*Unitat de Bioquímica, Departament de Ciències Fisiològiques I, Facultat de Medicina, Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Universitat de Barcelona, Spain; °Unitat d'Eritropatologia, IDIBAPS, Hospital Clínic, Facultat de Medicina, Universitat de Barcelona, Spain

Funding: this work was supported by Ministerio de Ciencia y Tecnología (Grants 99PM0040 and SAF-2001-1205) and by FIS (Instituto de Salud Carlos III, Red de Centros RCMN C03/08). We thank Miss Christine O'Hara for language revision.

Key words: phosphoglycerate mutase, PGAM, BB isoenzyme.

Correspondence: Fernando Climent, Unitat de Bioquímica, Facultat de Medicina, Universitat de Barcelona, Casanova 143, 08036 Barcelona, Spain. E-mail: fcliment@ub.edu

References

1. Fothergill-Gilmore L. Watson, H. The phosphoglycerate mutases. Adv Enzymol 1989;62:227-313.
2. Toscano A, Tsujino S, Vita G, Shanske S, Messina C, Di Mauro S. Molecular basis of muscle phosphoglycerate mutase (PGAM-M) deficiency in the Italian kindred. Muscle Nerve 1996;19:1134-7.
3. Tsujino S, Shanske S, Sakoda S, Toscano A, DiMauro S. Molecular genetic studies in muscle phosphoglycerate mutase (PGAM-M) deficiency. Muscle Nerve 1995;Suppl 3:S50-S3.
4. Repiso A, Pérez de la Ossa P, Avilés X, Oliva B, Juncá J, Oliva R, et al. Red blood cell phosphoglycerate mutase. Description of the first human BB isoenzyme mutation. Haematologica 2003;88:ECR07.
5. Castellá J, Ureña J, Ludevid D, Carreras J, Climent F. Immunological properties of rat phosphoglycerate mutase isozymes. Biochim Biophys Acta 1988;956:97-102.
6. Minakami S, Suzuki C, Saito T, Yoshikawa H. Studies on erythrocyte glycolysis. Determination of the glycolytic intermediates in human erythrocytes. J Biol Chem 1965;58:543-50.
7. Andrés V, Carreras J, Cussó R. Regulation of muscle phosphofructokinase by physiological concentrations of bisphosphorylated hexoses: effect of alkalinization. Biochem Biophys Res Comm 1990;172:238-334.

Chronic Myeloproliferative Disorders

Clonality analysis by *HUMARA* assay in Spanish females with essential thrombocythemia and polycythemia vera

Analysis of the human androgen receptor gene (*HUMARA*) allows clonality to be assessed in essential thrombocythemia (ET) and polycythemia vera (PV). We studied clonality in 44 patients with ET, 18 with PV and in 64 healthy controls. The X-chromosome inactivation pattern was analyzed by HUMARA-polymerase chain reaction on DNA from purified granulocytes, T lymphocytes and the CD3⁻ fraction of mononuclear cells.

haematologica 2005; 90:259-261
(http://www.haematologica.org/journal/2005/2/259.html)

Essential thrombocythemia (ET) and polycythemia vera (PV) are clonal myeloproliferative disorders without consistent karyotypic and molecular markers. *HUMARA* analysis, an assay based on X-chromosome inactivation patterns (XCIP), allows clonality to be assessed in ET and PV. There is controversy over the percentage of clonal hematopoiesis in ET and little is known about this feature in PV patients.

The present study was conducted in a cohort of Spanish ET and PV patients to determine: (i) the percentage of clonality in these disorders; (ii) differences in frequencies of clonality depending on whether granulocytes or CD3⁻ mononuclear cells are taken as the pathologic tissues and, (iii) the relationship between clonality status and the appearance of thrombotic and hemorrhagic complications. One hundred and twenty-six people were included in this study; 44 had ET, 18 had PV and 64 were healthy, age-matched controls. The diagnoses of ET and PV were established according to the PVSG criteria.

