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Expression of RAN, ZHX-2, and CHC1L genes in multiple myeloma patients and in myeloma cell lines treated with HDAC and Dnmts inhibitors

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Real time PCR is a powerful tool for studying the expression of genes involved in the pathophysiology of human diseases. Recent studies of the RAN (6p21), ZHX-2 (8q24.3), CHC1L (13q14.3) loci highlight the importance of these genes in multiple myeloma (MM) prognosis and therapeutic applications. Here, we described a detailed Real-Time PCR method for the detection of RAN, ZHX-2, and CHC1L expression, which could be applied in clinical situations. The expression profiles of these genes were studied in peripheral blood lymphocytes of healthy individuals, patients suffering from MM, and in the myeloma cell line, MOLP-8. Low expression levels of RAN, ZHX-2, and CHC1L were observed in myeloma patients, compared with peripheral blood lymphocytes and MOLP-8 cells. An inhibitor of histone deacetylases (TSA) had the ability to decrease expression of CHC1L and ZHX-2 in MOLP-8 cells, while expression of RAN was relatively stable in peripheral blood lymphocytes, control MOLP-8, and TSA- or 5-azacytidine treated MOLP-8 cells. In myeloma patients, we observed significant decreases in the expression of selected genes, but it was patient-specific. Our experiments illustrate new methodological approaches and troubleshooting for conducting gene expression studies in clinical laboratories.

Key words: Multiple myeloma, RAN, ZHX-2, and CHC1L gene expression, qRT-PCR

Multiple myeloma (MM) is a malignant plasma-cell tumor, which usually progresses through a stepwise process and has a median survival of 2 to 3 years. It is a slow proliferating tumor that may or may not originate from a common benign plasma cell tumor, called monoclonal gammopathy of uncertain significance (MGUS) [1]. MM is associated with a constellation of disease manifestations, including osteolytic bone lesions, anemia, immunosuppression, and end-organ damage. The karyotypes of multiple myeloma cells are complex and more similar to those found in epithelial tumors and the blast phase of chronic myelogenous leukaemia than those of other haematopoietic tumors [2]. Karyotypic instability is seen at the earliest stages of the disease and increases with disease progression. Patients with MM have abnormal karyotypes, usually with highly complex changes, involving both structural and numerical abnormalities [3, 4].

Gene expression analysis has become a routine method for the detailed study of gene activity in normal cell development and disease processes. These studies can facilitate the management of MM, not only by identifying prognostic subgroups, but also by defining molecular pathways that are associated with these subgroups and that are possible targets for future therapies [5]. MM pathogenesis is accompanied by changes in karyotype and altered expression patterns of some genes. For example, the c-myc gene becomes dysregulated by secondary translocations in myeloma tumor cells, in which c-myc translocates to the immunoglobulin locus (IgH) [6]. Pre-malignant stage MGUS and MM cells are characterized by genetic changes of NRAS or KRAS2 proto-oncogenes [7]. Multiple myeloma cells have also been connected to expression profile changes for genes encoding cyclin D1, cyclin D3, and fibroblast growth factor receptor 3 (FGFR3) [8]. Recent studies on MM revealed the important diagnostic potential of the RAN (6p21), ZHX-2 (8q24.3), and CHC1L/RCBTB2 (13q14.3) loci. Expression studies of these genes may prove an important therapeutic approach for MM [5, 9]. For example, patients with high RAN expression have increased risk of MM events, while individuals with high expression of ZHX-2 or CHC1L have a decreased risk. The dysregulated expression of RAN, ZHX-2, and CHC1L is associated with rapid relapses [10]. RAN is a member of the Ras superfamily that regulates nuclear transport, cell-cycle progression, chromosome condensation, mitotic spindle formation, and post-mitotic nuclear assembly [11]. CHC1L maps close to the retinoblastoma RB1

gene, which is deleted in about 50% of MM cases, leading to poor survival rates [12]. ZHX-2 encodes a transcription factor that promotes cell-cycle repression and maps near the c-myc proto-oncogene. It is thought that ZHX-2 may be a tumor suppressor gene that can be silenced by hypermethylation [13]. Quantitative analysis of RAN, ZHX-2, and CHC1L genes by real-time reverse transcription PCR showed that ZHX-2 is weakly expressed in high-risk proliferative MM disease, while high ZHX-2 expression is associated with a better response and longer survival after high-dose chemotherapy. Decreased CHC1L expression has been observed in hyperdiploid samples of patients, in comparison with hypodiploid cases. On the other hand, RAN is highly expressed in symptomatic MM and myeloma cell lines [9].

