Upregulation of Dicer is more frequent in monoclonal gammopathies of undetermined significance than in multiple myeloma patients and is associated with longer survival in symptomatic myeloma patients

María E. Sarasquete, Norma C. Gutiérrez, Irena Misiewicz-Krzeminska, Bruno Paiva, María C. Chillón, Miguel Alcoceba, Ramón García-Sanz, Jesús M. Hernández, Marcos González, and Jesús F. San-Miguel

Servicio de Hematología, Hospital Universitario, Centro de Investigación del Cáncer-IBMCC (USAL-CSIC), Salamanca, Spain

ABSTRACT

Dicer and Drosha are key enzymes in the miRNA-processing pathway which is altered in many human cancers.

We analyzed Dicer and Drosha expression levels by quantitative PCR in 151 patients with monoclonal gammopathies: 102 symptomatic myeloma patients, 23 smoldering myelomas and 26 monoclonal gammopathy of undetermined significance. We found that Dicer expression values were significantly higher in monoclonal gammopathy of undetermined significance than in smoldering myelomas and symptomatic myeloma (mean ± SD, 0.84±0.36 *vs*. 0.60±0.23 and 0.62±0.51; $P<0.01$). Moreover, the median progression-free survival was significantly longer in symptomatic myeloma patients with high expression of Dicer (not reached *vs.* 23.6 months; $P=0.02$). By contrast, no differences in the expression of Drosha among these groups of patients were observed.

Our data suggest that Dicer expression may play an important role in the progression and prognosis of monoclonal gammopathies.

(Clinicaltrials.gov identifier: NCT00461747 for MM patients under 65 years of age and NCT00443235 for MM patients over 65 years of age)

Key words: monoclonal gammopathyof undeterminated significance, multiple myeloma, smoldering myeloma and gene expression.

Citation: Sarasquete ME, Gutiérrez NC, Misiewicz-Krzeminska I, Paiva B, Chillón MC, Alcoceba M, García-Sanz R, Hernández JM, González M, and San-Miguel JF. Upregulation of Dicer is more frequent in monoclonal gammopathies of undetermined significance than in multiple myeloma patients and is associated with longer survival in symptomatic myeloma patients. Haematologica 2011;96(03):468-471. doi:10.3324/haematol.2010.033845

Introduction

MicroRNAs (miRNAs) are small non-coding RNAs that regulate gene expression at the post-transcriptional level and are involved in critical biological processes. MiRNA processing starts in the nucleus, where long endogenous double-stranded RNA segments (pri-miRNA) are cut into short hairpin-shaped double-stranded RNA precursors (pre-miRNA) by the RNAase III enzyme Drosha. Pre-miRNAs then move to the cytoplasm, where they are cleaved by Dicer to yield mature miRNAs, which produce mRNA degradation or translational repression after being activated through the RNA-induced silencing complex (RISC). We and other researchers have shown abnormal expression of miRNAs in many types of human tumors, including multiple myeloma (MM).[1-6] The potential role of Dicer and Drosha as master regulators of the RNA interference machinery in tumorigenesis has also been investigated in several malignancies.[7-11] Low levels of Dicer and Drosha expression were associated with advanced tumor stage and suboptimal tumor cytoreduction, respectively, in ovarian cancer.[9] Likewise, a low level of these enzymes has been found in high-risk neuroblastoma tumors with poor outcome.[11] Similar observations have been made in lung cancer in which upregulation of Dicer was observed in precursor lesions while decreased levels were found in invasive lung adenocarcinoma.[7] By contrast, in prostate cancer a high level of Dicer expression was associated with metastatic disease.[12] As far as hematologic malignancies is concerned, Dicer expression has only been investigated in acute myeloid leukemia.[13]

These findings prompted us to investigate Dicer and Drosha expression levels in patients with monoclonal gammopathies. This term covers a wide spectrum of plasma cell (PC) disorders: from the pre-malignant conditions, monoclonal gammopathy of undetermined significance (MGUS) to the smoldering myeloma (SMM) and, finally, the more advanced and aggressive disease, the symptomatic multiple myeloma. This progressive model of transformation provides a unique opportunity to investigate the potential role of Dicer and Drosha in the pathogenesis of monoclonal gammopathies. In addition, we have also investigated the correlation of Dicer expression with cell cycle distribution and dis-

Acknowledgments: we are grateful to "Grupo Español de Mieloma" clinicians for providing MM samples, and to I. Isidro, T Prieto, A. Anton and V. Gutierrez for technical assistance.

