Curriculum vitae

PERSONAL INFORMATION

Mauro Di Ruvo

Sex Male | Date of birth 12 Mar 1989 | Nationality Italian

POSITION

Biomedical scientist

WORK EXPERIENCE

7 May 2012–10 May 2014

Biomedical scientist

University of Ferrara, Ferrara (Italy)

Participation in the molecular diagnostics activity of the Endocrinology Laboratory. Specifically, I was involved in the analysis of MEN1/MEN2 AND B-RAF gene mutations using direct sequencing techniques and High Resolution Melting.

Management and use of diagnostic tools (thermocyclers, automatic sequencer, electrophoresis equipment) and software for data analysis, including international databases available on the internet.

Participation in the Laboratory's genetic-molecular research activities aimed at the publication of scientific articles on international magazines. I was involved in scientific research about MEN1 gene and glucocorticoid-like activity of phytocosmetic procucts and phytosterols.

Business or sector Endocrine phisiopatology laboratory

3 Jun 2014–Present

Biomedical scientist

U.L.S.S. 6 Euganea, Cittadella hospital
40, via Casa di Ricovero, 35013 Cittadella (Italy)

- Execution of blood counts, coagulation and clinical chemistry analysis;
- Work done on rotating periods of 24 hours and 7 days a week;
- Execution of specialistic analysis og Emoglobin electrophoresis and serum protein electrophoresis;
- Cytofluorimetry examinations for the diagnosis of leukemia diseases.

Business or sector Clinical pathology Laboratory

EDUCATION AND TRAINING

Oct 2008–30 Nov 2011

Degree in Biomedical Laboratory Technics — EQF level 6

University of Ferrara, Ferrara (Italy)

Nov 2017–Mar 2019

Master in Management and coordination of healt professions — EQF level 7

Unitelma Sapienza University, Rome (Italy)

PERSONAL SKILLS

Mother tongue(s): Italian

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Foreign language(s)

	UNDERSTANDING		SPEAKING		WRITING
	Listening	Reading	Spoken interaction	Spoken production	
English	B2	C1	B2	B2	B2

Levels: A1 and A2: Basic user - B1 and B2: Independent user - C1 and C2: Proficient user
Common European Framework of Reference for Languages

Communication skills

Ability to work in a group gained during my work as a laboratory technician, in situations where collaboration between different work figures was essential.

Organisational / managerial skills

Ability to work in stressful conditions, with deadlines and adapting to every type of task.

Job-related skills

Ability to solve unexpected problems in a situation of stress and in complete autonomy.

Ability to adapt to complex situations that occurred during night and solitary work shifts.

Digital skills

SELF-ASSESSMENT				
Information processing	Communication	Content creation	Safety	Problem-solving
Proficient user	Proficient user	Proficient user	Independent user	Proficient user

Digital skills - Self-assessment grid

Excellent knowledge of Microsoft applications and the Office package, in particular Word and Excel, used for statistical analysis. Good ability in Internet navigation. Knowledge of main molecular genetic databases available on the internet (NCBI, OMIM, HGMD, dbSNP short variations).

Other skills

Acquisition of molecular biology techniques: Preparation of PCR reaction and enzyme restriction, genomic DNA extraction from blood and tissues, automatic sequencing and cell cultures. Brief experience in the field of epigenetics and the study of chromatin.

Driving licence

B

ATTACHMENTS

- Scientific publications.pdf

Scientific publications.pdf

Familial Cancer (2014) 13:273–280
DOI 10.1007/s10689-014-9702-y

ORIGINAL ARTICLE

Deletion of exons 1–3 of the *MEN1* gene in a large Italian family causes the loss of menin expression

Maria Chiara Zatelli · Federico Tagliati · Mauro Di Ruvo · Emilie Castermans · Luigi Cavazzini · Adrian F. Daly · Maria Rosaria Ambrosio · Albert Beckers · Ettore degli Uberti

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Abstract Multiple endocrine neoplasia type 1 (MEN1) syndrome is an autosomal dominant disease, characterized by parathyroid adenomas, endocrine gastroenteropancreatic tumors and pituitary adenomas, due to inactivating mutations of the *MEN1* gene (chromosome 11q13). *MEN1* mutations are mainly represented by nonsense, deletions/insertions, splice site or missense mutations that can be detected by direct sequencing of genomic DNA. However, MEN1 patients with large heterozygous deletions may escape classical genetic screening and may be misidentified as phenocopies, thereby hindering proper clinical surveillance. We employed a real-time polymerase chain reaction application, the TaqMan copy number variation assay, to evaluate a family in which we failed to identify an *MEN1* mutation by direct sequencing, despite a clear clinical diagnosis of MEN1 syndrome. Using the TaqMan copy number variation assay we identified a large deletion of the *MEN1* gene involving exons 1 and 2, in three affected family members, but not in the other nine family members that were to date clinically unaffected. The same genetic alteration was not found in a group of ten unaffected subjects, without family history of endocrine tumors. The *MEN1* deletion was further confirmed by multiplex ligation-dependent probe amplification, which showed the deletion extended from exon 1 to exon 3. This new approach allowed us to correctly genetically diagnose three clinical MEN1 patients that were previously considered as MEN1 phenocopies. More importantly, we excluded the presence of genetic alterations in the unaffected family members. These results underline the importance of using a variety of available biotechnology approaches when pursuing a genetic diagnosis in a clinically suggestive setting of inherited endocrine cancer.

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Keywords MEN1 · Gene deletions · Quantitative PCR · Genetic analysis · MLPA

Introduction

Multiple endocrine neoplasia type 1 (MEN1) syndrome is an autosomal dominant disease, characterized by parathyroid adenomas, endocrine gastroenteropancreatic tumors and pituitary adenomas, due to inactivating mutations of the *MEN1* gene on chromosome 11q13. *MEN1* mutations are scattered within and around the menin open reading frame and are mainly represented by nonsense, deletions/insertions, splice site or missense mutations which can be detected readily by direct sequencing of genomic DNA [1, 2]. *MEN1* germline mutations are identified in 70 % of the

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European skills passport
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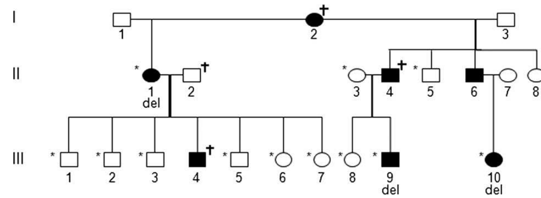

Fig. 1 Pedigree of the MEN1 kindred. *Closed* and *open symbols* represent symptomatic and asymptomatic individuals, respectively. * = tested patients; † = deceased family members; *del* = patients presenting the deletion encompassing exon 1–3

familial forms and in 10 % of sporadic cases, while in almost 20 % of clinically affected patients *MEN1* gene analysis fails to detect germline mutations (cases that are termed MEN1 phenocopies) [3]. Indeed, 5–25 % of MEN1 patients do not harbour mutations that can be identified by sequencing in the *MEN1* gene coding region, but have whole or partial gene deletions. Mutations in the promoter, 5′-untranslated and 3′-untranslated regions have rarely been screened for systematically [4–9].

