained CD19+ cells from a healthy individual. Cells were lysed and mRNA was directly transcribed to cDNA. Gene expression was analysed by multiplex, seminested PCR. Samples contained 500-2500 cells. cDNA was further amplified with specific gene targeting primers. Amplicons were separated on an agarose gel. Gel eletrophoresis image

was analyzed by densitometry, using ImageJ program. Band intensity was normalized according to control *HPRT* band intensity for samples 1-4 and according to control *GUSB* band intensity for samples 5-11.

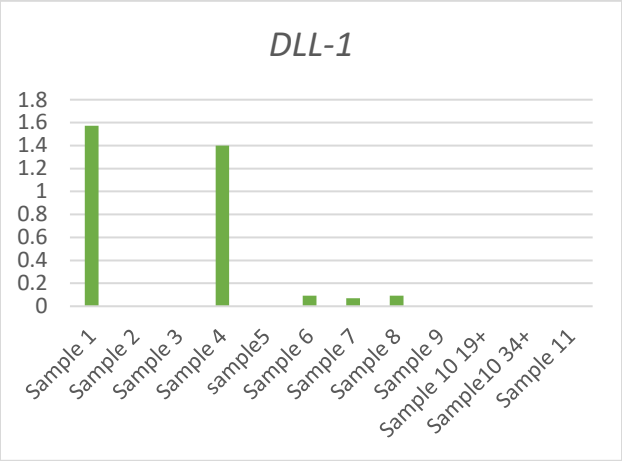

Figure 2 *DLL-1* expression in blood cell samples from patients in MM remission, obtained by PCR. Samples 1-4 included patients in MM remission and one healthy individual. White blood cells were separated from peripheral blood on a density gradient. RNA was isolated from white blood cells, reversely transcribed to cDNA and amplified by PCR, using specific gene targeting primers For samples 5-11, white blood cells were separated from peripheral blood on a density gradient. Blood cells were separated by flow cytometry depending on the presence of CD19 or CD34 antigens. 6 samples contained CD19+ and one, sample 10, CD34+ cells from multiple myeloma patients in remission, one contained CD19+ cells from a healthy individual. Cells were lysed and mRNA was directly transcribed to cDNA. Gene expression was analysed by multiplex, seminested PCR. Samples contained 500-2500 cells. cDNA was further amplified with specific gene targeting primers. Amplicons were separated on an agarose gel. Gel eletrophoresis image was analyzed by densitometry, using ImageJ program. Band intensity was normalized according to control *HPRT* band intensity for samples 1-4 and according to control *GUSB* band intensity for samples 5-11.

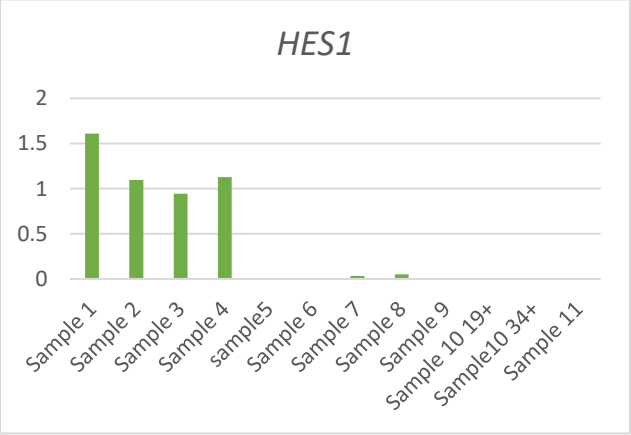

Figure 3 *HES1 e*xpression in blood cell samples from patients in MM remission, obtained by PCR. Samples 1-4 included patients in MM remission and one healthy individual. White blood cells were separated from peripheral blood on a density gradient. RNA was isolated from white blood cells, reversely transcribed to cDNA and amplified by PCR, using specific gene targeting primers For samples 5-11, white blood cells were separated from peripheral blood on a density gradient. Blood cells were separated by flow cytometry depending on the presence of CD19 or CD34 antigens. 6 samples contained CD19+ and one, sample 10, CD34+ cells from multiple myeloma patients in remission, one contained CD19+ cells from a healthy

individual. Cells were lysed and mRNA was directly transcribed to cDNA. Gene expression was analysed by multiplex, seminested PCR. Samples contained 500-2500 cells. cDNA was further amplified with specific gene targeting primers. Amplicons were separated on an agarose gel. Gel eletrophoresis image was analyzed by densitometry, using ImageJ program. Band intensity was normalized according to control *HPRT* band intensity for samples 1-4 and according to control *GUSB* band intensity for samples 5-11.