Funding: this study was partially supported by grants from the Spanish FIS (PI080568 and PS0901450) and the Gerencia Regional de Salud, Junta de Castilla y León (GRS202/A08), and the Spanish Myeloma Network Program (RD06/0020/0006). MES and MCC were supported by the Ministerio de Sanidad y Consumo (Contrato de Técnicos de Apoyo a la Investigación, CA08/00212 and CA07/00077, respectively).

Manuscript received on September 24, 2010. Revised version arrived on November 8, 2010. Manuscript accepted on November 26, 2010.

Correspondence: Jesús F. San Miguel, Servicio de Hematología, Hospital Universitario, Paseo de San Vicente 58-182, 37007 Salamanca, Spain. Fax: international +34.923294624. E-mail: sanmigiz@usal.es or Maria E. Sarasquete, Servicio de Hematología, Hospital Universitario, Paseo de San Vicente 58-182, 37007 Salamanca, Spain. Fax: international +34.923294624; E-mail: a9136@usal.es

ease outcome in symptomatic multiple myeloma treated according to the Spanish Pethema trial GEM–2005.[14]

Design and Methods

The study included 151 patients: 102 symptomatic multiple myeloma, 23 smoldering myeloma and 26 monoclonal gammopathy of undetermined significance cases, and 5 healthy donors as normal controls. All patients provided written informed consent in accordance with the Declaration of Helsinki, and the research ethics committee of the University Hospital of Salamanca approved the study. Symptomatic multiple myeloma patients were treated according to the Spanish Pethema trial GEM-2005 (ClinicalTrials.Gov: NCT00461747 for MM patients under 65 years of age and NCT00443235 for MM patients over 65 years of age.[14]

CD138-positive plasma cell (PC) isolation was performed in all diagnostic bone marrow samples. The purity was above 95% in the smoldering myeloma and multiple myeloma and 90% in monoclonal gammopathy of undetermined significance and healthy donors. Total RNA was extracted from tumor plasma cells using an RNEasy Mini Kit (Qiagen, Valencia, USA) following the manufacturer's protocol. RNA quality and quantity were assessed with the RNA Nano LabChip (Agilent Tech. Inc., Palo Alto, CA, USA). The retrotranscription reaction was performed with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems Foster City, CA, USA) according to the manufacturer's recommendations. Finally, real-time quantitative PCR was performed using TaqMan gene expression assay kits (Applied Biosystems Foster City, CA, USA): Hs_00229023 for Dicer, Hs_00203008 for Drosha and Hs00245445_m1 for *ABL* as a control gene. Relative gene expression was calculated by the $2^{-\Delta Ct}$ method, $\Delta Ct = Ct_{(gene)} - Ct_{(ABL)}$.[15] The data were presented as $\log_{10}$ values of the relative quantity of each gene.

In addition, we used miRNA expression profile data assessed by quantitative PCR from a previously published manuscript; data available for 27 out of the 102 symptomatic multiple myeloma patients (GEO accession number: GSE16558).[2]

The cell cycle was analyzed by multiparametric flow using a CD38/CD138 propidium iodide double-staining technique, as previously described[16] in a subset of 80 symptomatic multiple myeloma patients.

The Mann-Whitney U test was used to identify statistically significant differences between groups. Progression-free survival (PFS) and overall survival (OS) distribution curves were plotted using the Kaplan-Meier method; the log rank test was used to estimate the statistical significance of differences between the curves. All statistical analyses were conducted using SPSS (version 15.0; SPSS Inc., Chicago, IL, USA).