Mononuclear cells and granulocytes were isolated from peripheral blood. CD3⁺ lymphocytes were then separated from the CD3⁻ fraction by magnetic beads. Genomic DNA was extracted from each fraction. The *HUMARA* assay was performed according to normal procedures in our laboratory. Clonality was calculated after correcting for the degree of lyonization in CD3⁺ cells. Cells were considered to be clonally derived when the corrected allele ratio was <0.25. The sensitivity and specificity of the *HUMARA* assay in our study were 39% and 94%, respectively. The rate of heterozygosity of the *HUMARA* gene in our Spanish women was 88%. Table 1 shows the clinical data and *HUMARA* results from the ET and PV patients and the controls. We found significant differences in clonality results between controls and patients ($p<0.001$). Clonality in normal controls has also been reported by other authors[1-9] (Table 2). Champion *et al.* suggested that this phenomenon could be more frequent in elderly women, probably due to constitutive skewing, age-related acquired skewing or genetic factors leading to selective pressure.[2] This might suggest that imbalanced myeloid XCIP in the presence of balanced T cell XCIP in elderly patients (>65 years) should not be certainly interpreted as clonal hematopoiesis.

A wide range of positivity for clonality has been reported in ET patients (18.7-68%).[1-9] These differences might be attributed to methodology, number of studied patients and criteria used to define a clonal pattern (allele ratios or the equation of Asimakopoulos *et al.*[10]) (Table 2). There are two studies reporting on clonality in PV. In one, 26/41 patients (8 ET, 30 PV and 3 primary myelofibrosis) had clonal proliferation,[2] and in the other, in which 31 PV patients were studied, hematopoiesis was clonal in 16/22.[6]

Clonality analysis in ET and PV has mainly been performed using granulocytes and platelets as target cells. A major limitation of platelets is that the study has to be performed on mRNA instead of DNA, using polymorphisms that are less heterozygous than the *HUMARA* gene. For this reason, we investigated not only granulocytes but also CD3⁻ mononuclear cells as these have been suggested to share a common abnormal hematopoietic precursor cell. Our data show that when CD3⁻ cells were tested instead of granulocytes, clonality was not detected in 13 patients and therefore a substantial number of clonal results could be missed ($p<0.001$) (Table 1).

Although no significant correlation was found between clonality status and the patients' age, there was a statistical trend to more clonal cases among elderly (>65 year) women than among younger women ($p=0.180$). This trend, not significant in our study, had been found to be statistically significant by other authors.[4,9] When skewed cases were considered, there was statistical trend of more skewed cases in elderly women ($p=0.075$).

It is known that the prevalence of thrombotic complications is strongly influenced by a patient's age and duration of follow-up. In addition, some authors observed that thrombosis occurred more frequently in clonal than in polyclonal ET patients.[5,7,9] In contrast, El-Kassar *et al.*[3]

Table 1. Clinical data and *HUMARA* results from ET and PV patients.

Disease	*Age in years (mean)*	*CD3⁺/CD3⁻*	*CD3⁺/granulocytes*	*Thrombohemorrhagic complications*	*Treatment**
Essential Thrombocythemia (n=44)	32-91 (61.4)	6 homozygous 4 clonal 27 polyclonal 7 skewed	6 homozygous 11 clonal 20 polyclonal 7 skewed	11 patients (2 homozygous, 3 clonal, 4 polyclonal, 2 skewed)	21 patients
Polycythemia Vera (n=18)	30-86 (62.6)	1 homozygous 1 clonal 14 polyclonal 2 skewed	1 homozygous 7 clonal 8 polyclonal 2 skewed	4 patients (1 homozygous, 3 clonal)	7 patients
Controls (n=64)	30-91 (62)	8 homozygous 2 clonal 50 polyclonal 4 skewed	8 homozygous 3 clonal 49 polyclonal 4 skewed	0 patients	0 patients

**Patients were receiving myelosupressive/platelet-lowering agents as follows: hydroxyurea ± acetylsalicylic acid (ASA) (n=19); anagrelide ± ASA (n=8) and busulfan (n=1). Twelve patients only received ASA. Phlebotomy was performed when packed red cell volume was higher than 0.42 L/L.*

Table 2. Review of the literature concerning clonality in ET and PV studied by the *HUMARA* assay.

