CLINICAL STUDY

Analysis of genetic variants of phosphodiesterase 11A in acromegalic patients

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Abstract

Objectives: Aberrant cAMP signaling is involved in the pathogenesis of somatotropinomas. The aim of the study was to screen acromegalic patients for the presence of variants of phosphodiesterase type 11A (*PDE11A*) gene, which have been recently identified in adrenocortical and testicular tumors.

Subjects and methods: We sequenced the *PDE11A* gene-coding region in 78 acromegalic patients and 110 controls. Immunohistochemistry for PDE11A was performed in a subgroup of adenomas and normal pituitary samples.

Results: We found 15 nonsynonymous germline substitutions in 13 acromegalic patients (17%), i.e. 14 missense variants (Y727C in six, R804H in one, R867G in four, and M878V in three) and one truncating mutation (FS41X), with a prevalence only slightly higher than that observed in controls (14%). Immunohistochemistry revealed PDE11A expression higher in somatotropinomas than in normal somatotrophs, without significant difference between tumors with or without PDE11A variants, with the exception of two tumors (one with loss of heterozygosity (LOH) at the *PDE11A* locus and one with FS41X mutation) showing markedly reduced PDE11A staining. No significant differences in hormonal and clinical parameters between patients with or without PDE11A variants were observed, although patients with PDE11A changes showed a tendency to have a more aggressive tumor compared with patients with wild-type sequence (extrasellar extension in 69 vs 45%).

Conclusions: This study first demonstrated the presence of PDE11A variants in a subset of acromegalic patients, which was only slightly more frequent than in controls. The normal expression of the enzyme in the majority of tumor tissues together with the lack of significant clinical phenotype suggests that these variants might only marginally contribute to the development of somatotropinomas.

European Journal of Endocrinology 161 687–694

Introduction

The search for novel candidate genes possibly involved in the pathogenesis of pituitary tumors is still a challenge. Despite the large number of screening studies carried out in the last decades, mutations of the gene encoding the α -subunit of Gs (*GNAS1*), leading to constitutive activation of adenylyl cyclase and cAMP production (*gsp* oncogene), are the only mutational change unequivocally associated with the development of sporadic GH-secreting tumors (1). Intracellular cAMP levels depend on the activity of adenylyl cyclase and cyclic nucleotide phosphodiesterases (PDEs), a superfamily of related phosphohydrolases that play a major role in the regulation of intracellular cAMP levels by hydrolyzing cAMP or cGMP and that display different biochemical and pharmacological properties (2, 3). Increases in cAMP levels result in PDEs activation

through a short-term phosphorylation process and a long-term induction of gene expression and protein synthesis. Accordingly, an increase in both PDE4 activity and expression has been demonstrated in GH-secreting tumors carrying *gsp* oncogene, this phenomenon probably representing a mechanism able to counteract, at least in part, the oncogenic potential of these mutations (4, 5).

The most recently discovered PDE enzyme family is PDE type 11A (*PDE11A*) (6). The *PDE11A* gene contains 23 exons. Until now, four splice variants of PDE11A have been identified (*PDE11A1–4*), with a dual specificity for both cAMP and cGMP. Of the four possible splice variants, *PDE11A4* coding starts from exon 3 and the protein (934 aa) includes, in addition to the catalytic domain (exon 14–22), two complete GAF domains and a putative phosphorylation site for protein kinase A and protein kinase G. *PDE11A4* transcripts are

particularly abundant in the prostate, but they are present also in endocrine tissues, including the adrenal cortex and pituitary (6–8). Germline inactivating or missense mutations of *PDE11A* gene have been identified in individuals with ACTH-independent macronodular adrenocortical hyperplasia, adrenocortical adenomas, and cancers (9–11), suggesting that these defects may confer susceptibility to the development of adrenocortical lesions. More recently, inactivating germline mutations in *PDE11A* have also been implicated in familial and bilateral testicular germ cell tumor susceptibility (12).

This study aimed to search for the presence of germline *PDE11A* variants in patients with acromegaly, based on the notion that somatotroph growth is largely cAMP dependent.