The recent Endocrine Society clinical practice guidelines for MEN1 recommend performing multiplex ligation-dependent probe amplification analysis (MLPA) for the detection of exonic deletions in patients in whom a *MEN1* mutation is not identified within the coding region and splice sites [1]. Alternatively, long-range polymerase chain reaction (PCR) amplification (LRPA) can be employed [10].

We here describe a *MEN1* deletion identified by employing a new method, based on a quantitative PCR assay, in a large Italian family with three family members clinically presenting MEN1, previously diagnosed as MEN1 phenocopies.

Materials and methods

Patients

We studied a large Italian family with 21 members, 17 of whom are living, including three subjects showing a clear MEN1 phenotype (see Fig. 1; Table 1). Among living family members, 12 consented to blood withdrawal for genetic testing and signed an informed consent, including the three affected patients. Among the four family members who were not alive, three died from malignant tumors not classically associated with the MEN1 phenotype (exocrine pancreatic cancer (n = 2); laryngeal cancer (n = 1)), while the fourth family member died from metastatic spread of a bronchial carcinoid. This latter subject also had a history of primary hyperparathyroidism, a prolactin-secreting pituitary adenoma, papillary thyroid carcinoma, bilateral adrenal hyperplasia, with a clinical MEN1 phenotype similar to the other affected family members (see Table 1). Genetic testing of this subject was not possible.

Table 1 Clinical phenotype and history of the three affected family members

II-1 (F, 74 years)	
56 years	Left parathyroid adenoma
58 years	Right parathyroid adenoma
	PRL-secreting pituitary macroadenoma
62 years	Left adrenal gland macronodular hyperplasia
65 years	Pancreatic glucagonoma
III-9 (M, 48 years)	
38 years	ACTH-secreting pituitary adenoma
42 years	Multiple parathyroid adenomas
48 years	Pancreatic neuroendocrine carcinoma with lymphnode metastases
	Bilateral diffuse adrenal gland macronodular hyperplasia
III-10 (F, 23 years)	
15 years	Left parathyroid adenoma
	PRL-secreting pituitary microadenoma
23 years	Left adrenal gland macronodular hyperplasia
	Pancreatic insulinoma

DNA isolation and direct sequencing

Genomic DNA (gDNA) was isolated from each subject's whole blood by using the QIAamp DNA Blood Mini Kit (QIAGEN, Milano, Italy) on the QIAcube automated system (QIAGEN). DNA from somatic cells was isolated by using the QIAamp DNA FFPE Tissue Kit (QIAGEN) from paraffin-embedded parathyroid adenoma and pancreatic neuroendocrine carcinoma from patient III-9. At least 100 ng of DNA were used for each application.

Direct DNA sequencing of the *MEN1* coding region and intron–exon boundaries using sequence-specific primers was performed as described previously [11–13]. A pool of normal human gDNAs from six male and six female subjects was employed as control for genetic analysis (referred to as "normal control"). Similarly, a group of eight unrelated patients undergoing *MEN1* genetic analysis were studied.

TaqMan® gene copy number assays

The pre-designed TaqMan® gene copy number assays (Hs01998305_cn; Hs02189358_cn; Hs01221989_cn; Hs03796573_cn; Hs03794510_cn; Hs03785674_cn; Hs00736968_cn; Hs03768072_cn; Hs03793578_cn; Hs03800380_cn; Hs03773377_cn; Hs01778293_cn; Hs01920054_cn; Hs01699461_cn; Hs01957966_cn; Hs00823207_cn; Life

Technologies, Milano, Italy), covering the *MEN1* sequence almost completely, were used to detect gene copy number in quadruplicate gDNA samples.

A sample not containing DNA template (no template control, NTC) was used as negative control, while normal control with a known copy number for the gene of interest was used as calibrator in quadruplicate. Briefly, 5 ng of gDNA were amplified by adding target-specific forward and reverse primers and the genotyping master mix, containing AmpliTaq Gold® DNA Polymerase (Life Technologies), according to the manufacturer's instructions. FAM™ dye-labeled MGB probes covering the MEN1 encoding sequence and the flanking regions were used. Samples were then analyzed on the ABI PRISM® 7900 Sequence Detection Systems (Life Technologies), using PCR cycling conditions according to user's manual (40 cycles = 95 °C for 15 s and 60 °C for 60 s). The assays were run in duplex with VIC dye-labeled TaqMan copy number reference assays. These reference genes are known to be present in two copies in a diploid genome, regardless of the copy number of the target of interest and are used to normalize sample input and minimize the variation between the targets of the test and reference assays. The TaqMan copy number reference assay (RNase P) containing the reference sequence-specific forward and reverse primers and the VIC® dye-labeled TAMRA™ probes was employed. Data from 7900HT were analyzed by SDS software and then exported on copy caller software as described by manufacturer (see TaqMan® copy number assays protocol applied biosystem and CopyCaller™ Software User Guide Applied Biosystem).

Multiplex ligation-dependent probe amplification

Deletions within the *MEN1* gene were assessed in gDNA samples using the SALSA MLPA probemix P017-C1 MEN1 (version 15) (MRC-Holland, Amsterdam, The Netherlands). Reference and control probe-pairs that were specific to unrelated genetic regions were also included in the probe-mix. All probes had amplification products from 142 to 373 nucleotides in length and had an annealing temperature >70 °C as per the RAW probe program (MRC-Holland, Amsterdam, The Netherlands). PCR products were analyzed on an AB3130 XL capillary electrophoresis apparatus (Applied Biosystems, Lennik, Belgium). Copy number quantification involved normalization of the peak area of the *MEN 1*-specific MLPA probe by dividing it by the combined areas of the control probes. This ratio was compared with the similar ratio obtained from control DNA. Deletion was observed when the wild-type signal was reduced by 35–50 % for each *MEN 1*-specific probe.

Immunohistochemistry

Immunohistochemistry to detect menin reactivity was performed by employing the goat polyclonal anti-menin antibody (N-19 sc-8201; Santa Cruz Biotechnology, Heidelberg, Germany), as previously described [14, 15]. Normal parathyroid tissue was employed as positive control, while negative controls were performed by omission of the primary antibody.

Results

MEN1 mutation studies

Direct *MEN1* sequencing failed to identify any mutation. On the contrary, as shown in Fig. 2a, b, TaqMan® gene copy number assays using specific probes for *MEN1* exon 1 assessed that the three affected patients (II-1, III-9 and III-10) displayed half of the gDNA quantity as compared to the normal control (NC). In contrast, the nine family members that were clinically normal and without MEN1 features at the time of investigation, as well as the eight unrelated patients, displayed a normal gDNA copy number for exon 1. Similar results were found for the assay using a probe specific for exon 2 (Fig. 2c). On the contrary, the assay using a probe specific for *MEN1* regions different from exon 1 and exon 2 (exon 11 is shown in Fig. 2d) showed the same gDNA quantity for all the examined subjects. Therefore, these results indicate that *MEN1* exons 1 and 2 are deleted in the three affected patients, but not in the unaffected family members, as well as in the unrelated patients.