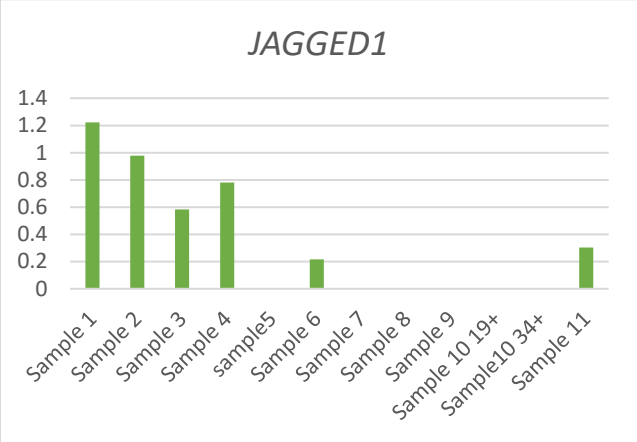

Figure 4 *JAGEED1* expression in blood cell samples from patients in MM remission, obtained by PCR. Samples 1-4 included patients in MM remission and one healthy individual. White blood cells were separated from peripheral blood on a density gradient. RNA was isolated from white blood cells, reversely transcribed to cDNA and amplified by PCR, using specific gene targeting primers For samples 5-11, white blood cells were separated from peripheral blood on a density gradient. Blood cells were separated by flow cytometry depending on the presence of CD19 or CD34 antigens. 6 samples contained CD19+ and one, sample 10, CD34+ cells from multiple myeloma patients in remission, one contained CD19+ cells from a healthy individual. Cells were lysed and mRNA was directly transcribed to cDNA. Gene expression was analysed by multiplex, seminested PCR. Samples contained 500-2500 cells. cDNA was further amplified with specific gene targeting primers. Amplicons were separated on an agarose gel. Gel eletrophoresis image was analyzed by densitometry, using ImageJ program. Band intensity was normalized according to control *HPRT* band intensity for samples 1-4 and according to control *GUSB* band intensity for samples 5-11.