Here, we studied the expression of RAN, ZHX-2, and CHC1L in patients suffering from MM. The expression data obtained from clinical samples were compared with expression levels of candidate genes in the myeloma cell line, MOLP-8, after treatment with inhibitors of histone deacetylases (HDACs) or DNA methyltransferases (Dnmts). We used Trichostatin A (TSA) as an HDAC inhibitor and 5-azacytidine as a Dnmts inhibitor. Similar inhibitors of epigenetic processes, such as vorinostat (Merc) and panobinostat (Novartis), are candidate epigenomic modifiers of multiple myeloma cells, indicating that these drugs are potential suppressors of tumor cell proliferation [14, 15].

Materials and methods

Cell cultivation and sample collection. Multiple myeloma MOLP-8 cells (DSMZ, Braunchweig, Germany) were cultured in RPMI-1640 medium supplemented with 20% fetal calf serum (PAN, Germany), 100 U/ml penicillin and 0.1 mg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO_2/95% air. The cells were treated with inhibitors of histone deacetylases, Trichostatin A (100 nM, dissolved in ethanol) and 5-azacytidine (3 μM dissolved in distilled water), 24 and 48 hours after plating. After the treatments, cells were cultured for an additional 24 hours and then harvested for analysis.

Clinical samples (bone marrow cells) were purchased from the Department of Internal Medicine-Hematooncology, Masaryk University Hospital in Brno. The plasma cells, $CD138^+$ fraction was obtained by magnetic separation (separator VarioMACS, no. 431-02). Samples from bone marrow (BM) biopsies of MM patients were used.

RNA isolation from the myeloma MOLP-8 cell line and clinical samples. Total RNA was prepared using the RNAqueous®-4PCR Kit (Ambion; #AM1914). According to the manufacturer's instructions, cells were pelleted by centrifugation at low speed and disrupted in the guanidinium lysis solution. The sample lysates were mixed with the ethanol solution and applied to a silica-based filter, which selectively and quantitatively binds mRNA and large ribosomal RNAs. The filter was then washed to remove residual DNA, proteins, and other contaminants, and the RNA was eluted in nuclease-free water. The concentration and purity of RNA solutions were verified using a NanoDrop 2000A Spectrophotometer. For analysis, we used samples with the following characteristics: A260/280 = 1.8-2.0 and A260/230 > 1.5. The quality of RNA isolated from clinical samples was analyzed using an Agilent 2100 Bioanalyzer. Only samples with an RNA integrity number (RIN) of 9-10 were used for real-time PCR reactions. For Agilent 2100 Bioanalyzer analysis, we used 1 μl of RNA containing at most 50 ng of RNA.

RNA isolation from peripheral blood leukocytes. Whole blood samples were collected, according to standard procedures, into 9- to 10-ml test tubes containing EDTA as an anti-coagulant. For isolation of total RNA from peripheral blood leukocytes, we used the LeukoLOCK™ Total RNA Isolation System (Ambion; #AM1923). According to the manufacturer's instructions, the whole blood was passed through a LeukoLOCK Filter that captured the total leukocyte population, followed by washing with PBS to remove residual red blood cells. RNA*later* (component of LeukoLOCK™ Total RNA Isolation System) was then used to stabilize the RNA from the leukocytes.

Real-time PCR in the myeloma MOLP-8 cell line, lymphocytes, and $CD138^+$ MM samples. We obtained clinical samples (total RNA or cDNA isolated from BM of MM patients) from the Department of Internal Medicine-Hematooncology, Masaryk University Hospital in Brno (Czech Republic) and the cell line, MOLP-8 (ACC 569), was obtained from the cell culture bank, DSMZ, and was used as a control. The iScript cDNA Synthesis kit (Bio-Rad, #170-8891) was used to synthesize cDNA from 1 μg of total RNA. Reactions were run on a DNA Engine® Peltier thermal cycler (Bio-Rad, Hercules, CA, USA). qRT-PCR amplification mixtures (20 μl) contained 10-50 ng of template cDNA, SYBR Premix Ex Taq™ (10.4 μl; Takara, #RR041A), and 200 nM forward and reverse primers. Reactions were run on a 7300 Real-Time PCR System (Applied Biosystems). The 2-step PCR cycling conditions for all genes tested were: 30 s of TaKaRa Ex TaqTM polymerase activation at 95°C, and 40 cycles of 95°C for 15 s, 60°C for 1 min. Each assay included standard curves of three serial dilutions of cDNA [in triplicate - 2 ng (or 25 ng), 10 ng (or 50 ng), and 50 ng (or 75 ng)]. Analysis of real-time PCR profiles was carried out using the detection software supplied with the 7300 Real-Time PCR System (include the name of the software). The quantity of each experimental sample was extrapolated from the standard curve.