Accordingly, MLPA analysis confirmed the presence of exon 1 and 2 deletions at the *MEN1* locus in the three affected family members, but not in the unaffected family members (Fig. 3). In addition, MLPA analysis also showed the presence of a deletion of exon 3 in the same subjects that was not detected by the TaqMan copy number assay, since the TaqMan probe set did not cover exon 3.

In addition, the TaqMan copy number assay was performed on pancreatic neuroendocrine carcinoma from an affected patient. This showed a copy number of 0 for exons 1 and 2, while the other exons covered by the commercial probes had a copy number of 2, which indicates that the loss of heterozygosity was limited to only part of the MEN1 gene.

Immunohistochemical study of menin

MEN1 deletions in MEN1-related tumors are likely to result in a reduced (or absent) menin staining. As shown in Fig. 4, menin staining was absent in the pancreatic

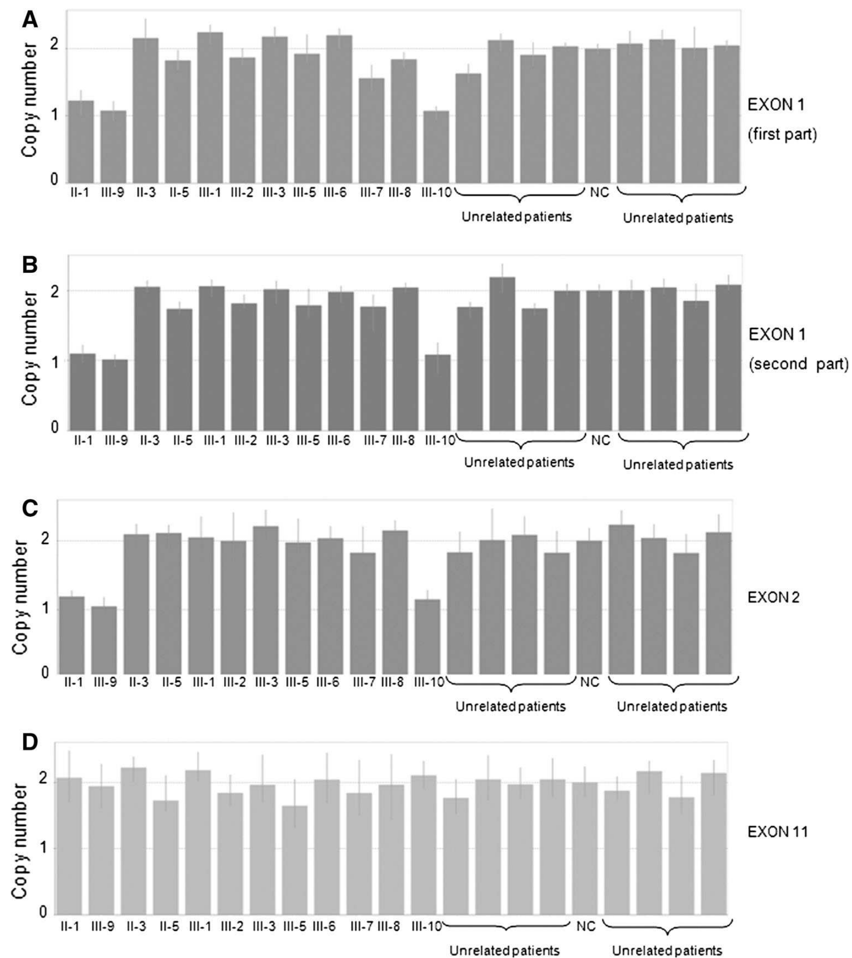

Fig. 2 MEN1 exon 1 and exon 2 copy number assay. Exon 1–11 of the MEN1 gene were amplified by using TaqMan® gene copy number assays, as described in the "Materials and methods". **a** MEN1 exon 1 (first part) copy numbers in affected (II-1, III-9, and III-10) and unaffected family members (II-3, II-5, III-1, III-2, III-3, III-5, III-6, III-7, III-8), as well as in the NC and in eight unrelated patients. **b** MEN1 exon 1 (second part) copy numbers in affected (II-1, III-9, and III-10) and unaffected family members (II-3, II-5, III-1, III-2, III-3, III-5, III-6, III-7, III-8), as well as in the NC and in eight unrelated patients. **c** MEN1 exon 2 copy numbers in affected (II-1, III-9, and III-10) and unaffected family members (II-3, II-5, III-1, III-2, III-3, III-5, III-6, III-7, III-8), as well as in the NC and in eight unrelated patients. **d** MEN1 exon 11 copy numbers in affected (II-1, III-9, and III-10) and unaffected family members (II-3, II-5, III-1, III-2, III-3, III-5, III-6, III-7, III-8), as well as in the NC and in eight unrelated patients

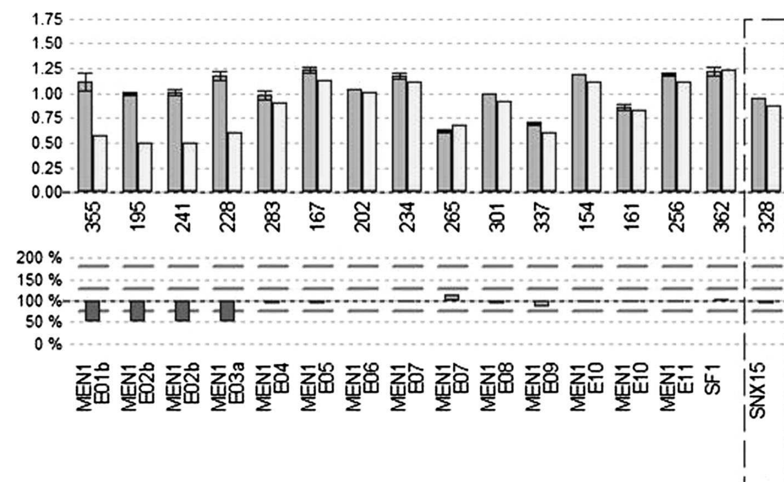

Fig. 3 Results of MLPA analysis of the MEN1 exons in the affected patients. A 50 % decrease in signal for exons 1, 2 (in both exon 2 probes) and 3 is seen in the *lower panel* of the figure. SF1 and SNX15 are unrelated reference genes that occur before and after the MEN1 gene region