Results and Discussion

The expression values of Dicer were significantly higher in monoclonal gammopathy of undetermined significance than in smoldering myeloma (mean±SD, 0.84±0.36 *vs.* 0.60±0.23; *P*=0.013) and symptomatic multiple myeloma (mean±SD, 0.84±0.36 *vs.* 0.62±0.51; *P*=0.007). However, no differences were found between smoldering myeloma and multiple myeloma (Figure 1). Dicer expression level in normal plasma cells (NPC) from healthy donors was very similar to monoclonal gammopathy of undetermined significance (mean±SD, 0.92±0.72 *vs.* 0.84±0.36). No relevant differences were found in Drosha expression between these entities, although the series of multiple myeloma patients analyzed was rather short (n=49). These data show that monoclonal gammopathy of undetermined significance, a pre-malignant condition, displays higher levels of Dicer than both smoldering and symptomatic myelo-

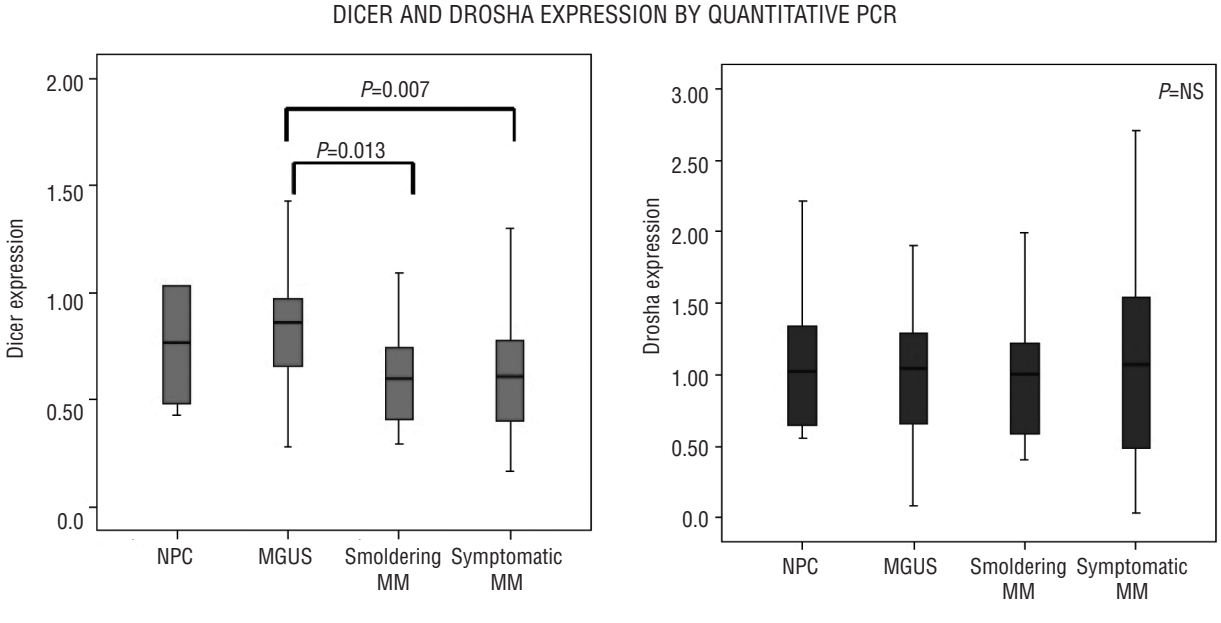

Figure 1. Expression of Dicer and Drosha by real-time quantitative PCR in Normal plasma cells (NPC), monoclonal gammopathy of undetermined significance (MGUS), smoldering myeloma (SMM) and symptomatic multiple myeloma (MM) patients. Relative values were calculated by the $2^{-\Delta Ct}$ method ($\Delta Ct = Ct_{(Gene)} - Ct_{(ABL)}$). The *ABL* gene was used as a control gene.

ma, and very similar to those in normal plasma cells. These findings are concordant with those described in lung cancer, which showed upregulation of Dicer in the precursor lesions and reduced expression in the more aggressive stages,[7] as well as in ovarian cancer, with low Dicer mRNA levels in advanced disease.