Primers used for real-time PCR reactions were designed using the Primer3Plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). Each primer pair spanned an exon-exon boundary, and the size of the final PCR product was 90-110 bp in length. Primers for RT-PCR reactions were the following: CHC1L: 5'-AGACAGCATCAGCCTTTCTCC-3' and 5'-GCCTTGCTTGATAGTTTGTTGG-3'; RANBP9: 5'-ATCAGCTTGACCCGATTCAG-3' and 5'-GAGGTTGCTTTGGCAGATTG-3'; ZHX-2: 5'-TGTCGGAACTGGCTGAATC-3' and 5'-CCGTTGATCAGCACAGCAG-3'; and primers for the house keeping gene, HPRT1, were:

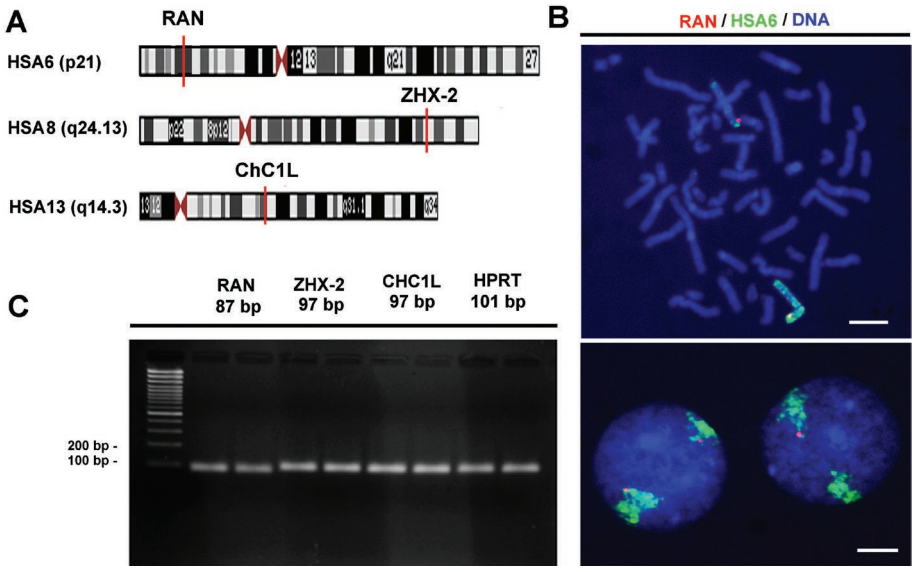

Figure 1. Characteristics of RAN (6p21), ZHX-2 (8q24.3), and CHC1L/RCBTB2 (13q14.3) A) Mapping of RAN, ZHX-2, and CHC1L/RCBTB2 within relevant human chromosomes 6, 8, and 13 (HSA 6, HSA8, HSA13). B) DNA probe for RAN was prepared using PAC clone RP3-503616 (red signals). The purified DNA fragments were labeled using DIG-Nick Translation Mix (Roche, Germany). The efficiency of DNA-FISH probe (6p21) hybridization (red signals) was verified on interphase nuclei and metaphase spreads in parallel with simultaneous hybridization of whole chromosome 6 probe (green). Nuclei of lymphocytes were acquired by Nipkow disc-based confocal microscopy. Bars indicate 0.5 μm. C) Results of RT-PCR analysis of RAN, ZHX-2, CHC1L, and HKG gene, HPRT1. Length of RT-PCR product was approximately 100 bp for each gene. PCR primers were designed to fulfill this condition.