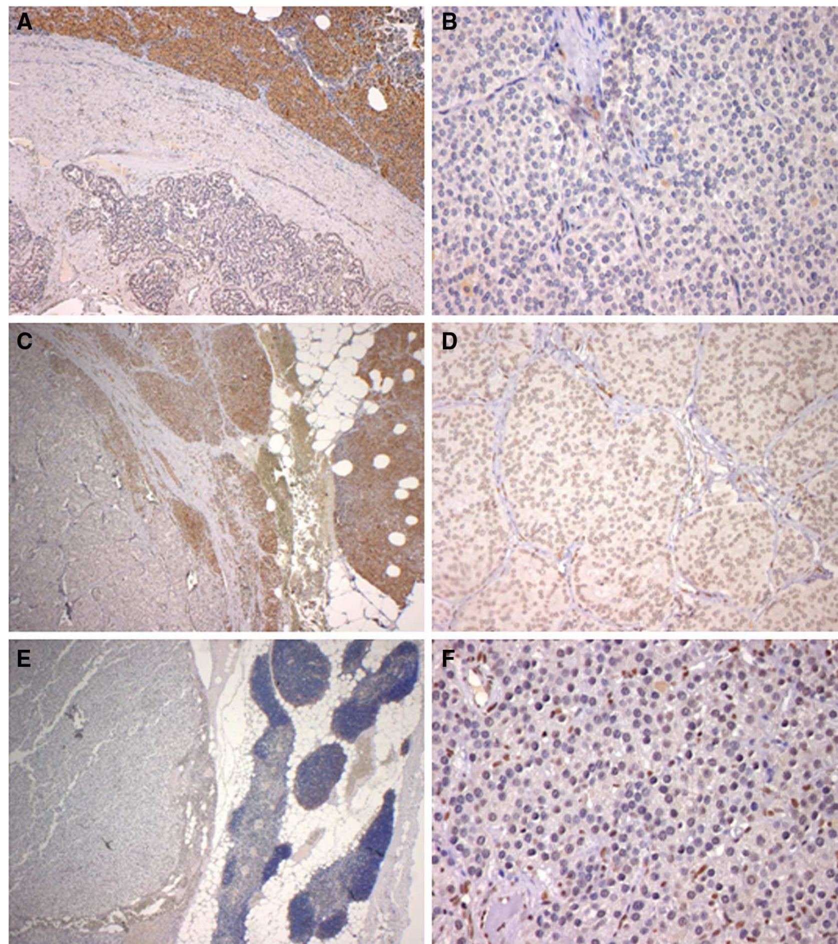

Fig. 4 Immunohistochemistry for menin. **a**, **b** Menin immunostaining of a representative area of the pancreatic glucagonoma (and adjacent normal pancreatic tissue) of patient II-1. Original magnification of ×200 for (**a**) and ×400 for (**b**). **c**, **d** Menin immunostaining of a representative area of the pancreatic neuroendocrine carcinoma (and adjacent normal pancreatic tissue) of patient III-9. Original magnification of ×200 for (**c**) and ×400 for (**d**). **e**, **f** Menin immunostaining of a representative area of parathyroid tumours (and adjacent normal thymic tissue) of patient III-10. Original magnification of ×200 for (**e**) and ×400 for (**f**)

glucagonoma of patient II-1 (Fig. 4a, b), as well as in the pancreatic neuroendocrine carcinoma of patient III-9 (Fig. 4c, d) and in a parathyroid tumour of patient III-10 (Fig. 4e, f).

Discussion

In this study, gross deletions in the *MEN1* gene were investigated by TaqMan copy number assay in a large family with three affected members showing MEN1 phenotype, who were negative for *MEN1* gene mutations on direct DNA sequencing. In these affected members we identified a gross deletion involving exon 1 and exon 2 of the *MEN1* gene, which was absent in the remaining nine living family members and in eight sporadic mutation-negative cases studied. The applied method allowed a definite diagnosis of MEN1 in these cases, with significant implications for both patients and their families. Indeed, the patients were correctly identified as *MEN1* mutation carriers (and not phenocopies) and their relatives as non-carriers. The latter classification allowed us to stop the intensive clinical follow-up they were undergoing, with a considerable psychological relief and sparing of clinical resources. While this approach was valuable in the current case, the TaqMan copy number assay may not be useful to

identify deletions located in *MEN1* gene regions different from the one detected in the presented family, that are not covered by this method. In addition, the latter cannot precisely identify the extent of the deleted exon 2 region (i.e. whether it extends to the whole exon 2 or to exon 3). This evidence is further underlined by the results of MLPA, showing that exon 3 is also deleted in the affected members of this family.

Previous published work reported alternative methods, such as MLPA, LRPA, reverse-transcription-PCR, gene dose assay and Southern blot, that allow the identification of *MEN1* deletions [16–23]. Our approach has some advantages as compared to other methodologies, such as Southern blot analysis [10], being much simpler and less labour-intensive similarly to MLPA, which is considered the gold standard for gene copy evaluation. While currently available commercial MLPA kits cover all exons of *MEN1*, additional methods, such as LRPA, may be required for complete gene evaluation. In addition, MLPA is highly sensitive to sample purity and to small changes in experimental conditions. Therefore, it should be performed in specialized reference centres that guarantee reliable results. These issues may hamper the routine clinical diagnostic application of such methods in non-specialized laboratories.

The TaqMan copy number assay may represent an alternative method to identify *MEN1* deletions and/or duplications. As compared with MLPA, TaqMan copy number variation assays has comparable costs in terms of reagents, requires less time and specialized equipment/expertise to be performed. Indeed, the employed technique provides reliable results after a single step, represented by a quantitative PCR, following gDNA isolation. In addition, the employed assay can also detect deletions extending to the minimal *MEN1* promoter region, allowing the identification of deletions in regions that are excluded from the routinely performed investigations. However, none of the commercially available kits, concerning both TaqMan copy number assay and MLPA, completely cover the entire *MEN1* gene region. As noted above, the MEN1 MLPA kit demonstrated that the deletion extended to exon 3, which indicates that the probe-mix used in the TaqMan assay would require significant optimization to perform well across all coding regions. Following such optimization, both techniques could be useful when searching for a large MEN1 deletion/insertion.

In our series, the phenotype of the affected patients was similar. All of them had developed hyperparathyroidism, a pituitary adenoma (Cushing's disease and PRL-oma), a pancreatic endocrine tumor (glucagonoma, non functioning well-differentiated endocrine carcinoma, insulinoma) and bilateral adrenal macronodular hyperplasia, even if occurring at different times along their clinical history. Another large deletion similar to the one we describe here was reported by Bergman et al. [25] in a patient with classical MEN1 phenotype. However, it must be pointed out that in that patient the deletion began upstream of the gene and terminated before exon 6, obliterating the start codon, being much more extensive than the one described here. Therefore, the functional consequences, not explored by Bergman et al., as well as the clinical phenotype may not completely overlap. A more recent report [24] describes a Spanish family with ten family members showing a deletion encompassing *MEN1* exons 1 and 2, detected by MLPA. Among gene mutation carriers, eight family members displayed at least one MEN1 clinical manifestation (hyperparathyroidism in eight, prolactinomas in two and gastrinomas in three) after 12 years of follow-up. Our findings are slightly different, since our patients with an exon 1–3 deletion displayed the full MEN1 phenotype. Therefore, as with point mutations, clinical appearance among family members showing a similar genetic abnormality may differ [25], supporting the hypothesis that the phenotypes of the MEN1 cases with gross deletions are not significantly different from those with other *MEN1* mutations [10], that lack a direct genotype-phenotype correlation even though a higher risk of death secondary to a MEN1 tumor has been reported in patients with mutations affecting the JunD interacting domain [26]. Further studies on new families will be necessary to assess the lack of correlations between *MEN1* mutations and clinical manifestations of the disorder also in *MEN1* mutation carriers characterized by the loss of exons 1–3.