Materials and methods

Patients and tumors

The study was performed on leukocyte samples from peripheral blood of 78 patients (32 males, 46 females, age 42 ± 15.1 years, mean $\pm$ s.d.) affected with acromegaly (27 microadenomas, 51 macroadenomas with suprasellar extension in 38 patients, mean serum GH levels 26.7 ± 23.7 mg/l). Acromegaly was diagnosed on the basis of clinical features, elevated insulin-like growth factor 1 (IGF1) plasma levels, and elevated GH levels not suppressible during oral glucose tolerance test. Data concerning the screening for *gsp* mutations in the adenoma tissue from operated patients ($n=68$) were available from our database. In order to evaluate a possible adrenocortical involvement, abdominal imaging was performed by CT scan in 66 patients. Leukocytes from 110 healthy volunteers with no clinical evidence of endocrine tumors were also collected as controls for *PDE11A* gene analysis. All patients signed an informed consent for DNA analysis and access to the data. Local ethical approval was obtained for the study. Pituitary tumor samples ($n=12$) were obtained at the time of surgery, immediately frozen at -80°C , and stored for DNA extraction.

DNA extraction and sequencing

Genomic DNA was extracted from peripheral blood leukocytes or surgically removed tumoral tissues by Nucleon BACC2 genomic DNA purification kit (GE Healthcare, Piscataway, NJ, USA), according to the manufacturer's instructions. The DNA obtained was amplified by PCR for 20 coding exons (exons from 3 to 23) and flanking intronic sequences of *PDE11A* gene (ENSG00000128655) using specific primers (available upon request). Direct sequencing of the amplified exons was then performed with the AmpliTaq BigDye Terminator kit and 310 Genetic Analyzer (Perkin-Elmer Corp., Applied Biosystems, Foster City, CA, USA).

Immunohistochemistry

Sections from paraffin-embedded tissues from a subgroup of 16 available GH-secreting adenomas surgically removed by the transsphenoidal route (eight wild-type tumors and eight tumors with *PDE11A* sequence variants) and from seven normal pituitary samples obtained by autoptic surgery within 8 h after death (kindly supplied by Prof. F Basolo, Pisa, Italy) were processed for immunohistochemistry, as previously reported (13). Specific polyclonal antibody for *PDE11A* was used under the conditions specified by the manufacturer (Acris Antibodies GmbH, Herford, Germany) with testis sample as positive control. This antibody detects *PDE11A* with no cross-reaction with other PDEs. To determine the phenotype of cells expressing *PDE11A*, double immunostaining was performed on normal pituitary samples, as previously described (13). In particular, after positive staining for *PDE11A* was observed, the same sections were tested in a second immunostaining step against GH antibody (1:50, mouse monoclonal GH antibody, Santa Cruz Biotechnology, Santa Cruz, CA, USA), and the antigen-antibody detection was performed with the DAKO ChemMate EnVision detection kit (DAKO A/S, Glostrup, Denmark), according to the manufacturer's instructions. As a positive control, normal human adrenal tissue was used. Negative controls were obtained by occulting the primary antibody or by using an unrelated antibody. At least two blinded readers graded the specimens for all stainings. Immunoreactivity was graded 0–3, with score 0 = absence of immunoreactivity, $1 \leq 10$, $2 = 10-50$, and $3 \geq 50\%$ in at least 400 cells in the main representative high-power field.

Statistical analysis

Continuous variables were expressed as mean $\pm$ s.d., while rates and proportions were calculated for categorical data. For continuous variables, differences were analyzed by means of the two-tailed Student's *t*-test, while for categorical variables differences were analyzed by means of the χ^2 test and Fisher's exact test when appropriate. Multiple regression analysis was performed as appropriate. Level of statistical significance was set at $P < 0.05$. Statistical analysis was performed using the SPSS 13.0 for Windows software package (SPSS Inc., Chicago, IL, USA).

Results

PDE11A sequencing analysis: nonsense mutation/missense variants in GH-secreting pituitary adenomas

We analyzed the *PDE11A* gene in germline DNA of 78 patients and 110 control subjects by direct sequencing. We found nonsynonymous substitutions in 13

Table 1 Frequency of phosphodiesterase 11A genetic variants found in 78 acromegalic patients and in 110 controls.