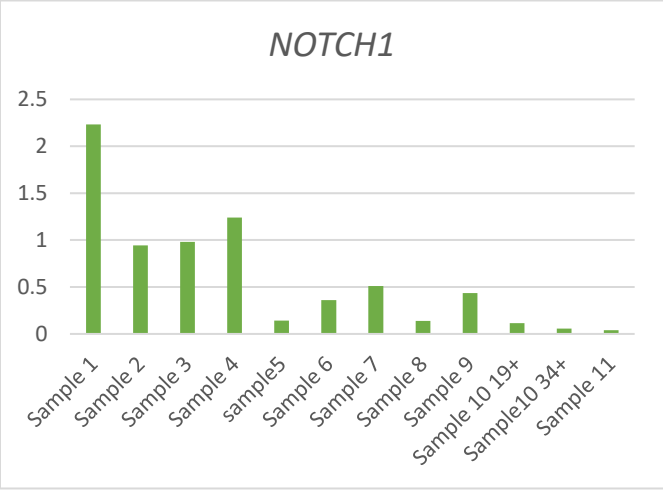

Figure 5 *NOTCH1* expression in blood cell samples from patients in MM remission, obtained by PCR. Samples 1-4 included patients in MM remission and one healthy individual. White blood cells were separated from peripheral blood on a density gradient. RNA was isolated from white blood cells, reversely transcribed to cDNA and amplified by PCR, using specific gene targeting primers For samples 5-11, white blood cells were separated from peripheral blood on a density gradient. Blood cells were separated by flow cytometry depending on the presence of CD19 or CD34 antigens. 6 samples contained CD19+ and one, sample 10, CD34+ cells from multiple myeloma patients in remission, one contained CD19+ cells from a healthy individual. Cells were lysed and mRNA was directly transcribed to cDNA. Gene expression was analysed by multiplex, seminested PCR. Samples contained 500-2500 cells. cDNA was further amplified with specific gene targeting primers. Amplicons were separated on an agarose gel. Gel eletrophoresis image was analyzed by densitometry, using ImageJ program. Band intensity was normalized according to control *HPRT* band intensity for samples 1-4 and according to control *GUSB* band intensity for samples 5-11.

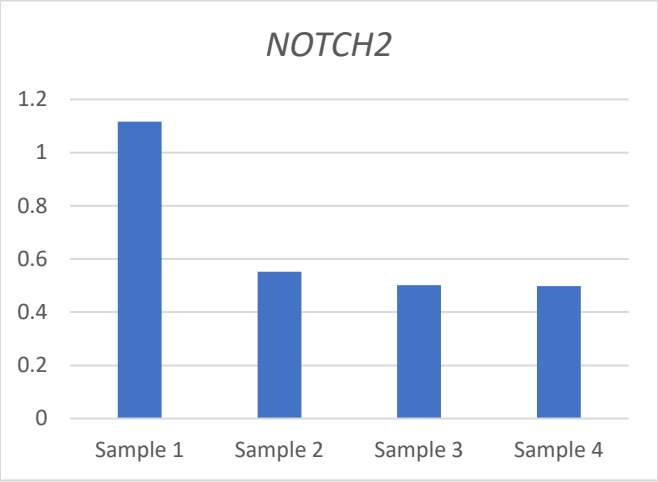

Figure 6 *NOTCH2* expression in blood cell samples from patients in MM remission, obtained by PCR. On a density gradient, white blood cells were separated from peripheral blood of patients in MM remission, samples 1-3 and from one healthy individual, sample 4. RNA was isolated from white blood cells, reversely transcribed to cDNA and amplified by PCR, using specific gene targeting primers. Amplicons were separated on an agarose gel. Gel eletrophoresis image was analyzed by densitometry, using ImageJ program. Band intensity was normalized according to control *HPRT* band intensity.

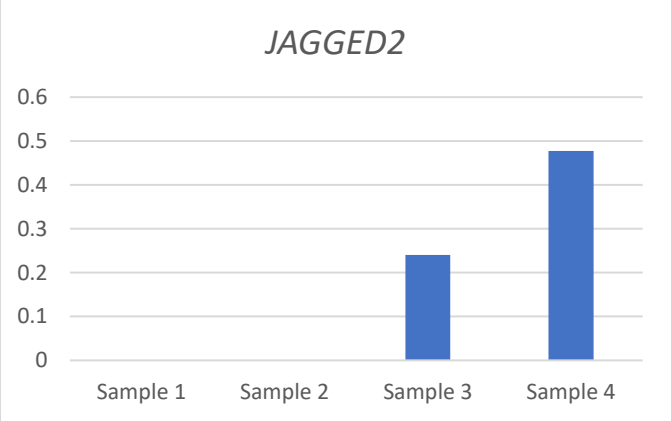

Figure 7 *JAGGED2* expression in blood cell samples from patients in MM remission, obtained by PCR. On a density gradient, white blood cells were separated from peripheral blood of patients in MM remission, samples 1-3 and from one healthy individual, sample 4. RNA was isolated from white blood cells, reversely transcribed to cDNA and amplified by PCR, using specific gene targeting primers. Amplicons were separated on an agarose gel. Gel eletrophoresis image was analyzed by densitometry, using ImageJ program. Band intensity was normalized according to control *HPRT* band intensity.