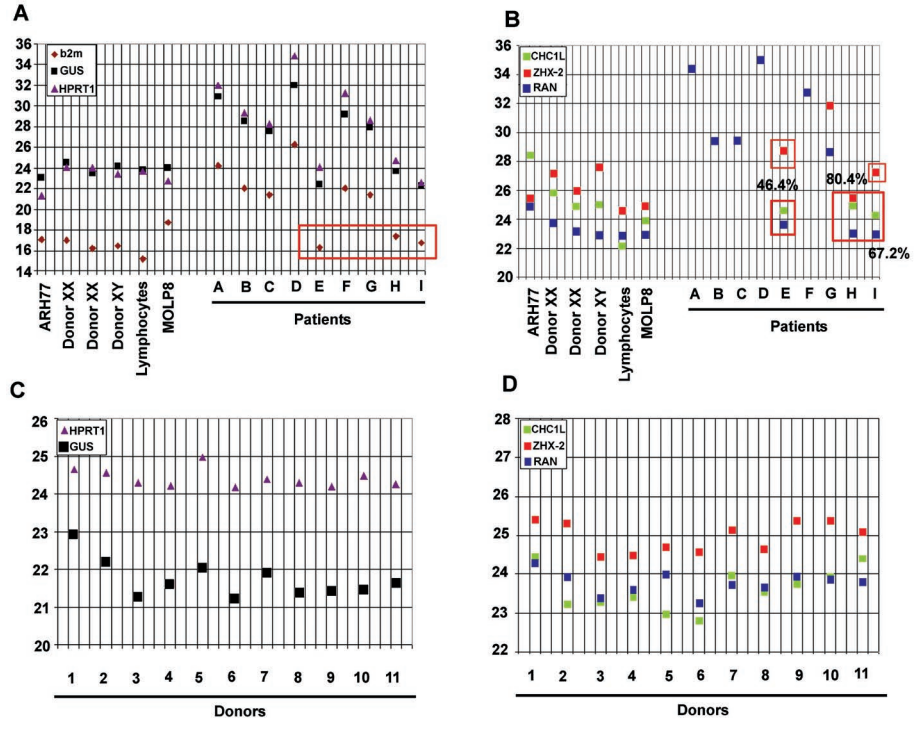

Figure 2. Analysis of RAN, ZHX-2, and CHC1L gene expression in healthy donors and MM patients. A) Optimization of house keeping gene. β2-microglobulin, GUS, and HPRT1 were selected for this analysis. HPRT1 gene was the best control for gene expression analysis in MM. Variability was observed in individual MM patients and only three of them were selected for RAN, ZHX-2, and CHC1L expression analysis (frames). B) Expression of RAN, ZHX-2, and CHC1L in selected MM patients (frames). The expression of ZHX-2 correlated with the level of infiltration of the malignant clone to bone marrow (frame and percentage). C) Detection of HKGs HPRT and GUS in healthy donors was used to optimize the real-time PCR protocol. D) Expression of RAN, ZHX-2, and CHC1L in 11 healthy donors. Analysis was performed to measure the variability of RAN, ZHX-2, and CHC1L expression in healthy individuals.

5'-ACACTGGCAAAACAATGCAG-3' and 5'-ACTTCGTGGGGTCCTTTTC-3'. β2-microglobulin: 5'-TTCTGGCCTGGAGGCTATC-3' and 5'-TCAGGAAATTTGACTTTCCATTC-3', GUS: 5'-TGGTTGGAGAGCTCATTTGG-3' and 5'-CCCTTTTTATTCCCCAGCAC-3'.

Fluorescence in situ hybridization (FISH). DNA/DNA hybridization for RAN (6p21), ZHX-2 (8q24.3), and CHC1L/RCBTB2 (13q14.3) (Fig. 1A) was optimized to provide comprehensive information for clinical laboratories. We constructed a method for analyzing these important genes in myeloma patients. One method is to study gene expression profiles, which can be compared with FISH data showing karyotype stability/instability. For FISH we used PAC clone RP3-503616 containing a RAN (6p21) insert (see Fig. 1B). The efficiency of DNA-DNA hybridization was verified on interphase nuclei and metaphase spreads by simultaneous hybridization of RAN (6p21) and whole chromosome 6 probes (Biotin-Labeled Human Paint Box, cat. No. #1088-B) purchased from Cambio, UK. For detection of the ZHX-2 gene, which maps near the c-myc gene, we used PAC clone RP11-968N11 and for CHC1L, which maps near the retinoblastoma gene RB1, we used clone RP11-893E5 obtained from the Resources for Molecular Cytogenetics (http://www.biologia.uniba.it/rmc/). The DNA from BAC clones was isolated using a standard anion-exchange procedure and a QIAGEN Large Construct Kit, and the whole FISH procedure was performed following Legartová et al. [16]. FISH-stained nuclei were acquired by Nipkow disc-based confocal microscopy and FISH 2.0 software was used for image analysis [17].

