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Cell cycle genes co-expression in multiple myeloma and plasma cell leukemia

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ABSTRACT

The objective of this study was to describe co-expression correlations of cell cycle regulatory genes in multiple myeloma (MM) and plasma cell leukemia (PCL).
Our results highlight the presence of dynamic equilibrium between co-expression of activator and inhibitor gene sets. Moreover inhibitor set is more sensitive to the activator changes, not vice versa. We have shown that CDKN2A expression is associated with short-term survival in newly diagnosed MM patients (survival was 30.3 ± 3.9 months for 'low' expressed and 7.5 ± 5.6 months for 'high' expressed group, $p < 0.0001$). Moreover low-expression CDKN2A group showed time-to-progression benefit in newly diagnosed patients (remission was 20.8 ± 3.6 months for 'low' and 8.4 ± 2.7 months for 'high' expressed group, $p < 0.0001$) as well as in whole studied cohort of MM patients (remission was 20.8 ± 2.8 months for 'low' and 9.8 ± 1.1 months for 'high' expressed group, $p < 0.0001$). The overexpression of inhibitors can be explained as a compensatory reaction to growing "oncogenic stress".

1. Introduction

The regulation of cell cycle is a dynamic and complex process, thus G1-to-S cell-cycle progression is determined not only by the absolute level of any given cell-cycle regulator, but principally by the levels of positive regulators (cyclin dependent kinases — CDKs and cyclins), the aggregate balance between positive and negative cell-cycle regulators (CDKIs) and the interplay among them [1]. Furthermore, majority of the changes in gene expression dynamics do not manifest in the form of differential expression [2].

In multiple myeloma (MM), the second most common haematopoietic cancer, unlike normal plasma cells, myeloma cells retain the self-renewing potential. Primary plasma cells do not longer divide as they are arrested in the G1 phase of cell cycle as intermediates in plasma-cell differentiation, which is a consequence of transformation [3]. Unlike other tumors, proliferation index in MM is predominantly low, but impaired mechanisms of myeloma cells proliferation and apoptosis lead to the accumulation of myeloma cells in bone marrow [1].

Despite a low proliferation index in monoclonal gammopathy of undetermined significance (MGUS) and most MM tumors, there is an increased expression of one of the three CCNDs genes in virtually all MGUS and MM tumors [4]. Over-expression of any of cyclin D genes—sometimes as a consequence of a primary IgH translocation but otherwise by presently unknown mechanisms—appears to be a unifying and early event [5]. However; a prominent over-expression of cyclin D1 is possibly redundant and does not lead to cell cycle progression because of quiescence of majority of myeloma cells.

Normal long-lived plasma cells that provide serological memory, residing in specialized niches in bone marrow where they can survive for many years, are arrested in the G1 phase of the cell cycle [6]. Majority of immortalized myeloma cells regardless of over-expression of cyclins D stay in the G1 phase due to pro-apoptotic and cell cycle regulatory capacity of p53 depended axis. Nevertheless, after leukemic transformation in secondary plasma cell leukemia (PCL) or *de novo* in case of primary PCL, bone marrow myeloma cells become highly proliferative and even presenting as circulating plasma cells in the peripheral blood in number of greater than 20% [7]. We suggest that after

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leukemic transformation the critical G1/S cell-cycle checkpoint is not able to be regulated by deterrent mechanisms and potentially increased levels of cyclin D proteins leading to complete mitosis.

Thus, we suspect that expression of cell cycle activators gene set is in dynamic equilibrium with cell cycle inhibitors gene set; nevertheless, accumulation of carcinogenic events during MM to PCL progression leads to insufficiency of inhibitors control. Such growing disbalance between cell cycle regulators gives to plasma cells potential to breakthrough mitotic restriction points.

On the assumption of this, the objective of current study was to describe co-expression correlations of cell cycle regulator genes in MM and PCL.

2. Design and methods

2.1. Patients & sample preparation

A total of 91 MM and 10 PCL patients enrolled at University Hospital Brno, Czech Republic, University Hospital Olomouc, Czech Republic and University Hospital Bratislava, Slovakia, were included in this study. Patients' baseline characteristics are summarized in Table 1. This study was approved by the institutional ethical review boards and all patients provided written informed consent. The bone marrow of patients was obtained during routine diagnostic procedure. Plasma cells in mononuclear cell fraction were enriched by anti-CD138 + immunomagnetic beads and sorted using AutoMACS Pro (MiltenyiBiotec). Purity of CD138 + fraction was measured by flowcytometry and/or cytospin and samples with >80% plasma cells were provided for total RNA isolation.

Table 1
Patients baseline characteristics.