The majority of reported *MEN1* mutations, such as frameshift and nonsense mutations are predicted to result either in a truncated protein, with the consequent loss of functional domains or in loss of the translated protein because of nonsense-mediated mRNA decay [5]. Deletion of the first three exons likely determines the lack of MEN1 mRNA transcription and therefore no menin protein in the tumour samples, with important consequences at cellular level. In the tumoral tissue samples of an affected family member we could demonstrate somatic loss of *MEN1* exons 1 and 2, indicating the absence of wild-type *MEN1* gene exon 1 and exon 2. Therefore, we supposed that menin protein was not expressed in these tissues. And indeed, no menin immunostaining in the pathological tissues could be found, supporting the hypothesis that the deletion prevents menin transcription and translation. The molecular mechanisms that lead to such large germline deletions, however, remain to be elucidated [18].

Our work underlines that direct sequencing is not sufficient for a complete genetic analysis in patients with MEN1 phenotype, since, even if in rare cases, it cannot identify the underlying genetic alteration in all patients with a high clinical suspicion of MEN1. New approaches, such as real-time PCR and MLPA, may help us to achieve

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a correct diagnosis also in patients not recognised by standard sequencing-based testing protocols.

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Conflict of interest The authors declare that they have no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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ENDOCRINE GENETICS/EPIGENETICS

Early onset acromegaly associated with a novel deletion in *CDKN1B* 5′UTR region

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Abstract Genetic alterations frequently are involved in the development of a pituitary adenoma in young age. We here characterize the functional role of a deletion in *CDKN1B* 5′-UTR region (c.-29_-26delAGAG) identified in an acromegalic patient that developed a growth hormone in pituitary adenoma during childhood. Our results show that the identified novel heterozygous deletion in the *CDKN1B* 5′-UTR region associates with a reduction in *CDKN1B* mRNA levels, a predicted altered secondary mRNA structure, and a reduced *CDKN1B* 5′-UTR transcriptional activity in vitro. The patient displayed loss of heterozygosity in the same *CDKN1B* 5′-UTR region at tissue level and the 5′UTR region containing the deleted sequence encompasses a GRE. These findings indicate that the identification of functional alterations of newly discovered genetic derangements need to be fully characterized and always correlated with the clinical manifestations.

Keywords Acromegaly · *CDKN1B* · Genetic derangements

Introduction

Pituitary adenomas are the most frequent intracranial tumors with a prevalence of 77–94 cases/100.000 inhabitants, equally distributed among sexes and ages [1, 2]. The development of a pituitary adenoma in young age is frequently associated with genetic alterations, including multiple endocrine neoplasia type 1 (MEN1), Carney complex (CNC), familial isolated pituitary adenomas (FIPA), and MEN type 4 (MEN4). The latter is caused by mutations in the cyclin-dependent kinase inhibitor 1B (*CDKN1B*) gene, encoding for the CDK inhibitor $p27^{Kip1}$ [3, 4], that are rare in the settings of FIPA [5, 6], but cause MEN4 [3]. Mutations in the *CDKN1B* gene are associated with the development of multiple endocrine tumors, displaying highly variable phenotypes that share features with both MEN1 and MEN2 syndromes in rats and show a MEN1-like phenotype in humans [4, 7]. Different types of pituitary adenomas have been described in MEN4 patients, including GH-secreting [3, 8, 9], ACTH-secreting [8, 10], and non-functioning pituitary adenomas [11]. On the contrary, *CDKN1B* mutations/rearrangements have not been reported in sporadic settings [6, 8, 12].

We here present a patient with a recurrent GH-secreting pituitary adenoma, which occurred at a very young age, and whose putative genetic causes have been intensively investigated.

Silvia Sambugaro and Mauro Di Ruvo have contributed equally to this work.

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The patient, a 30-year-old woman, came to our observation due to previous diagnosis of acromegaly. At the age of 5 years, the patient presented with excessive growth velocity and therefore underwent clinical investigation that disclosed GH hypersecretion due to a pituitary adenoma. At the age of 6 years, the patient underwent trans-nasal-sphenoidal adenomectomy with histological diagnosis of pituitary macroadenoma with positive immunohistochemistry for GH and a Ki-67 <1 %. Despite the lack of pituitary adenoma recurrence, IGF-1 levels were high and therefore Bromocriptine therapy was started. Biochemical control was not achieved, and medical therapy was changed to somatostatin analog (SSA) s.c. without benefit. Therefore, at the age of 7 years, the patient underwent external fractionated radiotherapy (29 sessions with total administered dose of 4640 Gy). Nevertheless, high GH and IGF-1 levels persisted and therapy with SSA was started again. Since then, the patient had been treated in the last 20 years with SSA due to the lack of GH suppression under glucose load and to increased IGF-1 levels when medical therapy was withdrawn. The patient developed gallbladder stones and central hypothyroidism.

Because of the young age at disease onset, a genetic predisposition was hypothesized and the presence of germline MEN1 and AIP mutations was investigated, with negative findings. In the search for other possible genetic causes and because of the lack of clinical signs and symptoms of CNC, *CDKN1B* was analyzed for the presence of mutations. We found a c.-29_-26delAGAG in the *CDKN1B* 5′-UTR region of the patient and failed to identify the same variant in 20 normal subjects and in 10 acromegalic patients. This deletion occurs next to a previously described *CDKN1B* deletion in the 5′-UTR region [13]. The latter has been found to be carrying different variants in patients with acromegaly [9]. The aim of this study is to characterize the functional role of the c.-29_-26delAGAG in *CDKN1B* 5′-UTR region.

Materials and methods

DNA extraction and sequencing

Genomic DNA was extracted using a QIAamp DNA Mini Kit (Qiagen, Milano, IT). *CDKN1B* gene coding regions and intron–exon boundaries were amplified by Polymerase Chain Reaction (PCR) using the thermal cycler GeneAmp PCR System 9700 (Life Technologies, Milano, IT), by applying the following thermal cycling conditions: incubation at 96 °C for 3 min; 35 cycles at 94 °C for 30 s, 66 °C for 1 min, and 72 °C for 1 min with a final extension at 72 °C for 7 min. Each reaction mixture was prepared using GoTaq green master mix (Promega, Milano, IT). PCR primers employed to amplify the 5′UTR region (5′UTR p27 for and 5′UTR p27 rev) and the coding sequence (exons 1–3) are described in Table 1. Direct sequencing was performed as reported [14].

DNA isolation from formalin-fixed, paraffin-embedded tissue sections

Formalin-fixed, paraffin-embedded (FFPE) tissue sections from the patient's pituitary adenoma were incubated with xylene to dissolve paraffin, and DNA was isolated using the QIAamp DNA FFPE Tissue Kit procedure (Qiagen).

Prediction of mRNA *CDNK1B* secondary structure

Secondary structure analysis of the 5′-UTR (-575/-1) *CDKN1B* mRNA was performed using the web application www.rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi.

cDNA synthesis and relative qPCR

RNA was isolated from peripheral blood leukocytes of the patient and of healthy controls. RNA quality and quantity were evaluated using the Experion automated electrophoresis system (Bio-Rad, Milano, Italy). RNA samples with a RNA Quality Indicator between 9.4 and 10 and a 28S/18S rRNA ratio >1.7 were processed. For cDNA synthesis, RNA was reverse transcribed using Superscript First Strand Synthesis System for RT-PCR (Life Technologies) with random hexamers, according to the manufacturer's instructions. The amount of *CDKN1B* mRNA was quantified by relative qPCR using Taq Man Gene Expression assay Hs01597588_m1 (Life Technologies). The qPCR assay was performed on 100 ng of reverse-transcribed RNA per replicate and each reaction mixture was prepared using 1X TaqMan Gene Expression Master Mix, 1X TaqMan Gene Expression Assay, for a total reaction volume of 20 μl. The samples were assessed in triplicate on the 7900HT FAST Real-Time PCR System (Life Technologies) and the program reaction was: 1 cycle at 95 °C for 10 min, 40 cycles at 95 °C for 15 s and at 60 °C for 1 min. Each qPCR reaction included a negative control (NTC). The reaction specificity was verified by amplicon sequencing (data not shown).