To validate these results, we evaluated Dicer expression levels generated by Affymetrix microarray platform. We used data for 60 symptomatic multiple myeloma patients and normal plasma cells from 5 healthy controls of bone marrow (data are available to the public on the NIH GEO [http://www.ncbi.nlm.nih.gov/geo/] under accession number GSE16558)[2] and a set of 20 monoclonal gammopathy of undetermined significance and 33 smoldering myeloma samples also analyzed in our institution (JF San Miguel, unpublished data, 2011). All the samples had been analyzed with the Human Gene 1.0 ST Array. We observed that the expression values of Dicer were significantly higher in normal plasma cells, monoclonal gammopathy of undetermined significance and smoldering myeloma than in symptomatic multiple myeloma (P=1.8×10^{-5}, P=5.2×10^{-6}, P=5×10^{-7}, respectively). No differences were found between monoclonal gammopathy of undetermined significance and smoldering myeloma (Figure 2). Maybe the different samples used for this comparison can explain these slightly different findings. Nevertheless, these results confirm that Dicer expression was down-regulated in symptomatic multiple myeloma compared to normal plasma cells and monoclonal gammopathy of undetermined significance.

A global increase of microRNA expression by Dicer upregulation has been suggested in several tumors.[11,12] To explore this possibility, we reviewed our recently published data on miRNA expression profile assessed by quantitative PCR in order to compare this among high and low Dicer multiple myeloma patients (n=27).[2] To define high and low Dicer expression levels, we selected a cut-off value of 0.66, since this was close to the median Dicer

Table 1. List of miRNAs significantly up-regulated in the group of MM patients showing high levels of Dicer expression (P<0.01).

MiRNA	*P* value
miR-7a	0.006
miR-7b	0.007
miR-7d	0.002
miR-7e	0.01
miR-7g	0.005
miR-93	0.009
miR-142-3p	0.007
miR-142-5p	0.011
miR-218	0.015
miR-223	0.009
miR-301	0.005
miR-26b	0.003
miR-103	0.001
miR-125a	0.006
miR-497	0.001
miR-30c	0.006
miR-206	0.009
miR-371	0.009
miR-198	0.009
miR-28	0.005
miR-32	0.003
miR-149	0.007
miR-196b	0.013
miR-325	0.008
miR-328	0.002
miR-342	0.0005
miR-500	0.008
miR-139	0.008
miR-145	0.004
miR-425	0.013
miR-591	0.008
miR-601	0.001

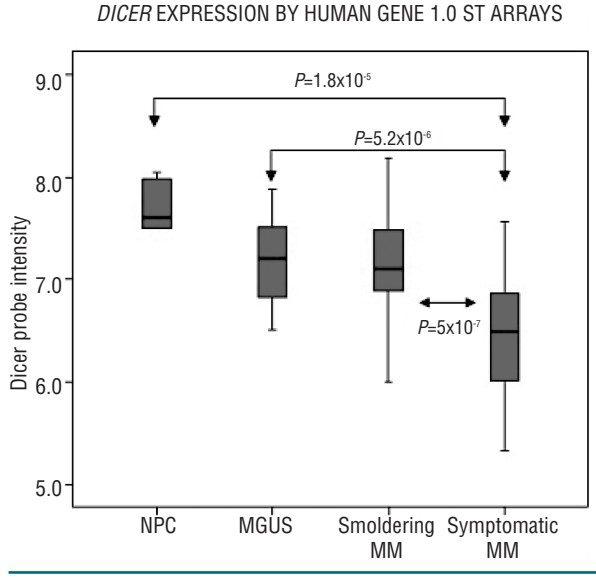

Figure 2. Expression of Dicer by Human Gene 1.0 ST Affymetrix Array in normal plasma cells (NPC), monoclonal gammopathy of undetermined significance (MGUS), smoldering myeloma (SMM) and symptomatic multiple myeloma (MM) patients.