	Multiple myeloma	Plasma cell leukemia
No. of patients	91	10
Follow-up median (min–max) [days]	409 (6–6024)	543 (163–1839)
Gender: males–females	50.0%–50%	40.0%–60%
Age median (range) [years]	69 (45–85)	60 (51–78)
ISS stage: I–II–III	25.9%–25.9%–48.1%	30%–10%–60%
Durie–Salmon stage: I–II–III	6%–15.5%–78.6%	20%–30%–50%
Durie–Salmon substage: A–B	76.5%–23.5%	60%–40%
Igisotype: IgG–IgA–FLC–Nonsecr–IgM–IgD	60.8%–25.3%–11.4%–2.5%–0%–0%	40%–10%–30%–0%–10%–10%
Light chains: kappa–lambda	64.6%–35.4%	50%–50%
No. of previous treatment lines		
None (first line treatment)	58 (63.7%)	4 (40%)
One	16 (17.6%)	2 (20%)
Two	8 (8.8%)	3 (30%)
More (>2)	9 (9.9%)	1 (10%)
Treatment regimen		
Bortezomib	40 (52.6%)	3 (37.5%)
Thalidomid	16 (21.1%)	4 (50%)
Lenalidomide	20 (26.3%)	1 (12.5%)
Treatment response		
CR (+sCR)	4 (7.7%)	0 (0%)
VGPR	15 (28.8%)	1 (10%)
PR	19 (36.5%)	0 (0%)
MR	2 (3.8%)	0 (0%)
SD	3 (5.8%)	0 (0%)
PG	9 (17.3%)	9 (90%)
Chromosomal abnormality		
RB1 deletion	40 (50%)	6 (60%)
TP53 deletion	7 (9.1%)	3 (30%)
Translocation t(4;14)	16 (21.6%)	2 (23.3%)
Translocation t(14;16)	11 (17.7%)	1 (10%)
1q21 gain	40 (50%)	9 (90%)
Hyperdiploidy	42 (51.2%)	1 (10%)

2.2. Fluorescence in situ hybridization (FISH)

FISH was performed as a part of routine diagnostic procedure according to protocol previously described [8]. The following aberrations were studied: 1q21 gain, 13q14 deletion, 17p13 deletion, translocation t(4;14) and translocation t(14;16). Hyperdiploidy status was determined with commercial probes mapping to chromosome 5 (LSI D5S23/D5S721), 9 (CEP9) and 15 (CEP15) (Abbott Molecular, Des Plaines, IL, USA).

2.3. RNA isolation

Total RNA was isolated using QIAGEN RNeasy Mini Kit. RNA isolation and purification were described previously [9]. Total RNA with purity ratio 260/280 > 1.7 and integrity (RIN) >7.5 (as measured by Agilent 2010 Bionalyzer) was used for further analyses.

2.4. Quantitative real-time PCR (qRT-PCR)

Quantitative RT-PCR was performed on a chosen list of genes, according to their role in cell cycle regulation, using the Applied Biosystems platform (Suppl. Table 1). Input of 100 ng of high-quality total RNA was reverse transcribed using High Capacity cDNA Reverse Transcription Kit and preamplified with TaqMan PreAmp MasterMix Kit (Applied Biosystems, Foster City, CA). Expression of each gene was evaluated in a duplicate reaction using TaqMan Gene Expression Assays and human glyceraldehydes 3-phosphate dehydrogenase (GAPDH) as an internal control on 7500 Real Time PCR System. Raw data were analyzed with SDS 1.4 software and relative fold change of expression for each gene was calculated using ΔΔCt approach.

2.5. Immunoblotting

Proteins from 4 MM patients PCs, 2 PCL patients PCs and Hela cell line (positive control) were extracted using RIPA buffer (2 M Tris, 5 M NaCl, 0.5 M EDTA, 0.5% NP-40, 0.2% NaF) containing 10% PhosSTOP and 10% Complete (Roche). Equal amounts of proteins were separated by SDS-PAGE, transferred onto PVDF membrane (Millipore) and incubated with primary mouse mAb p-16 (DCS-50), rabbit Ab p-14 ARF (H-132) (both Santa Cruz) and GAPDH Ab (Cell Signaling Technology) overnight at 4 °C in 5% milk in TBS-T buffer. Subsequently, membrane was washed in TBS-T and incubated with secondary antibodies conjugated with horseradish peroxidase (anti-mouse IgG and anti-rabbit IgG, 1:5000, Sigma-Aldrich) in TBS-T buffer and visualized by chemiluminescence (Immobilon Western Chemiluminiscent HRP Substrate, Millipore). The same exposition time (5 min) was used for all samples.

2.6. Statistical analysis

Standard descriptive statistics were applied in the analysis; median supplemented by min–max range for continuous variables and absolute and relative frequencies for categorical variables. Statistical significance of differences in continuous variables among groups of patients was analyzed using nonparametric Kruskal–Wallis or Mann–Whitney *U* test. For discrete variables, chi-square test or Fisher's exact test was used. For the robust analysis of continuous parameters relationship the Spearman correlation coefficient was adopted. Canonical correlation analysis was applied for the analysis of relationship between set of variables. Survival and progression rates were estimated using the Kaplan–Meier method. The overall survival (OS) and time-to-progression (TTP) were determined according to International Myeloma Working Group guidelines [10]. Differences in survival among subgroups of patients were compared using the log-rank test. Time-dependent receiver operating characteristic (ROC) was used for the identification of cut-off value of continuous variables for survival of