The best reference genes were chosen using the GeNorm program, as previously reported [14]. All samples were normalized against four different housekeeping genes: ACTB (β-actin), GUS (β-Glucuronidase), CYC (Cyclophylin), and HuPO (human acidic ribosomal protein). HuPO was used as reference gene (4326314E) [15]. The amplification efficiency (E) was calculated based on the slopes of the standard curves for our sample and reference gene ($E = 99$ %).

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Table 1 Primers employed to amplify *CDKN1B* promoter and coding regions

Name	Sequence
5′UTR p27_ChIP for	5′-TGTGTCTTTTGGCTCCGAGG-3′
5′UTR p27_ChIP rev	5′-CTCCCGTTAGACACTCGCAC-3′
5′UTR p27 for	5′-GAG GAG CGG GAG GGA GGT CG-3′
5′UTR p27 rev	5′-CTAGGGCTCCCGTTAGACACT-3′
p27ex1A for	5′-CGTCAGCCTCCCTTCCACCG-3′
p27ex1A rev	5′-CTCTTCGTGGTCCACCGGGC-3′
p27ex1B for	5′-GAGCCCTAGCCTGGAGCGGAT-3′
p27ex1B rev	5′-GCGGGGCCCCAAACACATTCT-3′
p27ex2 for	5′-CTGACTATGGGGCCAACTTC-3′
p27ex2 rev	5′-GCCAGCAACCAGTAAGATCAG-3′
p27ex3A for	5′-TGAACACTGGCTAAAGATAATTGCTATTTA-3′
p27ex3A rev	5′-TGCCAGGTCAAATACCTTGTTTG-3′
p27ex3B for	5′-GACCAAAGAACACAGCACAGAGGA-3′
p27ex3B rev	5′-CTGGGGAGGGCAGTGAGGAT-3′
5′UTR p27 BglII	5′ GAG GAG CGG GAG GGA GAT CTG GGC TT 3′
5′UTR p27 NcoI	5′ GAC ACT CGC ACG TTT GCC ATG GTT CTC 3′

The ΔΔCt method and RQ manager 1.2.1 (Life Technologies) were used to calculate the mRNA amounts. Data analysis was performed by SDS 2.4 software (Life Technologies).

Immunohistochemistry

Immunohistochemistry (IHC) was performed using a monoclonal anti-p27 antibody (BD Biosciences, CA, USA), as already reported [3].

Plasmid constructs

The *CDKN1B* 5′UTR region (-821/-1 to the start site of translation) displaying (5′-UTR-DEL) or not (5′-UTR-WT) the deletion (c.-29_-26delAGAG) was cloned into the pCR 2.1 vector (Life Technologies) using the TA Cloning Kit (Life Technologies). Ligation reaction was performed using T4 DNA Ligase Express Link (Life Technologies).

DNA was then amplified by PCR using primers containing a BglII and NcoI sequence (5′UTR p27 BglII and 5′UTR p27 NcoI, see Table 1). After enzymatic digestion with BglII and NcoI restriction enzymes (Euroclone, Milano, IT), the 5′-UTR-WT and 5′-UTR-DEL *CDKN1B* sequences were cloned in the pGL4.10 [luc2] vector (Promega) containing the firefly luciferase reporter gene. The following three constructs were generated: pGL4 basic (promoterless), pGL4-5′-UTR-WT, and pGL4-5′-UTR-DEL.

Cell culture

GH3, MCF-7, AtT-20/D16v-F2, and Hela cells were obtained from ATCC (American Type Culture Collection, Manassas, VA, USA) and cultured as previously described [11, 15, 16]. The human cervix adenocarcinoma cell line, Hela, was cultured in the DMEM high glucose medium (Gibco by Life Technologies) enriched with 10 % FBS.

Transfection and luciferase gene reporter assays

GH3, MCF-7, and AtT-20/D16v-F2 cells were transfected with a 100 ng/well pGL4 construct (pGL4 basic, pGL4-5′-UTR-WT, or pGL4-5′-UTR-DEL) using Lipofectamine LTX PLUS Reagents (Life Technologies). The cells were co-transfected with pRL-TK vector (Promega) in triplicates. Luciferase activity assays were performed using the Dual-Glo Luciferase Assay System (Promega). The readings were carried out on EnVision Multilabel Reader (Perkin Elmer, Monza, IT).

Chromatin immunoprecipitation (ChIP) assays

ChIP assays were carried out using the SimpleChIP® Enzymatic Chromatin IP Kit Agarose Beads #9002 (Cell Signaling Technology, Danvers, Massachusetts, USA) after incubating Hela cells with or without 10^{-6} M Dexamethasone (Sigma-Aldrich, Saint Louis, MO, USA) for 24 h. Primer sequences for amplification of *CDKN1B* 5′-UTR region encompassing the c. -29_-26delAGAG (5′-UTR p27_ChIP for and 5′-UTR p27_ChIP rev) are described in Table 1. The following PCR reaction program was employed: 95 °C for 5 min, 34 cycles at 95 °C for 30 s, then 59 °C for 30 s, and 72 °C for 30 s with a final extension at 72 °C for 5 min.

Statistical analysis and informed consent

Data were expressed as mean ± standard error of the mean (SEM). We used the paired or unpaired Student *t* test to

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evaluate individual differences between means. P values <0.05 were considered significant. Patient gave written informed consent for molecular analysis and data collection.

Results

CDKN1B sequencing results

A germ-line heterozygous deletion in the *CDKN1B* gene 5′-UTR region (c.-29_-26delAGAG) was found in the patient (Fig. 1b) and in her mother, who has always been asymptomatic. The father displayed a wild-type *CDKN1B* gene 5′-UTR region.

Detection of loss of heterozygosity (LOH) in the patient's pituitary adenoma

The *CDKN1B* 5′-UTR region encompassing the deleted sequence was amplified by PCR from DNA isolated from FFPE pituitary adenoma sections, using the 5′UTR p27_ChIP for and 5′UTR p27_ChIP rev primers, which amplify a 160 bp fragment. *CDKN1B* 5′UTR region amplification from the DNA of the patient's pituitary adenoma shows a single 156 bp band on a 10 % acrylamide gel. On the contrary, we obtained two bands of 160 and 156 bp, respectively, from the patient's germ-line DNA, demonstrating LOH in the patient's pituitary adenoma (Online Resource 1). These findings have been confirmed by direct sequencing.