Sequence change (amino acid change)	Patients (%)	Controls (%)	P
A2180G (Y727C)	7.7	6.4	0.775
G2411A (R804H)	1.3	2.7	0.643
C2599G (R867G)	5.1	3.6	0.720
A2638G (M878V)	3.8	0.9	0.309
171delT (FS41X)	1.3	0	0.415

acromegalic patients (17%). In particular, as shown in Table 1, we found four missense variants, i.e. Y727C in exon 17 in six patients, R804H in exon 19 in one patient, R867G in exon 22 in four patients, and M878V in exon 22 in three patients (Table 1). The prevalence of Y727C, R867G, and M878V substitutions was higher in acromegalic patients in comparison with the control group, but the difference did not reach statistical significance. Finally, we found a truncating mutation in exon 3 (FS41X) in one patient, which was not detected in the controls (Table 1).

Eleven patients had one heterozygous substitution, while patient with the truncating mutation in exon 3 (no. 13) also had a heterozygous R867G variant and patient no. 10 showed one homozygous substitution (M878V) together with a heterozygous substitution (Y727C), which ruled out the presence of deletions as the cause of homozygous appearance (Table 2).

To investigate the possible loss of the wild-type allele in tumoral tissues of patients with heterozygous substitutions, we analyzed the sequence of DNA extracted from the tumor samples available ($n=6$). In five cases, both the wild-type and the variant sequences were observed, whereas one case (patient no. 11) showed LOH at *PDE11A* locus with retention of the mutant allele in the tumoral tissue (Fig. 1).

PDE11A sequencing analysis: intronic and synonymous polymorphisms in GH-secreting pituitary tumors

We found several synonymous and intronic *PDE11A* polymorphisms (single nucleotide polymorphisms (SNPs), ins/del, variable number of tandem repeats (VNTR)) and we tested their frequency in our cohort of patients and controls (Table 3). The allelic variants c.1072-3 C>T in intron 4, c.1263 A>C/p.E421E in exon 6, c.1577 T>C in intron 10, c.1626 A>G/p.A542A in exon 11, c.1644+26insGTTTATA in intron 11, and c.2758_2760 insTCC/p.S920ins in exon 23 have already been reported in literature (11, 12), while others, i.e. c.1501-107 A>G in intron 9, c.1576+67 A>CCT in intron 10, c.2424-46 C/T in intron 19 have only been recorded in online polymorphism databases (www.ensembl.org). We examined Hardy-Weinberg equilibrium for all detected

Table 2 Clinical characteristics and genotype of acromegalic patients with phosphodiesterase 11A nonsense/missense substitutions.

Patient nos	Sex	Age (years)	Exon nos	Genotype	Amino acid change	gsp ^a	Tumor dimension ^b	Tumor extension ^c	Serum GH (mg/l)	Serum IGF1 (nmol/l) ^d	Adrenal involvement
1	M	45	17	A2180G/wt	Y727C	-	Macro	Intra	57	107 (13-39.9)	No
2	F	50	17	A2180G/wt	Y727C	-	Macro	Extra	19	148 (12.6-38.2)	Yes
3	M	29	17	A2180G/wt	Y727C	-	Macro	Extra	22	123 (15-44.3)	No
4	F	35	17	A2180G/wt	Y727C	-	Micro	Intra	8	69 (14-42)	No
5	M	25	17	A2180G/wt	Y727C	+	Macro	Extra	8.3	129 (15-44.3)	No
6	F	66	19	G2411A/wt	R804H	-	Macro	Extra	51	83 (11.8-36.9)	No
7	F	33	22	C2599G/wt	R867G	-	Macro	Extra	40	78 (14-42)	No
8	M	50	22	C2599G/wt	R867G	+	Micro	Intra	8	125 (12.6-38.2)	No
9	F	60	22	C2599G/wt	R867G	+	Micro	Intra	10	86.3 (11.8-36.9)	No
10	M	29	22	A2632G/A2632G	M878V	+	Macro	Extra	38	124 (15-44.3)	No
11	M	18	22	A2180G/wt	Y727C	-	Macro	Extra	4.9	70 (30-64.9)	No
12	M	36	22	A2632G/wt	M878V	+	Macro	Extra	15	103.5 (14-42)	Yes
13	F	64	3	171delT/wt	FS41X	-	Macro	Extra	46	126 (11.8-36.9)	Yes
			22	C2599G/wt	R867G						

—, Wild-type; +, mutations at codon 201 or 227.

^agsp, mutations of Gsx gene.