Study	*Cases*	*Age in years*	*CD3 separation methods*	*Definition of clonality*	*Cut-off level of clonality*	*Homozygous*	*Treated*	*Clonal*	*Non informative*	*Clonal controls*	*Complications[1]*
El-Kassar[1]	26 ET[2]	29-81	No separation	Allele ratios[8]	80%	10	10/16	3/16	9/16	5/23	NA
Champion[2]	8 ET 30 PV 3 PM	60-88	Magnetic beads	Asimakopoulos equation[7]	>50% (Rg <0.33 and Rt= 1.0)[7]	0	NA	26/41	1/41	15/65	NA
El-Kassar[3]	17 ET3	13-70	Magnetic beads	Allele ratios[8]	80%	0	11/17	9/17	4/17	4/44	5/9 clonal 2/4 polyclonal
El-Kassar[4]	53 ET	13-80	Magnetic beads	Allele ratios[8]	80%	4	18/30	31/49	5/49	NA	NC
Harrison[5]	43 ET[4]	11-89	Magnetic beads	Allele ratios[8]	80%	0	NA	10/43	20/43	0/9	6/10 clonal[5] 2/13 polyclonal
Mitterbauer[6]	23 ET 31 PV	27-88	NA	Allele ratios[8]	75% (allele ratio >3:1)	3 ET 9 PV	NA	17/20 16/22	0/20 0/22	22/242[6]	NA
Chiusolo[7]	40 ET	20-63	Magnetic beads	Allele ratios[8]	75% (allele ratio >3:1)	0	34/40	17/40	8/40	NA	7/17 clonal[5] 1/15 polyclonal
Shih[8]	73 IT	18-92	RBC rosetting	Asimakopoulos equation[7]	>50%(Rg <0.33 and Rt= 1.0)[7]	9	NA	42/64	10/64	12/43	NA
Shih[9]	89 ET	15-92	RBC rosetting	Asimakopoulos equation[7]	>50%(Rg <0.33 and Rt= 1.0)[7]	10	NA	54/79	10/79	NA	24/54 clonal[5] 1/15 polyclonal
Present study	44 ET 18 PV	32-91 30-86	Magnetic beads	Allele ratios[8]	75% (allele ratio >3:1)	6 ET 1 PV	21/44 9/18	11/38 7/17	7/38 1/17	3/64	3/11 clonal ET 4/20 polyclonal ET 3/7 clonal PV 0/8 polyclonal PV

[1]Thrombotic or hemorrhagic; [2]we excluded 16 patients because the HUMARA assay had been performed in DNA from unfractionated blood; [3]we excluded 29 patients because polymorphisms (P55, IDS, G6PD) other than the HUMARA gene were used to study clonality in these patients; [4]we excluded 3 patients because polymorphisms other than the HUMARA gene were used to study clonality in these patients; [5]thrombotic complications were significantly higher in clonal ET ($p<0.05$); [6]control subjects included healthy women, those with secondary neutrophilia and reactive thrombocytosis; [7]percentage of clonal granulocytes (Gc) was calculated using the following equation,[10] when Rt (T-cell ratio) > Rg (granulocyte ratio); Gc: Rt-Rg / Rt(Rg+1) × 100; when Rt < Rg Gc: Rg-Rt / Rg+1 × 100. This equation divides XCIP into 3 categories: 1) clonal, when Gc>50% (corresponds to a Rg <0.33 in the presence of an Rt: 1.0); 2) polyclonal, when Gc<50% and Rt>0.33; 3) ambiguous, when Gc<50% and Rt<0.33; [8]Expression of the lower allele after correcting for the degree of lyonization in T-lymphocytes. ET: essential thrombocythemia; PV: polycythemia vera; PM: primary myelofibrosis; IT: idiopathic thrombocytosis; NA: not available; NC: not conclusive.

found no correlation between the incidence of ischemic or hemorrhagic episodes and XCIP. Our data do not show a significant relationship between clonality status and retrospectively analyzed thrombohemorrhagic events in ET (p=0.676). These discrepant results are probably due to differences in the size of the populations studied. Among PV patients, we found a statistical trend of more clinical complications in clonal than in polyclonal patients (p=0.075). This observation has not been reported before and should be confirmed in future larger series. In addition, for the whole group (ET and PV patients), no relationship was found between clonal or polyclonal hematopoiesis and platelet lowering treatments. Further studies of clonality in ET and PV patients should be prospectively done in order to determine the pathophysiology of these diseases in detail and to ascertain the possible clinical implications of clonal or polyclonal hematopoiesis.