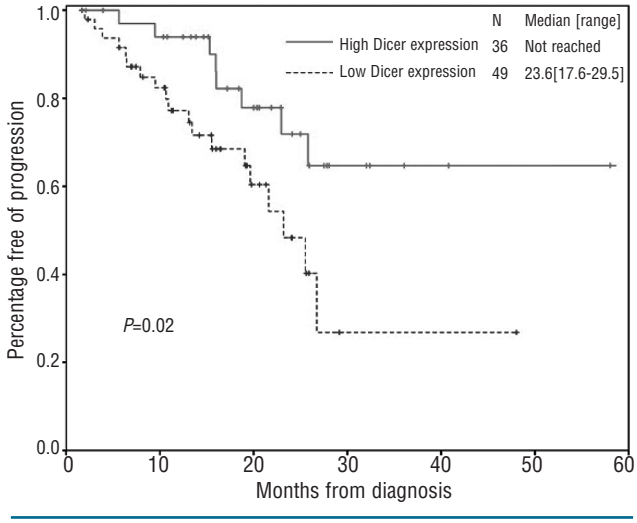

Figure 3. Progression-free survival in symptomatic multiple myeloma patients (n=85) with respect to Dicer gene expression level.

expression in the multiple myeloma samples. We analyzed the 368 miRNA, previously normalized with the median values of RNU44-RNU48 control miRNA (Delta CT values). We found significant differences ($P<0.01$) in 32 miRNAs (8.7%), whose expression profile displayed higher values in the group of patients with high Dicer levels (Table 1).

Zhou *et al.* have recently reported that the silencing of Dicer is associated with significantly enhanced G_0– to G_1–phase accumulation and greatly increased apoptosis.[17] To assess whether the high levels of Dicer expression are related to cell-cycle activation, we compared the distribution of plasma cells among G_0-G_1, S and G_2-M phases in the symptomatic multiple myeloma patients divided into two groups: high *versus* low levels of Dicer expression. We failed to find differences in cell-cycle distribution between the two groups, and there was no cell-cycle arrest in those patients with the lowest expression levels of Dicer; these results apparently differ from those of Zhou *et al.* but the discrepancies can be explained by differences in the sample material analyzed (cell lines and fresh tumor cells from patients), and also because the silencing *in vitro* of Dicer expression may have different effects than its pathophysiological downregulation.

Finally, we analyzed the impact of Dicer expression levels in the clinical outcome of 85 multiple myeloma patients treated according to the GEM05 trial. We found no significant differences in the clinical or biological features of multiple myeloma patients between the high and low Dicer expression level groups. Additionally, we evaluated the expression of Dicer according to the ploidy status, and we could not find any noteworthy differences among the hyperdiploid and non-hyperdiploid patients. The most relevant genetic abnormalities (*IGH* translocations, *RB* and *P53* deletions) were also evaluated, but there was no association between these genetic features and Dicer status. However, the median progression-free survival was significantly longer in multiple myeloma patients with a high expression level of Dicer (not reached *vs.* 23.6 months; $P=0.02$) as compared with those with weak Dicer expression (Figure 3). When we selected only those patients under 65 years of age who received high-dose melphalan followed by autologous stem cell transplantation, progression-free survival tended to be longer in patients with high levels of Dicer (not reached *vs.* 26), although the differences were not statistically significant probably because of the small number of patients (*data not shown*). As mentioned above, the prognostic impact of the enzymes involved in the miRNA processing machinery has been widely explored in solid tumors.[7-9;11] By contrast, to the best of our knowledge, the prognostic influence of Dicer expression in hematologic malignancies has only been analyzed in a group of 71 acute myeloid leukemia patients, in whom no influence in outcome was apparent.[13]

Our results show that Dicer expression levels are upregulated in monoclonal gammopathy patients with a premalignant condition compared with smoldering and symptomatic multiple myeloma. Moreover, in line with this finding, we observed that in symptomatic multiple myeloma patients the progression-free survival is longer for cases with high levels of Dicer.

Authorship and Disclosures

The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.

Financial and other disclosures provided by the authors using the ICMJE (www.icmje.org) Uniform Format for Disclosure of Competing Interests are also available at www.haematologica.org.