CDKN1B mRNA secondary structure prediction and expression

As shown in Fig. 1c, the wild-type *CDKN1B* 5′-UTR region (575 nucleotides) displays a 'stem and loop' secondary structure, that includes the -29/-26 AGAG element. This region contains a 'U-rich' sequence which interacts with mRNA-binding proteins and modulates ribosome recruitment [17–19], being involved in *CDKN1B* mRNA stability regulation. The deleted *CDKN1B* 5′-UTR region has a different predicted secondary structure (Fig. 1d) as compared to the wild-type *CDKN1B* 5′-UTR, indicating that the c.-29_-26delAGAG modifies the predicted 'stem and loop' structure of the 5′UTR region.

CDKN1B mRNA expression levels were analyzed by qPCR in peripheral blood T lymphocytes of the patient and

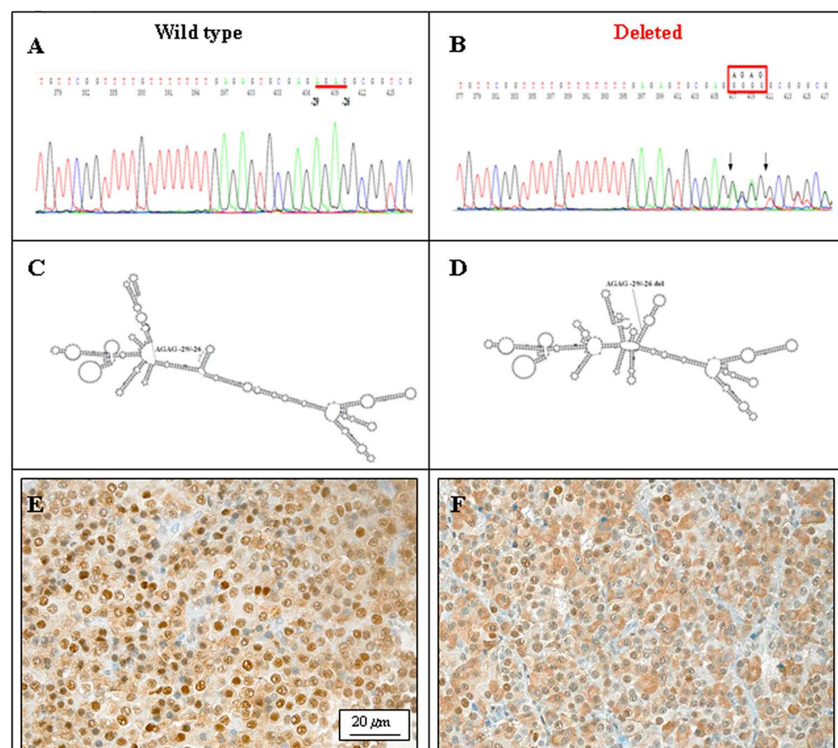

Fig. 1 Germ-line mutation in patient's *CDKN1B* gene, *CDKN1B* mRNA secondary structure. **a** Wild-type sequence (Wild type) of the *CDKN1B* 5′-UTR region with the -29/-26 AGAG nucleotides underlined. **b** Deleted sequence (Deleted) of the *CDKN1B* 5′-UTR region with the c.-29_-26delAGAG. (*red box*). **c** Predicted secondary structure of the wild-type 5′-UTR (-575/-1) *CDKN1B* mRNA performed using the web application: www.rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi. **d** Predicted secondary structure of the deleted 5′-UTR (-575/-1) *CDKN1B* mRNA. **e** Immunohistochemical staining for p27^{Kip1} in a representative GH-secreting pituitary adenoma tissue from a *CDKN1B* deletion-negative patient, showing prevalent nuclear p27^{Kip1} immunoreactivity. **f** Immunohistochemical staining for p27^{Kip1} in the patient's pituitary adenoma, showing prevalent cytoplasmic p27^{Kip1} distribution

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we found a 70 % reduction in *CDKN1B* mRNA expression as compared to 3 normal unrelated subjects. ($P < 0.01$).

p27^{Kip1} expression in patient's pituitary tumor

IHC shows that p27^{Kip1} protein staining is reduced and mainly cytoplasmic in the patient's pituitary adenoma (Fig. 1f) as compared to a representative GH-secreting pituitary adenoma tissue from a *CDKN1B* deletion-negative patient (Fig. 1e), where p27^{Kip1} protein staining is higher and predominantly nuclear.

Functional characterization of the c.-29_-26delAGAG in *CDKN1B* 5′-UTR region

The luciferase gene reporter assays showed that pGL4-5′-UTR-DEL luciferase activity was significantly reduced in all the evaluated cell lines (GH3, MCF7, AtT-20/D16v-F2) as compared to pGL4-5′-UTR-WT luciferase activity ($P < 0.01$) (Online Resource 2).

Identification of a Glucocorticoid response element (GRE) in the *CDKN1B* 5′-UTR region

Glucocorticoids induce p27^{Kip1} protein expression in lymphoid and non-lymphoid cells [20]. To explore the hypothesis that the *CDKN1B* 5′-UTR region encompassing the deleted sequence contains a glucocorticoid response element (GRE), a ChIP assay was performed. We found that the *CDKN1B* 5′-UTR region was amplified from the DNA immunoprecipitated with an anti-GR antibody, both before and after dexamethasone treatment of HeLa cells (Online Resource 3). In addition, the band obtained after PCR from the immunoprecipitated DNA isolated from HeLa cells treated with dexamethasone is stronger, indicating that treatment with glucocorticoids induces GR-DNA interaction at the investigated *CDKN1B* 5′-UTR region. These results are validated by the presence of adequate positive controls, such as a good-quality chromatin (input sample), correctly immunoprecipitated by the Anti-H3 antibody, but not by an anti-rabbit antibody. The positive controls are not modified by treatment with dexamethasone, indicating that the employed experimental conditions guarantee the success of immunoprecipitation.

Discussion

Our study underlines the importance of evaluating the functional effects of genetic alterations identified in light of specific clinical features in a case of recurrent GH-secreting pituitary adenoma, which occurs at a very young age.

Indeed, our results show that the identified novel heterozygous deletion in the *CDKN1B* 5′-UTR region, consisting of c.-29_-26delAGAG, causes a reduction in *CDKN1B* mRNA levels, a predicted altered secondary mRNA structure, and a reduced *CDKN1B* 5′UTR region transcriptional activity in vitro. We also found a LOH in the same *CDKN1B* 5′-UTR region at tissue level, possibly accounting for the reduced p27^{Kip1} protein levels in the pituitary adenoma of the affected patient and the lack of clinical manifestations in the patient's mother, who displayed the same germ-line deletion in heterozygosity. Moreover, the 5′UTR region containing the deleted sequence encompasses a GRE.

