The association of increased p14^{ARF}/p16^{INK4a} and p15^{INK4b} gene expression with proliferative activity and the clinical course of multiple myeloma

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p14/p16 and *p15* gene expression was assessed by quantitative polymerase chain reaction in purified plasma cells (PC) from 52 patients with symptomatic multiple myeloma (MM) and seven with smoldering MM in order to clarify the impact of these genes on the proliferative activity of tumor cells and patients' outcome. *p15* expression was lower in symptomatic MM than in smoldering SMM (-1.80 vs.1.51, *p*=0.026); similar results were observed for *p14/p16*. MM patients whose PC displayed high *p15* and/or *p14/p16* expression had a lower percentage of S-phase PC than the remaining cases (1·79%±1.35 vs. 3.04%±1.42, *p*=0.028), favorable prognostic factors and longer survival (100% vs. 49 %at 2.5 years; *p*=0.007).

Key words: p14^{ARF}, p15^{INK4b}, p16^{INK4a}, gene expression, real time quantitative PCR, multiple myeloma.

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Multiple myeloma (MM) is a clonal neoplasm that involves terminally differentiated B cells (plasma cells, PC), in which cell cycle activity is related to tumor development and progression.[1] Cell cycle-related parameters are currently important prognostic indicators of the outcome of MM .[2] Their key role in MM has recently been emphasized by gene expression profiling, which adds support to the concept that cyclin D over-expression is an early and unifying pathogenic event of MM.[3]

CCell cycle progression is controlled by the overall balance between positive and negative regulators. p15 and p16 are two cell cycle regulators involved in the inhibition of G1 phase progression, since they are CDK4/6 inhibitors that block their interaction with D cyclins.[4] p14 protein is also a cell cycle inhibitor and shares part of its genomic structure with p16, although the final gene product is different.[5] All these tumor suppressor genes can be inactivated in cancer by homozygous deletion, point mutations and promoter hypermethylation, at a frequency that varies greatly from one tumor to the other.[6] Although discrepant results can be found in the literature the only common form of inactivation in MM is aberrant methylation of the gene promoter.[7,8] The differences could be attributed to a variety of causes such as the use of non-separated bone marrow cells, inclusion of patients at different disease stages, and technical pitfalls. In order to clarify these discrepancies it would be useful to perform a direct study of gene expression in purified PC obtained in a series of uniformly treated patients. Until now, analysis of mRNA levels of these tumor suppressor genes has not led to the genes being attributed an important role in MM. However, the analyses were carried out using qualitative methods or within global gene expression profiling studies without sufficient sensitivity to detect small quantities of mRNA.[9] Real time quantitative polymerase chain reaction (RQ-PCR) is a quantitative, highly sensitive and reproducible method for assessing gene expression.[10] In the present study, we used RQ-PCR to assess the expression of *p14, p15* and *p16* genes in purified PC from patients with untreated MM and smoldering in order to clarify whether the genes have an impact on the proliferative capacity of the tumor cells and patients' outcome.

Design and Methods

Patients and controls

Fifty-two patients with untreated symptomatic MM diagnosed according to standard criteria and seven with smoldering MM were included in the present study. Patients were treated according to the protocol of the Spanish Cooperative Group GEM-2000, which included front line polychemotherapy (VBCMP/VBAD) followed by high-dose melphalan and autologous stem cell support.[11] Disease characteristics documented at diagnosis in these patients included conventional clinical and laboratory parameters as well as bone marrow PC and their cell cycle distribution assessed by flow cytometry. In addition, patients were grouped into clinical stages according to the recently developed International Staging System (ISS).[12] The response was considered to be complete, partial, minor or null according to the standard criteria of the European Group for Blood and Marrow Transplant.[13]

Plasma cell purification and RNA isolation

Bone marrow PC freshly obtained from patients were purified by immunomagnetic bead selection with monoclonal anti-CD138 antibodies using the AutoMacs automated separation system (Miltenyi Biotec, Auburn, CA, USA). The purity of the PC, assessed by morphology, was higher than 90%. RNA was extracted using the Rneasy Kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions.