^bMicro, microadenoma (tumor measuring <10 mm in diameter); macro, macroadenoma (tumor measuring ≥10 mm).

^cIntra, intrasellar extension assessed by CT or NMR; extra, extrasellar.

^dNormal range.

Figure 1 Representative sequences of *PDE11A* variants found in leukocytes or in tumoral tissues DNA of acromegalic patients: 2599 C>G (R867G; no. 7), 2632 A>G (M878V; no. 11), FS41X (no. 13). In the tumoral sample of patient no. 11, the wild-type allele is lost and only the mutated allele is present, whereas in the other two representative samples both wild-type and mutated alleles are retained in the tumoral tissue.

polymorphisms in affected and control groups, and no deviation has been revealed. We found an association between polymorphisms in intron 9-intron 10-exon 11-intron 11, as previously reported (11) and between intron 4 and exon 6 polymorphisms. The difference of frequency of these intronic and synonymous polymorphisms between patients and control group was not statistically significant.

Immunohistochemistry

Immunohistochemistry for *PDE11A* was performed on sections of GH-secreting pituitary tumors from eight patients with *PDE11A* genetic variants (patient nos. 2, 4, 6, 7, 9, 11–13), eight patients without these changes in *PDE11A* sequence (four *gsp* positive and four *gsp* negative), and normal pituitaries ($n=7$). In Fig. 2, representative stained sections from normal and tumoral samples are shown. In the normal tissues, *PDE11A* was present in about 10% of cells positive for GH (score=1). *PDE11A* staining was both cytoplasmatic and nuclear in most normal and tumor samples. All tumors showed *PDE11A* immunostaining higher (score=2–3) compared with normal somatotrophs without any significant difference between tumors with and without *gsp* mutation (data not shown). *PDE11A* expression was similar or slightly reduced in six tumors with *PDE11A* substitutions (score=2 in three and score=3 in three) in comparison with those without *PDE11A* changes. Conversely, *PDE11A* immunopositivity was reduced in tumor no. 11 with LOH at *PDE11A* locus and in tumor no. 13, bearing both exon 3 truncating mutation and R867G substitution.

Clinical features

Comparing patients with or without *PDE11A* non-synonymous variants, no significant differences were found in sex (46 vs 62% of females), age at diagnosis (42 ± 16 vs 42 ± 15 years), duration of symptoms before diagnosis (5.2 ± 3.4 vs 5.9 ± 4.0 years), serum GH levels (25.2 ± 18.6 vs 27.1 ± 24.6 mg/l), IGF1 s.d. score (9.3 ± 3.7 vs 9.6 ± 5.5), and responsiveness to somatostatin analogs (54 vs 40% of well controlled) as well as frequency of *gsp* mutations (38 vs 34%; Table 2). Moreover, patients bearing germline *PDE11A* substitutions and *gsp* mutations did not show different clinical characteristics, compared with patients with only *PDE11A* variants (data not shown). However, a tendency towards a clinical phenotype characterized by greater tumor dimension and suprasellar extension was seen in patients with *PDE11A* variants, compared with patients without these changes (77 vs 63% of macroadenomas, $P=0.525$; 69 vs 45% of suprasellar extension, $P=0.134$).

By simple and multiple linear and logistic regression analysis, we looked at possible correlations between the presence of missense variations and the clinical phenotype, but no statistically significant model was found (data not shown).

Searching for a possible adrenal involvement, we found bilateral adrenocortical hyperplasia or adrenocortical nodule in three out of 13 patients with nonsense/missense substitutions (no. 12, 2, 13 respectively, Table 2), with a frequency that was slightly higher than that observed in the 53 patients without *PDE11A* variants (23 vs 6%, $P=0.08$).

Table 3 Frequency of phosphodiesterase 11A synonymous or intronic polymorphisms found in 78 acromegalic patients and in 110 controls.