Lurdes Zamora,[°#] Blanca Espinet,[*#@] Lourdes Florensa,[#@^] Carles Besses,[#^] Beatriz Bellosillo,[*#] Francesc Solé[*@]*

*Laboratori de Citogenètica i Biologia Molecular, Servei de Patologia, Hospital del Mar, Barcelona; °Unitat d'Antropologia, Departament de Biologia Animal, Vegetal i Ecologia, Universitat Autonoma de Barcelona, Bellaterra; #Unitat de Recerca Translacional en Neoplasies Hematològiques (URNHE-PRBB); @Escola de Citologia Hematològica S. Woessner-IMAS; ^Laboratori de Citologia Hematològica, Servei de Patologia; §Servei d'Hematologia Clínica, Hospital del Mar, Barcelona, Spain

Key words: HUMARA, essential thrombocythemia, polycythemia vera, clonality.

Acknowledgments: the authors wish to express their gratitude to Dr. M. Albitar (University of Texas MD Anderson Cancer Center, Houston) for his valuable advice on the development of the HUMARA assay and to Mercè Musset, Rosa M. Vila, Carme Melero and Rosa Navarro for their valuable technical contribution to the study. Finally, Joan Vila (IMIM) helped in the statistical analysis.

Funding: this work was supported by grants PI030345 from the "Ministerio de Sanidad y Consumo", SAF-2001-4947 from the "Ministerio de Ciencia y Tecnología", and Red de Centros de Cáncer C03/07 from the "Instituto de Salud Carlos III", Spain.

Correspondence: Lurdes Zamora, Laboratori de Citogenètica i Biologia Molecular, Servei de Patologia, Hospital del Mar, Pg Marítim, 25-29, Barcelona 08003, Spain. Phone: international +34.93.248.30.35. Fax: international +34.93.2483131. E-mail: lurdes.zamora@wanadoo.es

References

1. El-Kassar N, Hetet G, Li Y, Briere J, Grandchamp B. Clonal analysis of haemopoietic cells in essential thrombocythaemia. Br J Haematol 1995;90:131-7.
2. Champion KM, Gilbert JG, Asimakopoulos FA, Hinshelwood S, Green AR. Clonal haemopoiesis in normal elderly women: implications for the myeloproliferative disorders and myelodysplastic syndromes. Br J Haematol 1997;97:920-6.
3. El-Kassar N, Hetet G, Briere J, Grandchamp B. Clonality analysis of hematopoiesis in essential thrombocythemia: advantages of studying T lymphocytes and platelets. Blood 1997; 89: 128-34.
4. El-Kassar N, Hetet G, Briere J, Grandchamp B. Clonality analysis of hematopoiesis and thrombopoietin levels in patients with essential thrombocythemia. Leuk Lymphoma 1998; 30:181-8.
5. Harrison CN, Gale RE, Machin SJ, Linch DC. A large proportion of patients with a diagnosis of essential thrombocythemia do not have a clonal disorder and may be at lower risk of thrombotic complications. Blood 1999;93:417-24.
6. Mitterbauer G, Winkler K, Gisslinger H, Geissler K, Lechner K, Mannhalter C. Clonality analysis using X-chromosome inactivation at the human androgen receptor gene (HUMARA). Evaluation of large cohorts of patients with chronic myeloproliferative diseases, secondary neutrophilia, and reactive thrombocytosis. Am J Clin Pathol 1999;112:93-100.
7. Chiusolo P, La Barbera EO, Laurenti L, Piccirillo N, Sora F, Giordano G, et al. Clonal hemopoiesis and risk of thrombosis in young female patients with essential thrombocythemia. Exp Hematol 2001;29:670-6.
8. Shih LY, Lin TL, Dunn P, Wu JH, Tseng CP, Lai CL, et al. Clonality analysis using X-chromosome inactivation patterns by HUMARA-PCR assay in female controls and patients with idiopathic thrombocytosis in Taiwan. Exp Hematol 2001; 29: 202-8.
9. Shih LY, Lin TL, Lai CL, Dunn P, Wu JH, Wang PN, et al. Predictive values of X-chromosome inactivation patterns and clinicohematologic parameters for vascular complications in female patients with essential thrombocythemia. Blood 2002; 100:1596-601.
10. Asimakopoulos FA, Gilbert JG, Aldred MA, Pearson TC, Green AR. Interstitial deletion constitutes the major mechanism for loss of heterozygosity on chromosome 20q in polycythemia vera. Blood 1996;88:2690-8.