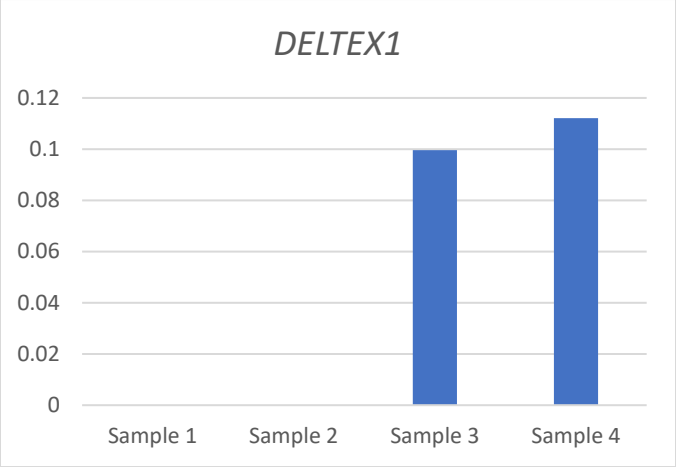

Figure 8 *DELTEX1* expression in blood cell samples from patients in MM remission, obtained by PCR. On a density, white blood cells were separated from peripheral blood of patients in MM remission, samples 1-3 and from one healthy individual, sample 4. RNA was isolated from white blood cells, reversely transcribed to cDNA and amplified by PCR, using specific gene targeting primers. Amplicons were separated on an agarose gel. Gel eletrophoresis image was analyzed by densitometry, using ImageJ program. Band intensity was normalized according to control *HPRT* band intensity.

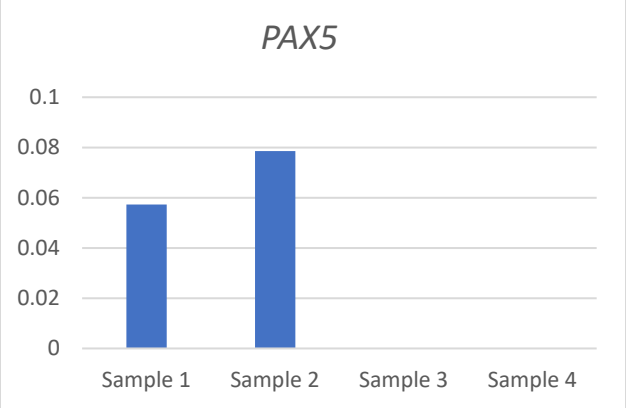

Figure 9 *PAX5* expression in blood cell samples from patients in MM remission, obtained by PCR. On a density gradient, white blood cells were separated from peripheral blood of patients in MM remission, samples 1-3 and from one healthy individual, sample 4. RNA was isolated from white blood cells, reversely transcribed to cDNA and amplified by PCR, using specific gene targeting primers. Amplicons were separated on an agarose gel. Gel eletrophoresis image was analyzed by densitometry, using ImageJ program. Band intensity was normalized according to control *HPRT* band intensity.

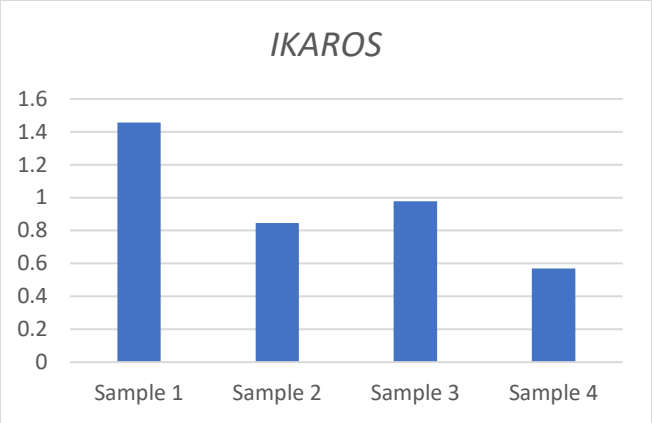

Figure 10 *IKAROS* expression in blood cell samples from patients in MM remission, obtained by PCR. On a density gradient, white blood cells were separated from peripheral blood of patients in MM remission, samples 1-3 and from one healthy individual, sample 4. RNA was isolated from white blood cells, reversely transcribed to cDNA and amplified by PCR, using specific gene targeting primers. Amplicons were separated on an agarose gel. Gel eletrophoresis image was analyzed by densitometry, using ImageJ program. Band intensity was normalized according to control *HPRT* band intensity.