C-DNA synthesis and RQ-PCR

c-DNA was synthesized according to the EAC protocol.[14] RQ-PCR was carried out on cDNA using the Assays-on-Demand gene expression mixes specific for *p14/p16* (Hs-00233365-m1) and *p15* (Hs-00793225-m1) and the TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA). Reactions were carried out in the ABI PRISM 7700 Sequence Detection System using 2 ng of cDNA in a final volume of 25 μL. The PCR consisted of 1 min AmpErase Uracyl N-Glycosylase activation at 50°C, 10 min at 95°C for Ampli TaqGold activation and 50 cycles of PCR of 15s denaturation at 95°C followed by 1 min at 60°C for annealing/extension. RQ-PCR amplification of the *ABL* gene was used to assess RNA quality and quantity as well as to normalize the gene expression in the experiments.

Quantification of the gene expression

The relative quantification of the gene expression was performed using the cycle threshold (CT) increment method. The final expression was assessed by comparison of the ΔCT with respect to that observed in the peripheral blood mononuclear cells obtained from healthy donors. Before using this method, a validation experiment was performed in order to demonstrate that efficiency of the target and control genes were equal. In addition, a mathematical modification was included in order to transform the expression into times higher (positive value) or lower (negative values) instead of the logarithmic expression in which values lower than control range between 0 and 1.

Immunophenotypic characterization of PC and DNA measurements

After morphological analysis, bone marrow PC were immunophenotypically characterized by flow cytometry using previously described methods.[15] DNA was assessed by flow cytometry, according to standard methods.[15] In this technique, bone marrow PC are identified by staining with both CD38 and CD138-FITC while the DNA is simultaneously stained with propidium iodide. The PC DNA index and the proportion of cells in the different phases of the cell-cycle was assessed according to previously described methods using the MODFIT software (Verity Software House, Topsham, ME, USA).

Statistical analysis

The statistical significance of the differences between means for continuous variables was assessed with the t-test and Mann Witney U-test with SPSS statistical software (SPSS Inc, Chicago, IL, USA). The χ^2 test (SPSS) was used to compare dichotomous variables. Survival curves were plotted according to the method of Kaplan and Meier and compared using the log-rank test (survival SPSS).

Results and Discussion

Gene expressions in MM patients

The expression of both *p14/p16* and *p15* genes in bone marrow PC from MM patients was heterogeneous. Thus, *p14/p16* expression ranged from –2.7 (2.7 times lower) to 32 (32 times higher) than the expression of the *ABL* control gene. *p15* was expressed at slightly lower levels, ranging from undetectable levels to 3 times higher than expression of the control gene. When gene expression in different MM clinical forms of MM was compared, the expression of *p15* gene was significantly lower in symptomatic MM than in smoldering MM (-1.8 vs. 1.51, p=0.026). Similar differences were seen for *p14/p16* gene expression between symptomatic MM and smoldering MM, although these differences were not statistically significant (38 vs. 54.5; p=0.19).

Correlation between the expression of p14/p16 and p15 gene and clinical and biological parameters

To evaluate possible correlations between gene expression and biological characteristics, patients were classified into different groups according to gene expression values. The threshold that we used was established as the 75th, since this provided the most appropriate classification of patients. This threshold means that patients with high levels of expression were part of the 25% of patients with the highest expression values and the remaining were those with a lower value than that. As expected, patients whose PC expressed high levels of *p15* and/or *p14/16* had a slow cell cycle progression, since the percentage of S-phase PC was lower in these cases (1.79±1.35 vs. 3.04±1.42; p=0.028) (Figure 1). In addition, high *p15* and/or *p14/p16* expression was statistically significantly associated with several favorable prognostic factors such as a low serum levels of β_2-microglobulin and C-reactive protein, age under 60 years and a less infiltration of the bone marrow by PC (Table 1). Interestingly, the expression of the two genes was not exactly parallel. When simultaneous *p14/p16* and *p15* gene expression was analyzed, it was found that 54% of patients showed no expression at all or expressed low levels of both genes while the remaining 46% of patients expressed one or both genes above the threshold value.

Tumor suppressor gene expression and outcome

As far as the response to treatment was concerned, ten patients were still under therapy when the study was closed, so response information was available for 42 patients. There was a significant correlation between a favorable response (complete and partial responses) and

