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Clonal chromosome anomalies and propensity to myeloid malignancies in congenital amegakaryocytic thrombocytopenia (OMIM 604498)

Congenital amegakaryocytic thrombocytopenia (CAMT, OMIM 604498) is an autosomal recessive disorder characterized by absent or reduced number of megakaryocytes in the bone marrow (BM) since birth, elevated serum levels of thrombopoietin (TPO), and very low platelet count. Prognosis of CAMT patients is poor, because all develop in childhood a tri-linear marrow aplasia that is always fatal when untreated.[1,2] Mutations of the *MPL* gene (OMIM 159530), coding for the TPO receptor,[3] are responsible for CAMT.[4,5] We report the cytogenetic investigations and the results of analysis by fluorescent *in situ* hybridization (FISH) on 5 unrelated Italian patients whose clinical characteristics and *MPL* gene mutations have already been reported.[5] Three patients were females and two males, age at diagnosis ranging between 16 and 49 months. All children developed pancytopenia at an age comprised between 22 and 49 months. Patients' designation (CAMT1-CAMT5 in Table 1) is as in Savoia *et al*.[5]

Chromosome analyses were repeatedly performed on BM and peripheral blood (PB) PHA-stimulated cultures with routine methods. Skin fibroblasts (SF) were cultured with routine methods in patient CAMT2. QFQ-banding technique was used for all chromosome analyses. FISH analyses on interphase nuclei were repeatedly performed on all patients' BM, on PB of CAMT4, and on SF of CAMT2 with centromere-specific probes for chromosomes 7 (D7Z1), and 8 (D8Z2) (Cytocell Technologies, Cambridge, UK) either with single fluorochromes or in dual color combination. Nuclei from healthy subjects were used as control.

All the results are detailed in Table 1. The karyotype was consistently normal in patients CAMT1, CAMT3, and CAMT5, and parallel FISH analysis on interphase nuclei from BM confirmed the normal disomies 7 and 8. DEB test performed on PB of CAMT1, CAMT2, and CAMT3 excluded Fanconi anemia (FA).

Patient CAMT2 progressed to pancytopenia, with normal BM and PB karyotype, at the age of 30 months (January 2005), but in a subsequent analysis on BM, in May 2006, trisomy 8 was found in 1 mitosis out of 24 and 5 nuclei out of 616. Analysis on fibroblasts from a skin biopsy excluded a constitutional trisomy 8 mosaicism (Table 1). In patient CAMT4, the karyotype was normal in BM cells in 2001, at the age of 12 months, and in 2004 when progression to pancytopenia was observed, but in May 2006 a BM clone with monosomy 7 was found which persisted in the following analyses (Table 1).

A risk of evolution into myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML) has often been assumed for CAMT, but in a search for *bona fide* CAMT patients who developed MDS/AML, we found only 3 such cases in the literature, and none of these was proved to carry mutations of the *MPL* gene: they are one refractory anemia with excess of blasts (RAEB) reported by King *et al*.,[2] and 2 cases mentioned by Alter,[6] a male with acute myelomonocytic leukemia (AMML) developed after an aplastic anemia phase, and a female with a pre-leukemic condition. These 2 latter cases were never reported in more detail, and were studied some decades ago.[6] In addition, a report is available of a CAMT patient with MPL mutations who developed a pre-B acute lymphoblastic leukemia.[7]

A review of CAMT cases with chromosome anomalies is even more difficult to carry out since cytogenetic results in the literature are often incorrectly mentioned, incomplete, and probably questionable. Among the patients with MDS/AML mentioned above, clonal chromosome changes in the BM were reported to be present in 2: the child with RAEB[2] with trisomy 21 in 10-15% of the cells, and the patient with AMML[6] with different anomalies of chromosome 19 (monosomy, trisomy, deletion) in 11 cells out of 55. As to CAMT without MDS/AML, King *et al*.[2] reported 2 patients with possible chromosome anomalies in PB, but the case identified as CAMT9 showed a translocation only in one cell out of 19, whereas CAMT13 showed a supernumerary marker (not better defined) in 94% of the cells. The only case from the literature with a reliable clonal anomaly in the BM was reported by Steele *et al*.,[8] who found monosomy 7 in 12 out of 20 mitoses and 91 out of 200 interphase nuclei. Mutations of the *MPL* gene were identified in these 3 patients.[4,8]

In our 5 patients, BM clonal anomalies were found in 2, in the absence of MDS/AML: one case of monosomy 7 and one of trisomy 8; the latter was demonstrated not to be a constitutional mosaicism, as is the case in 15-20% of patients with MDS/AML and trisomy 8.[9] While no exhaustive cytogenetic study on CAMT is available, our small group of patients was monitored over time for possible clonal chromosome anomalies in BM. We suggest that clonal chromosome changes are frequently acquired in the BM of CAMT patients: they often seem to be the most typical of MDS, monosomy 7 and trisomy 8. Interestingly, in both our patients the abnormal clones were found when the disease had already progressed to pancytopenia (Table 1), and the patient reported by Steele and co-workers showed monosomy 7 when CAMT had evolved to BM aplasia.[8]

In both our patients, the abnormal clone was detected not at the first chromosome analysis but after and showed a trend to expansion (Table 1). In particular, if we take into account evaluations by interphase FISH, in patient

Table 1. Results of chromosome analyses and FISH on interphase nuclei.