Results and discussion

Here, we analyzed the expression of RAN (6p21), ZHX-2 (8q24.3), and CHC1L/RCBTB2 (13q14.3) (Fig. 1C) in myeloma MOLP-8 cells, in peripheral blood lymphocytes of healthy individuals, and in $CD138^+$ bone marrow fractions of patients with diagnosed MM. ZHX-2 (8q24.3) a transcription factor that regulates the cell cycle, is a tumor suppressor gene that is down-regulated by hyper-methylation of the DNA in a majority of cancers [18]. CHC1L (13q14.3) is also a transcription factor, which maps close to RB1, while RAN (6p21) is a member of the Ras super-family responsible for nuclear transport, cell-cycle progression, chromosome condensation, and mitotic spindle formation [19, 20]. First, we optimized RT-PCR conditions for house keeping genes, including β2-microtubulin (b2m), GUS, and HPRT1 (Fig. 2A). Compared to lymphocytes and MOLP-8 cells, which are characterized by relatively stable levels of housekeeping genes, we have observed significant variations between individual clinical samples of $CD138^+$ separated cells (Fig. 2A). This variability was probably caused by distinct therapeutic applications of cytostatic treatments, which likely influenced the expression of housekeeping genes. This effect should be taken into account in analyses of gene expression in tumor cell samples, if patients have already been treated with chemotherapy. In this set of patients, we selected only three individuals, whose expression profiles for housekeeping genes were similar to those observed in relevant control lymphocytes and myeloma MOLP-8 cells (see frame in Fig. 2A). The RNA samples derived from these patients were used for gene expression analysis of candidate genes (frames in Fig. 2B). In these cases, we found that the ZHX-2 gene in particular had an altered expression profile; this gene was significantly down-regulated, which correlated with the percentage of infiltration of malignant clones in the bone marrow (see Fig. 2B, frames and data). However, RAN and CHC1L expression was relatively stable in the selected patients when compared to lymphocytes and MOLP-8 myeloma cells (Fig. 2B).

For our analysis, we used RNA isolated from peripheral blood lymphocytes of healthy individuals as a relevant control. We verified the purity and quality of RNA samples obtained from clinics and then measured the expression level of a housekeeping genes (HKGs), necessary for optimal analysis of RAN, ZHX-2, and CHC1L gene expression by real-time PCR (Fig. 2C). We observed relatively little heterogeneity in the expression of the selected HKGs in control samples (Fig. 2C). Moreover, the level of HPRT1 expression was more stable compared to GUS. A similarly small variability in expression was observed for RAN, ZHX-2 and CHC1L in healthy donors (Fig. 2D). These data served as a relevant control that was necessary for evaluating gene expression in clinical samples. The most stably expressed and appropriate control HKG in MM appears to be HPRT1, which was used for data normalization in studies of RAN, ZHX-2, and CHC1L expression in other sets of clinical samples (Fig. 3). We measured low expression levels for these genes in MM patients, compared to peripheral blood lymphocytes and MOLP-8 cells. Moreover, when MOLP-8 cells were treated by TSA and 5-azacytidine (5-AZA), only TSA was able to decrease the expression of CHC1L and ZHX-2 (Fig. 3). In clinical samples, the variance between individuals (Fig. 3) was similar to what was observed in healthy donors that were characterized by higher expression of RAN, ZHX-2, and CHC1L (Fig. 2D) compare to MM samples (Fig. 3). We tried to correlate our expression data to clinical outcomes: Patients 6 and 7, who had the lowest expression levels of CHC1L (in Fig. 3), were diagnosed during 2008 and survived. However, one was treated with melphalan, prednisone, and thalidomide (MPT), while the other was treated with cyclophosphamide, thalidomide, and dexamethazone (CTD). Similarly, patient 8, who had the highest level of CHC1L expression, was treated with CTD. When we analyzed the patients with the lowest levels of RAN expression (CTD-treated patients 13 and 14, Fig. 3), and compared them with those of the highest RAN expression (CTD-treated patient 12), we did not observed any connection between the level of expression of the candidate genes and therapeutic application. Similar conclusions were made when we analyzed possible correlations between ZHX-2 expression and CTD-related clinical approaches (Fig. 3).