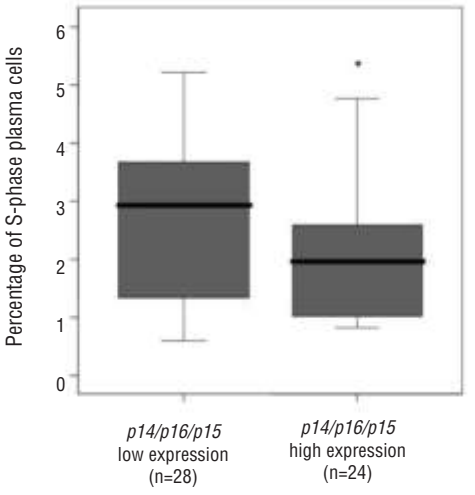

Figure 1. Percentage of S-phase plasma cells in symptomatic myeloma patients. These patients were classified into two groups according to *p14/p15* and *p16* gene expression values. The box plot shows the distribution of the percentages of bone marrow plasma cells in S-phase; the black line in the centre of the box corresponds to the median value; the box includes the two central percentiles (25 to 75%) and the two extremes correspond to the maximum and minimal values according to a normal distribution. The asterisk signifies a value that, although real, is out of the expected normal distribution. According to this analysis, those patients with high *p15* or *p14/p16* gene expression had a lower percentage of S-phase bone marrow plasma cells (median 1.86) than did patients with low *p15* or *p14/p16* gene expression (median 2.92). The Mann-Whaitney U-test for mean comparisons was statistically significant (p=0.028).

Table 1. Clinical characteristics of MM patients according to the levels of expression of *p14/p16* and *p15* genes.

Characteristics	*p15*		*p14/p16*		*p15 or p14/p16*	
	Low (n=36)	*High (N=16)*	*Low (n=36)*	*High (N=16)*	*Both Low (N=28)*	*High[a] (n=24)*
S phase Plasma cells ≥3%	31%	4%*	44%	19%	27%	8%*
Age (>60 years)	50%	16%	43%	20%	40%	24%*
Performance status (ECOG>2)	16%	2%	15%	4%	14%	4%
β2-Microglobulin ≥3 (mg/L)	46%	9%	46%	13%	39%	16%*
C-reactive protein ≥3 (mg/dL)	38%	3%*	31%	8%	30%	11%*
PC by morphology ≥40%	34%	14%	34%	14%	29%	19%
PC by flow cytometry ≥20%	40%	7%*	33%	10%	33%	13%*
Clinical stage						
I	7%	4%	10%	–	7%	4%
II	27%	18%	25%	19%	18%	27%
III	36%	9%	31%	15%	27%	18%

[a]*High expression of one or both of the two genes. *p< 0.05.*

high expression of the tumor suppressor genes (*p14/p16* and/or *p15*), since most patients with high values had favorable responses (89% vs. 58%; p=0.013). Progression-free survival and duration of response both tended to be slightly shorter in patients with low expression of *p15* and *p16/p14*, although the differences were not statistically significant. In addition, although the mean follow-up was relatively short (19 months), the overall survival of patients with high *p16* and/or *p15* expression was significantly longer since none of them had died by the time of the analysis. This provided a projected 2.5-year survival of 100% vs. 49% for patients with high versus low tumor suppressor gene expression, respectively (p=0.002; Figure 2). In the analysis of prognostic factors, nine other traditional variables were shown to be related with the overall survival: age, performance status, hemoglobin concentration, serum levels of lactate dehydrogenase, albumin, creatinine, serum calcium, β2-microglobulin, and ≥3% S phase PC). In a very preliminary multivariate analysis, tumor suppressor gene expression was shown to be independently associated with prognosis, as were S-phase PC, β2-