Sequence change	Patients (%)	Controls (%)	P
c.1072–3C>T (intron 4)			
c.1263A>G/p.E421E (exon 6)			
C/C	70.5	79.1	0.185
C/T	23.1	19.1	
T/T	6.4	1.8	
C allele proportion	82.0	88.6	
T allele proportion	18.0	11.4	0.073
c.1501–107A>G (intron 9)			
c.1576+67insCC&A>T (intron 10)			
c.1577–3T>C (intron 10)			
c.1626A>G/p.A542A (exon 11)			
c.1644+26insGTTTATA (intron 11)			
A/A	39.1	47.3	0.402
A/G	42.2	39.1	
G/G	18.7	13.6	
A allele proportion	60.2	66.8	
G allele proportion	39.8	33.2	0.158
c.2424–46T>C (intron 19)			
C/C	73.1	71.8	
C/T	26.9	25.5	
T/T	0	2.7	0.658
C allele proportion	86.5	84.6	
T allele proportion	13.5	15.4	
c.2758_2760insTCC/p.S920ins (exon 23)			
2758_2760delTCC/	16.7	11.8	0.228
2758_2760delTCC			
2758_2760delTCC/	35.9	48.2	
2758_2760insTCC			
2758_2760insTCC/	47.4	40.0	0.827
2758_2760insTCC			
2758_2760delTCC allele proportion	34.6	35.9	
2758_2760insTCC allele proportion	65.4	64.1	

None of the subjects with *PDE11A* variants reported a personal or familial history of cancer in other cAMP-dependent organs.

Discussion

This study first demonstrates the presence of nonsynonymous substitutions of *PDE11A* gene in a subset of acromegalic patients. Despite the crucial role of the cAMP pathway in the development of somatotropinomas, few key components of this pathway have been identified so far as targets for mutational changes, i.e. *GNAS1* gene which is activated in a subset of sporadic somatotropinomas and in the McCune–Albright syndrome (14), and *PRKARIA* gene that is inactivated in the Carney complex (15, 16). Since intracellular cAMP levels are the result of a steady state of synthesis by adenylyl cyclase and degradation by PDEs, genes encoding PDEs might represent good candidates for cAMP-related endocrine diseases, such as acromegaly.

Genetic alterations of PDEs have been found to cause disease in humans; in particular, *PDE6* mutations have been linked to several vision-related diseases (17–19), while a possible association of *PDE11A* variants with major depression and response to antidepressant treatment has been reported (20). Recently, a whole genome association study carried out on ten individuals with ACTH-independent Cushing's syndrome demonstrated the involvement of *PDE11A* in the tumorigenesis of the adrenocortical gland, which, similarly to the pituitary gland, has been linked to dysregulation of cAMP signaling pathway (9). More recently, nonsense and missense substitutions of this gene were found in individuals with ACTH-independent macronodular adrenocortical hyperplasia, adrenocortical adenomas, and cancers (9–11), suggesting that these defects may confer susceptibility to the development of adrenocortical lesions. Subsequently, nonsynonymous substitutions of *PDE11A* have also been detected in patients with familial and bilateral testicular germ cell tumors, consistent with the involvement of the cAMP signaling pathway in reproductive organ functions and diseases (12).

In the present study, we detected the presence of nonsynonymous substitutions in a significant subset of acromegalic patients, with a frequency only slightly higher than that observed in controls. Indeed, we confirmed the wide spectrum of *PDE11A* variants, including nonsynonymous substitutions, which have been previously reported in population studies. Admittedly, although healthy subjects of this series were not affected with clinically relevant endocrine disease, the presence of individuals with very mild and unrecognizable endocrine alterations in the control group cannot be excluded. Taking into account these limitations that are common to the other association studies, the frequency of *PDE11A* variants in acromegalic patients (17%) was found to be slightly lower in comparison with that reported for both adrenocortical and bilateral testicular germ cell tumors (20%) (11, 12).

All nonsynonymous substitutions found in acromegalic patients, which have also been detected in the other endocrine tumors so far analyzed, are located in the catalytic domain of the enzyme and are associated with functional defects. The most frequent missense variation found in acromegalic patients and in controls was the Y272C substitution, a relatively frequent polymorphism listed in the databases. Although frequent in the general population, it has been recently reported that when transfected in murine Leydig tumor cell line 1, this variant was associated with higher cAMP levels and lower PDE activity in comparison with the wild-type enzyme (12). Moreover, we found three other sequence variants, i.e. R804H, R867G, and M878V, associated with a reduced PDE activity *in vitro*, further suggesting that these variants, also present in the general population, could contribute to a genetic predisposition