Patient	*Date*	*T/P*[1]	*Material*[2]	*Karyotype*	*FISH on nuclei: D7Z1 probe, D8Z2 probe*[3]
CAMT1	03.11.04	T	BM	46,XX[20]	D7Z1: 450/467 normal[4] (96.3%); D8Z2: 557/571 normal (97%)
	06.12.04	T	PB-PHA	46,XX[100]	
	20.01.05	T	PB-DEB	46,XX[100]	
			BM	46,XX[7]	D7Z1: 450/458 normal (98.2%); D8Z2: 500/513 normal (97.4%)
CAMT2	11.01.05	T	BM	46,XX[8]	
			PB-PHA	46,XX[100]	
			PB-DEB	46,XX[100]	
	18.11.05	T	BM	46,XX[12]	
	23.05.06	P	BM	47,XX,+8[1]/46,XX[23]	D8Z2: 5/616 with trisomy 8 (0.8%)
	06.07.06	P	BM	47,XX,+8[5]/46,XX[22]	D8Z2: 9/332 with trisomy 8 (2.7%)
			SF	46,XX[56]	D8Z2: 1314/1342 normal (97.9%)
CAMT3	12.01.06	T	PB-PHA	46,XY[100]	
			PB-DEB	46,XY[100]	
			BM	46,XY[22]	D7Z1: 450/461 normal (97.6%); D8Z2: 400/415 normal (96.4%)
	24.11.06	P	BM	46,XY[35]	D7Z1: 400/458 normal (98.2%); D8Z2: 500/515 normal (97%)
CAMT4	17.05.01	T	BM	46,XY[67]	D7Z1 + D8Z2[5]: 390/400 normal (97.5%)
	01.06.04	P	BM	46,XY[26]	D7Z1 + D8Z2[5]: 11/400 with monosomy 7 (2.7%)
	24.05.06	P	BM	45,XY,-7[22]	D7Z1 + D8Z2[5]: 121/400 with monosomy 7 (30.2%)
	30.08.06	P	PB-PHA	46,XY[248]	D7Z1 + D8Z2[5]: 325/350 normal (92.9%)
	06.07.06	P	BM	45,XY,-7[55]/46,XY[4]	D7Z1 + D8Z2[5]: 161/400 with monosomy 7 (40.2%)
	18.10.06	P	BM	45,XY,-7[14]/46,XY[3]	
	04.01.07		BM	46,XY[16][6]	
CAMT5	14.03.02	T	BM	46,XX[116]	D7Z1 + D8Z2[5]: 459/472 normal (97.2%)
	14.05.02	P	BM	46,XX[48]	D7Z1 + D8Z2[5]: 397/400 normal (99.5%)
	02.10.03	P	BM	46,XX[18]	

[1]T: thrombocytopenia; P: evolution into pancytopenia; [2]BM: bone marrow direct preparations and 24-48h cultures; PB-PHA: peripheral blood PHA stimulated culture; PB-DEB: peripheral blood PHA stimulated cultures, with diepoxybutane (DEB); SF: skin fibroblast culture; [3]FISH on normal controls showed an average of 97.2% nuclei with two signals for D7Z1, and of 97.4% for D8Z2; [4]normal result means that no evidence of monosomy 7 and of trisomy 8 was reached in the few nuclei not showing two signals, with data comparable to controls; [5]dual color FISH with probes D7Z1 and D8Z2; [6]after hematopoietic stem cell transplantation.

CAMT2 the difference between BM samples of 23.05.06 and 06.07.06 only indicates this trend ($0.02<p<0.05$), while in patient CAMT4 the difference between the BM samples of 24.05.06 and 06.07.06 is significant ($p<0.01$).

The expansion of a BM clone with a chromosome anomaly typical of MDS/AML, should be considered with respect to the risk of hematologic malignancies in CAMT patients. A plausible hypothesis is that a risk of progressing into MDS/AML is really part of the CAMT phenotype, although the short life expectancy and the use of hematopoietic stem cell transplantation make it difficult to demonstrate this evolution in most patients. If this is the case, the patients with clonal anomalies might be those who will progress to MDS/AML. We postulate that *MPL* mutations might cause karyotype instability through a mutator effect, with emergence of abnormal clones in the BM possibly characterized by chromosome changes typical of MDS: this mechanism was suggested also for other Mendelian BM failure syndromes, such as Shwachman syndrome (SS), familial platelet disorder with propensity to acute myeloid leukemia (FPD/AML, OMIM 601399), and FA.[10,11] In CAMT, the BM abnormal clone might stay quiescent even for long periods, as in SS, a full-blown MDS picture being the consequence of its expansion. In conclusion, we suggest that an appropriate cytogenetic follow-up of BM should be part of the clinical management of patients with CAMT, because the early detection of clonal anomalies might be crucial for treatment choice.