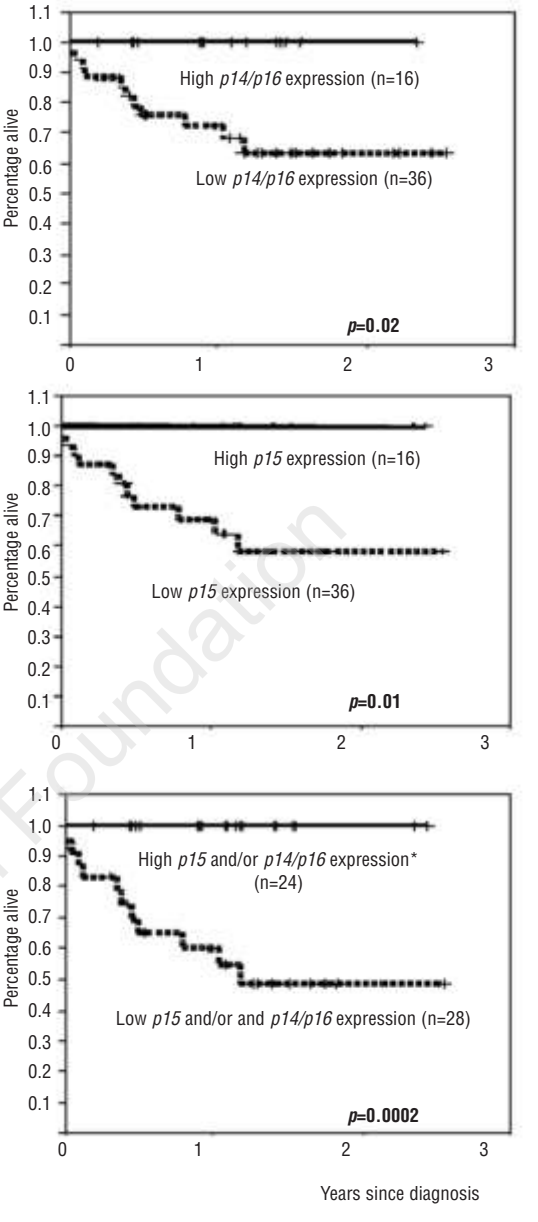

Figure 2. Overall survival curves of symptomatic MM patients estimated according to p15 and *p14/p16* gene expression.

microglobulin and lactate dehydrogenase level. In this study we used RQ-PCR to assess the expression of *p15* and *p14/p16* genes in separated PC from patients with symptomatic MM and smoldering MM in order to explore the relationship of these genes with clinical and biological characteristics. Our results show that high expression of at least one of these genes is associated with a low plasma cell proliferation index and favorable outcome, since patients with high gene expression levels had good prognostic features and longer survival.

This quantitative assay, and the use of separated PC, provides specific data on mRNA gene expression within tumor cells, overcoming some of the potential pitfalls inherent to previous studies based on DNA analysis (deletions, mutations, hypermethylation).[7,8] With this approach, we observed that the expression of *p15* and *p14/p16* was higher in smoldering MM than in symptomatic MM patients, although these results should be taken with caution since only seven patients with smoldering MM could be analyzed. Since *p14/p16* and *p15* are cell cycle inhibitors, these findings are in line with the notion that smoldering MM and monoclonal gammopathies of undetermined significance have a lower proliferative activity than symptomatic MM.[16] In the same way, within symptomatic MM patients, we found a relationship between *p15* and *p14/p16* RNA levels in MM cells and disease characteristics at diagnosis. Accordingly, high expression of one or both of these two gene clusters correlates with low percentages of PC in S-phase, which also supports the notion that tumor suppressor genes closely control the cell cycle progression in neoplastic PC, as previously suggested by different studies exploring the methylation status and function of these genes.[17] This is also in accordance with the fact that high expression of tumor suppressor genes was associated with favorable prognostic factors such as low levels of β_2-microglobulin and C-reactive protein, as well as with a favorable response and longer survival. It should be noted that gene expression analysis using microarrays have not shown an association between expression of *p14/p16* and *p15* genes and disease outcome.[9,18] Since gene arrays evaluate several thousands of genes, it is reasonable to suppose that the pathogenic relevance of some genes can be underestimated during the analysis of such an enormous amount of data. In summary, the present study suggests that MM patients with high levels of tumor suppressor genes may have a relatively indolent form of the disease, resembling smoldering MM, with good prognostic features and long overall survival. This clinical finding could be explained by the fact that over-expression of tumor suppressor genes helps to arrest cell cycling so that the myeloma clone becomes a slowly proliferating population with a less aggressive behavior.

MES participated in the design of the study, carried out molecular studies and prepared the database for the final analysis. She prepared the initial version of the paper; RG-S made the conception and design of most of the work, reviewed the database and made the statistical analysis. He re-wrote the paper and provided the pre-approval of the final version; AA participated in the initial conception and design, opf the study. She carried out the cell separation and made part of the molecular studies; MCC and AB participated in RNA extraction, carried out tyhe thecnical standardization and participated in the generation of the molecular results; MA, PM-J participated in the production of molecular results, database preparation and statistical analysis; MF, MS, FO, JMH, AS, LP, RG-S were the clinicians responsible for the patients who took care of the protocol accomplishment, sampling and collection of the clinical data. MG, JFSM were the promoters of the study and were responsible for getting the financial support and they carried out the final revision of the draft; JFSM was the main responsible of the group and was the person responsible last revision of the draft as well as the person who gave the final approval of the version to be published.

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