Figure 2 PDE11A immunohistochemistry. (A) Normal pituitary (score=1); (B) double immunostaining showing both GH (red) and PDE11A (brown) localization in normal somatotrophs; (C) tumor sample from patient no. 7 with R867G variant (score=3); (D) tumor sample from patient without *PDE11A* variant (score=3); (E) tumor sample from patient no. 11 with M878V variant and LOH (score=1); (F) tumor sample of patient no. 13 with R867G variant and FS41X-truncating mutation (score=0).

to endocrine lesions (10–12). Contrary to what has been reported in adrenal and testicular tumors, we did not detect any nonsynonymous substitutions in GAF A and GAF B, the cGMP binding domain of the enzyme. Finally, one patient had a frameshift mutation (171delT/FS41X, exon 3) that generates a truncated protein and that was not found in our series of healthy subjects. This mutation has been reported in one patient with adrenal tumor and in three of 1612 previously screened normal controls (10–12).

Frequent synonymous or intronic polymorphisms were also found in both acromegalic samples and controls. The exon 6 (E421E) and intron 10/intron 11 variants were found to play a role in the predisposition to adrenocortical cancer development (11). Our data show that, although the allelic frequency of the minor allele is slightly higher in acromegalic patients in comparison with the control group, the difference did not reach statistical significance.

Consistent with the hypothesis that *PDE11* may play a role as a suppressor gene, it has been reported that adrenal and testicular tumors expressing PDE11A variants also showed loss of the wild-type allele, thus resulting in a significant reduction of the enzyme levels in the affected tissues (9, 11, 12). This study shows that *PDE11A* LOH was a rare event in somatotropinomas.

Indeed, although tested in a limited series of tumors due to the lack of tissues available for DNA amplification and sequencing, the wild-type allele was lost only in one tumor, thus suggesting that PDE11A does not act as an anti-oncogene in most somatotropinomas.

In agreement with previous studies reporting the expression of PDE11A transcript (6, 7) and protein (8) at pituitary level, we demonstrated that PDE11A was expressed in normal and tumoral somatotrophs, where it localized both at cytoplasmatic and nuclear level. Consistent with the retaining of both the variant and the wild-type allele, tumors expressing the PDE11A variants showed a similar or slightly reduced PDE11A staining in comparison with tumors without these changes, whereas a significant reduction in PDE11A immunopositivity was present in two tumors, i.e. the one tumor with LOH and the other tumor showing both the truncating mutation 171delT/FS41X and the heterozygous R867G variant.

It is of note that PDE11A was overexpressed in tumoral somatotrophs in comparison with the normal cell counterpart and that this induction was not related to the presence of *gsp* oncogene. Taking into account that an increased expression of PDE4C, PDE4D and PDE8B transcripts has been observed in somatotropinomas carrying *gsp* mutations (4, 5), the present data

suggest different mechanisms of PDE11A upregulation, which seem to operate also in tumors without known mutations affecting the cAMP pathway.

As far as the clinical characteristics of our cohort of patients were concerned, patients with PDE11A substitutions did not significantly differ in hormonal and clinical parameters, although a tendency to have tumors with greater dimension and suprasellar extension, compared with patients without these changes in PDE11A sequence, was observed. Since increased activity and expression of PDEs have been proposed to be effective in limiting cAMP-dependent growth of somatotrophs, it is tempting to speculate that these PDE11A variants might, at least in part, limit this feedback mechanism. Finally, patients with PDE11A variants showed adrenal lesions with a frequency that was slightly higher than that observed in patients without variants. Although the excess of GH and IGF1 may *per se* cause adrenocortical hyperplasia and adenomas (21), the present data are consistent with the hypothesis that PDE11A variants may contribute to genetic predisposition to the development of adrenal tumors (11).

In conclusion, this study first demonstrated the presence of nonsense/missense PDE11A variants in about a fifth of acromegalic patients, a frequency slightly higher than that observed in control subjects. However, the retaining of the wild-type allele resulting in normal expression of the enzyme in most tumors together with the lack of significant clinical phenotype suggests that these variants might only marginally contribute to the development of somatotropinomas.

Declaration of interest

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was partially supported by AIRC (Associazione Italiana Ricerca Cancro, Milan), PRIN 2006060982_002 to A Spada, and Ricerca Corrente Funds of Fondazione Ospedale Maggiore Policlinico Mangiagalli e Regina Elena IRCCS (Milan).

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Received 5 August 2009

Accepted 9 August 2009