References

1. Calin GA, Croce CM. MicroRNA signatures in human cancers. Nat Rev Cancer. 2006;6(11):857-66.
2. Gutierrez NC, Sarasquete ME, Misiewicz-Krzeminska I, Delgado M, De Las RJ, Ticona FV, et al. Deregulation of microRNA expression in the different genetic subtypes of multiple myeloma and correlation with gene expression profiling. Leukemia. 2010;24 (3):629-37.
3. Li Z, Lu J, Sun M, Mi S, Zhang H, Luo RT, et al. Distinct microRNA expression profiles in acute myeloid leukemia with common translocations. Proc Natl Acad Sci USA. 2008;105(40):15535-40.
4. Ji J, Shi J, Budhu A, Yu Z, Forgues M, Roessler S, et al. MicroRNA expression, survival, and response to interferon in liver cancer. N Engl J Med. 2009;361(15):1437-47.
5. Yanaihara N, Caplen N, Bowman E, Seike M, Kumamoto K, Yi M, et al. Unique microRNA molecular profiles in lung cancer diagnosis and prognosis. Cancer Cell. 2006;9(3):189-98.
6. Iorio MV, Ferracin M, Liu CG, Veronese A, Spizzo R, Sabbioni S, et al. MicroRNA gene expression deregulation in human breast cancer. Cancer Res. 2005;65(16):7065-70.
7. Chiosea S, Jelezcova E, Chandran U, Luo J, Mantha G, Sobol RW, et al. Overexpression of Dicer in precursor lesions of lung adenocarcinoma. Cancer Res. 2007;67(5):2345-50.
8. Chiosea SI, Barnes EL, Lai SY, Egloff AM, Sargent RL, Hunt JL, et al. Mucoepidermoid carcinoma of upper aerodigestive tract: clinicopathologic study of 78 cases with immunohistochemical analysis of Dicer expression. Virchows Arch. 2008;452(6): 629-35.
9. Merritt WM, Lin YG, Han LY, Kamat AA, Spannuth WA, Schmandt R, et al. Dicer, Drosha, and outcomes in patients with ovarian cancer. N Engl J Med. 2008;359(25): 2641-50.
10. Sugito N, Ishiguro H, Kuwabara Y, Kimura M, Mitsui A, Kurehara H, et al. RNASEN regulates cell proliferation and affects survival in esophageal cancer patients. Clin Cancer Res. 2006;12(24):7322-8.
11. Lin RJ, Lin YC, Chen J, Kuo HH, Chen YY, Diccianni MB, et al. microRNA signature and expression of Dicer and Drosha can predict prognosis and delineate risk groups in neuroblastoma. Cancer Res. 2010;70(20): 7841-50.
12. Chiosea S, Jelezcova E, Chandran U, Acquafondata M, McHale T, Sobol RW, et al. Up-regulation of dicer, a component of the MicroRNA machinery, in prostate adenocarcinoma. Am J Pathol. 2006;169(5): 1812-20.
13. Martin MG, Payton JE, Link DC. Dicer and outcomes in patients with acute myeloid leukemia (AML). Leuk Res. 2009;33(8): e127.
14. Mateos MV, Oriol A, Martinez-Lopez J, Gutierrez N, Teruel AI, de Paz R, et al. Bortezomib, melphalan, and prednisone versus bortezomib, thalidomide, and prednisone as induction therapy followed by maintenance treatment with bortezomib and thalidomide versus bortezomib and prednisone in elderly patients with untreated multiple myeloma: a randomised trial. Lancet Oncol. 2010;11(10):934-41.
15. Sarasquete ME, Garcia-Sanz R, Armellini A, Fuertes M, Martin-Jimenez P, Sierra M, et al. The association of increased p14ARF/p16INK4a and p15INK4a gene expression with proliferative activity and the clinical course of multiple myeloma. Haematologica. 2006;91(11):1551-4.
16. Almeida J, Orfao A, Ocqueteau M, Mateo G, Corral M, Caballero MD, et al. High-sensitive immunophenotyping and DNA ploidy studies for the investigation of minimal residual disease in multiple myeloma. Br J Haematol. 1999;107(1):121-31.
17. Zhou Y, Chen L, Barlogie B, Stephens O, Wu X, Williams DR, et al. High-risk myeloma is associated with global elevation of miRNAs and overexpression of EIF2C2/AGO2. Proc Natl Acad Sci USA. 2010;107(17):7904-9.