Chronic Myeloproliferative Disorders

Safety profile of hydroxyurea in the treatment of patients with Philadelphia-negative chronic myeloproliferative disorders

The efficacy of hydroxyurea (HU) in myeloproliferative disorders is well documented. HU controls thrombocytosis both in polycythemia vera (PV) and in essential thrombocythemia (ET), while reducing the risk of thrombosis.[1] Despite many anectodal reports, no evaluation of the prevalence and type of side effects of HU exists in large series of patients.

haematologica 2005; 90:261-262
(http://www.haematologica.org/journal/2005/2/261.html)

This is a retrospective study of 75 patients with ET and 54 with PV (53 males, 76 females, mean age 58,12±14,68 years) diagnosed in agreement with the Polycythemia Vera Study Group criteria[2,3] and treated with HU (median follow-up 7.18 years) at our Department over the last 20 years. HU (Oncocarbide®, 500 mg) was given to reduce platelet count (induction dosage 30 mg/kg/day and maintenance 15 mg/kg/day), for one or more of the following reasons: (i) age over 60 years; (ii) previous major thrombotic event; and (iii) platelet count over $1,500\times10^9$/L.[1] The achievement of a platelet count $<600\times10^9$/L was considered a complete response (CR), while, if the platelet count remained $>600\times10^9$/L within 6 months of HU therapy, the drug treatment was considered to have failed. All side effects and toxic effects of HU were recorded; if a side effect did not require drug withdrawal it was considered a minor effect.

One hundred and twenty patients (93%) achieved a CR in a median time of 1 month (range 1-13 months). In 9 cases the drug treatment failed (7%). In 106 patients (88% of CR) HU was successfully continued without relevant complications over a median follow-up of 6.74 years to date (median 3.67 years). No hemorrhages but 15 thrombotic complications (4 cerebrovascular, 5 coronary, 1 peripheral arteries and 5 deep vein thrombosis in 2 cases with pulmonary embolism) were documented during HU therapy.

Minor effects. An increase of MCV (>98 fL) over normal levels occurred in 54 patients (45% of responders) within the first 6 months of therapy; this is a well known and usually negligible side effect of HU. However, 19 females and 13 males developed severe macrocytosis (MCV > 110 fL) within one year of treatment. One of these patients had black nail pigmentation which disappeared within three months after drug discontinuation; two more patients had black nail pigmentation, which was stable over time. This is an uncommon finding of not paramount importance.

Major effects. In 2 patients the drug was withdrawn because of symptomatic anemia (Hb less than 85 g/L) with MCV >130 fL occurred.[4] In both cases, more than 20 mg/kg/day HU was given. Twelve patients came off the drug because of toxic effects certainly or probably due to HU (2 fevers, 2 allergies, 4 leg ulcers, 3 acute leukemias, 1 cancer) (Table 1). This rate of necessary HU discontinuation was 1.6×100 patients/year.

Fever occurred after 2 and 3.5 days of therapy in 2 patients. In both cases, we made another treatment attempt which again resulted in fever. Fever can be