We followed up on the study by Armellini et al. [9], which showed that ZHX-2 is weakly expressed in high-risk prolifera-

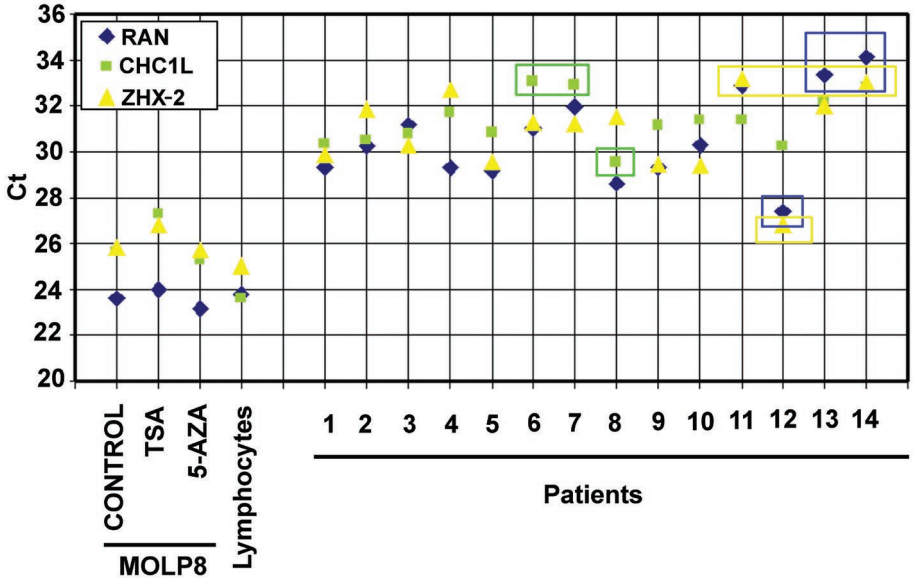

Figure 3. Analysis of RAN, ZHX-2, and CHC1L expression in MM patients and myeloma cell line, MOLP-8, treated with HDAC and Dnmts inhibitors. The expression of RAN, ZHX-2, and CHC1L was evaluated in relation to the HKG HPRT1 gene. MOLP-8 cells and peripheral blood lymphocytes of healthy individuals were characterized by higher expression levels of the RAN, ZHX-2, and CHC1L compared to samples from BM of myeloma patients. HDAC inhibitor TSA decreased the expression levels of CHC1L and ZHX-2. Differences in the expression of selected genes in MM patients were not significantly influenced by distinct therapeutic applications (see Results): Green frames indicate comparison of the highest and lowest expression of CHC1L in patients diagnosed in 2008 and treated with MPT or CTD. Blue frames are related to RAN gene expression in CTD-treated patients. Yellow frames showed CTD-treated patients with distinct expression levels of the ZHX-2 gene.

tive MM disease, while high expression of ZHX-2 is associated with better responses and longer survival after high-dose therapy. Our data reproduced the changes in the expression of ZHX-2 in MM patients; however, a certain amount of variability in expression was also observed for the other two genes analyzed (Fig. 3). Our data, along with those of Armellini et al. [9], document that the ZHX-2 gene, which maps close to the c-myc proto-oncogene, is highly significant for multiple myeloma malignancy. In addition, the c-myc gene is one potential proto-oncogene that is dysregulated in MM [2]. Therefore, it seems likely that more complex genomic regions, mapping to 8q24, may be key players in MM pathophysiology. Some data showed that c-myc cytogenetic rearrangement is observed as a late progression event associated with MM, and monoallelic expression of c-myc has been found in some myeloma cell lines (summarized by Kuehl and Bergsagel [8]). However, monoallelic c-myc gene expression does not seem to be MM specific, because c-myc is also monoallelically expressed in other tumor cells, including colon cancer cells, often characterized by c-myc amplification [21, 22].

Taken together, our data support the conclusion that changes in the expression of ZHX-2 play a fundamental role in the pathophysiology of multiple myeloma. Thus, ZHX-2 appears to be an important prognostic marker for MM. Our methods highlight some critical considerations for performing real-time PCR with clinical samples and show that the quality of RNA significantly influences the detection of gene expression [23–25].

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