Emanuela Maserati,[1] Claudio Panarello,[2] Cristina Morerio,[2] Roberto Valli,[1] Barbara Pressato,[1] Francesco Patitucci,[1] Elisa Tassano,[2] Alessandra Di Cesare-Merlone,[3] Chiara Cugno,[3] Carlo L. Balduini,[4] Francesco Lo Curto,[1] Carlo Dufour,[2] Franco Locatelli,[3] Francesco Pasquali[1]

[1]Biologia e Genetica, Dipartimento di Scienze Biomediche Sperimentali e Cliniche, Università dell'Insubria, Varese; [2]Dipartimento di Ematologia ed Oncologia Pediatrica, IRCCS Istituto G. Gaslini, Genova; [3]Oncoematologia Pediatrica, Università di Pavia, Fondazione IRCCS Policlinico S. Matteo, Pavia; [4]Clinica Medica III, Università di Pavia, Fondazione IRCCS Policlinico S. Matteo, Pavia, Italy

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Key words: congenital amegakaryocytic thrombocytopenia, MDS, AML, trisomy 8, monosomy 7, mutator effect.

Correspondence: Emanuela Maserati, Dipartimento di Scienze Biomediche Sperimentali e Cliniche, Università dell'Insubria, via J.H. Dunant 5, 21100 Varese, Italy. Phone: international +39.0332.217181; Fax: international +39.0332.217119. E-mail: emanuela.maserati@uninsubria.it

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Variability of clinical manifestation of factor VII-deficiency in homozygous and heterozygous subjects of the European *F7* gene mutation A294V

Inherited factor VII deficiency (FVIID) is a rare autosomal bleeding disorder. Subjects with reduced FVII activity and identified *F7* gene mutation are registered in the International Greifswald registry of FVIID.[1] The mutation A294V is the most frequent among 717 FVIID patients in Central and North-Eastern Europe. The high prevalence of FVIID homozygous and heterozygous subjects, who share the same mutation (A294V), offers the unique possibility to study the clinical variability in a relatively large number of individuals with the identical defect in the *F7* gene. In the reported study we have analyzed the clinical variability of 14 homozygous and 99 heterozygous subjects with *F7* gene mutation A294V. Subjects with combinations with other bleeding disorders such as von Willebrand Disease, deficiency of FIX, FII etc., were excluded.

We collected data on factor VII clotting (FVII:C) activity, FVII antigen (FVII:Ag), date of initial onset of bleeding and bleeding symptoms.[1,2]

FVII:C was assayed locally with standard one-stage methods using thromboplastin (mostly recombinant and human-derived, Thromborel S® Dade Behring). FVII:Ag was measured at some of the centers using an immunoenzymatic method (Asserachrom, Diagnostica Stago, Asnieres, France). The following bleeding symptoms were evaluated according to published criteria:[1-4] intracranial hemorrhage (ICH), gastrointestinal (GI) hemorrhage, hemarthrosis, subcutaneous hematoma as well as epistaxis, oral bleeding, and menorrhagia in accordance with Peyvandi and Mannucci.[5] Endogenous thrombin potential (ETP) was measured in platelet poor plasma from controls and subjects homozygous for A294V in the presence of activated protein C.[6] Citrated plasma samples from 8 control subjects without FV_{Leiden} or FVHR2 (factor V wild type; FV_{wt}) and without A294V mutation were used as a reference. We used the mutation H1299R as marker for FVHR2.[7] The FV_{Leiden} mutation was determined according to Bertina *et al.*[8]

The 14 homozygous subjects for the F7 gene mutation A294V are characterized by mean levels of FVII:C of 10±6% and of FVII:Ag of 52±11%. Nine of the homozygous patients (64%) had spontaneous bleeding symptoms (classified as symptomatic) and 5 (36%) did not (classified as asymptomatic). There was no significant difference in FVII level between symptomatic and asymptomatic homozygous A294V subjects.

The spontaneous bleeding profile of the 9 symptomatic subjects was characterized by GI (22%) bleeds, hemarthrosis (22%), epistaxis (33%), easy bruising (11%), gingival bleeding (22%), subcutaneous hematoma (11%),

Table 1. Endogenous thrombin potential, bleeding symptoms and co-inheritance of FVHR2 in nine patients homozygous for the mutation A294V with available plasma samples.

Patient	Age	FVII:C [%]	FV HR2	GI Bleed	Hemarthrosis	Easy Bruising	Epistaxis	Gum Bleed	Hematuria	Menorrhagia	Hematoma	Severity*	Endogenous thrombin potential compared to the references (reference (100%))	
G-9125	6	8	hz									asympt.	203%	
G-9297	18	25	ho									asympt.	146%	
G-9130	15	4	wt						x			mild	93%	
G-9623	32	8	hz								x	mild	82%	
G-13255	10	7	wt					x				mild	59%	
G-9773	59	6	wt				x	x		x		moderate	56%	
G-13325	74	11	wt		x							severe	65%	
G-12990	44	9	wt	x								severe	57%	
G-10833	57	3	wt		x		x		x			severe	47%	