Our findings strengthen the association of *CDKN1B* 5′UTR variants with a peculiar clinical phenotype, i.e., GH-secreting pituitary adenomas, as previously reported [6, 9]. Furthermore, a recent study identified a GAGA (-32/-29) germ-line heterozygous deletion in the *CDKN1B* 5′-UTR in a patient affected by gastric carcinoid tumor and hyperparathyroidism, providing evidence that this germ-line deletion alters *CDKN1B* transcription and *CDKN1B* mRNA levels [13], similar to what here reported. Indeed, we found a 26–68 % reduction in the transcriptional activity of the deleted *CDKN1B* 5′UTR region in different cell lines. These results show that the reduction in *CDKN1B* 5′UTR region transcriptional activity is not tissue-specific, since it has been found in cell lines originating from different tissues and species. In addition, we found a 70 % reduction in *CDKN1B* mRNA expression in the patient's leukocytes as compared to normal controls. The latter finding, however, could also be due to mRNA decay of the c.-29_-26delAGAG carrier allele in vivo. All together, our results and those from Malanga et al. suggest that the *CDKN1B* 5′UTR region from nucleotide -32 to nucleotide -26 with respect to the translation start site is relevant for the regulation of *CDKN1B* transcription. These results are in keeping with the finding that the human *CDKN1B* 5′-UTR contains a U-rich element involved in regulating *CDKN1B* mRNA stability and translation efficacy [17–19]. And indeed the identified c.-29_-26delAGAG modifies the predicted *CDKN1B* mRNA 'stem and loop' secondary structure, possibly impairing ribosome entry and subsequent mRNA transcription. In addition, p27^{Kip1} protein levels are reduced and the protein is mainly cytoplasmic in the patient's pituitary adenoma as compared to a GH-secreting pituitary adenoma from a patient lacking the c.-29_-26delAGAG. It has been previously reported that, when in the nucleus, p27^{Kip1} binds and inhibits cyclin/CDK complexes, acting as a cell cycle inhibitor [7]. On the contrary, there is evidence that a cytoplasmic localization impairs its function as a cell cycle inhibitor and correlates with high tumor grade [21]. Therefore, the finding that in our patient's pituitary adenoma p27^{Kip1} is mainly

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cytoplasmic, together with a reduced protein amount, suggests that this protein might exert a reduced oncosuppressor function. These findings are in keeping with the clinical aggressiveness of the GH pituitary adenoma in this patient, who needed to undergo two surgeries and external radiation therapy without reaching disease control.

It has been previously demonstrated that glucocorticoids induce p27^{Kip1} protein expression in lymphoid and non-lymphoid cells [22], indicating that the *CDKN1B* 5′-UTR region encompasses a GRE. Indeed, we could amplify the *CDKN1B* 5′-UTR region from DNA immunoprecipitated with an anti-GR antibody in a ChIP assay. The immunoprecipitated DNA region contains the -29/-26 AGAG sequence, that was found to be deleted in our patient. These results provide indirect evidence as to the presence of a GRE in the deletion site, suggesting that the deleted *CDKN1B* 5′-UTR region might be less responsive to glucocorticoids, with further possible functional consequences.

These findings indicate that the identification of functional alterations of newly discovered genetic derangements need to be fully characterized and always correlated with the clinical manifestations. However, the presence of other mutations (somatic or germ line) cannot be excluded, possibly contributing to the development of an aggressive and early onset acromegaly in our patient.

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Conflict of interest EdU received consulting fees from Novartis and Pfizer. MCZ received consulting fees from Novartis and Genzyme. The other authors have nothing to disclose and have no conflict of interest.

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ORIGINAL ARTICLE

CUSHING IN A LEAF: ENDOCRINE DISRUPTION FROM A NATURAL REMEDY

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Background: Information regarding the safety of herbal drugs is often not reported. We describe the case of a 65-year-old woman referred to us for a iatrogenic hypercortisolism, who denied any previous steroid consumption. She reported only a chronic application of a phytocosmetic cream, containing ethanol extract of the Cardiospermum halicacabum (CH) plant. Adrenal insufficiency occurred after the cream application was stopped. CH is used in traditional and Western medicine for its documented anti-inflammatory properties. Once the presence of synthetic glucocorticoids was ruled out in the phytocosmetic product, we investigated whether and how its chronic application could have caused the iatrogenic hypercortisolism.

Methods: LC/HRMS was performed to exclude the presence of known glucocorticoids in the cream. ELISA assay and Western blot analysis were employed to assess ACTH secretion and the glucocorticoid receptor expression respectively in murine ACTH-secreting pituitary adenoma cells AtT-20/D16v-F2, treated with dexamethasone, CH tincture and mifepristone alone or in combination. To detect specific interaction of CH extract with the glucocorticoid receptor we performed a dual-luciferase reporter assay in HEK293 cells.

Results: In AtT-20/D16v-F2 cells CH extract showed to significantly reduce basal and CRH-induced ACTH secretion and the glucocorticoid receptor expression, similarly to dexamethasone; these effects were counteracted by mifepristone. In HEK293 cells dexamethasone significantly induced luciferase activity after 24 and 36 hour treatment, CH tincture only after 36 hours and these effects were antagonized by mifepristone.

Conclusions: CH extract displays a glucocorticoid-like activity, by means of a direct binding to the glucocorticoid receptor.

Herbal drugs have been used since the beginning of human history as remedies for health disturbances. Early in the 19th century the knowledge about the active principles derived from medicinal plants allowed the development of modern pharmacology leading gradually to the discovery and development of new synthetic drugs, finally causing herbal drugs to be confined to herbal laboratories. Nonetheless, the herbal drug market has rapidly increased in the last decades, both in the US and Europe (1–3), along with the expansion of the so called "complementary and alternative medicine (CAM)" (4). Part of the success of the therapies based on herbal drugs (or "phytotherapy") is most likely due to the belief that "natural is safer".

Nevertheless, in Western medicine recourse to synthetic glucocorticoids is more and more common (5), since they are used as anti-inflammatory and immunosuppressive agents in several settings. Chronic treatment with glucocorticoids also represents the major known cause of hypercortisolism, a condition more likely to develop during

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oral therapy, but also after a prolonged topical administration (ie, inhaled, transcutaneous, endo-nasal, intra-joint). Clinical signs of Cushing syndrome caused by chronic exposure to exogenous glucocorticoids typically develop faster than in endogenous hypercortisolism, and are proportionally related to treatment potency and duration (6).

Besides several cases of adulteration of traditional and herbal remedies with synthetic glucocorticoids, to date no case of exogenous hypercortisolism due to herbal drug exposure has been described.

In this study we describe a case of iatrogenic hypercortisolism during chronic exposure to a topical phytocosmetic product.

THE CASE

A 65-year-old woman referred to our clinic for typical clinical features of Cushing syndrome (facial plethora, buffalo hump, reddish purple abdominal striae, central obesity). Her clinical history was significant for arterial hypertension, moderate aortic stenosis, previous carotid endarterectomy, type 2 diabetes mellitus and autoimmune thyroiditis. At presentation therapy included sartan, potassium canrenoate, bisoprolol, torasemide, ASA, atorvastatin, L-thyroxine.

Laboratory tests showed a hypothalamic-pituitary-adrenal (HPA) axis suppression, suggesting iatrogenic hypercortisolism (Table 1). Abdominal CT, showing a significant hepatomegaly, was negative for adrenal lesions and pituitary magnetic resonance imaging (MRI) was normal.

The patient denied any previous steroid use and, carefully questioned about this, she denied the use of intranasal sprays or joint injections containing steroids. However she reported a daily application of a phytocosmetic cream for the treatment of a submammary erythema for approximately 3 years. The active principles of the cream were: 10% ethanol extract of Cardiospermum halicacabum (CH) plant, 0.5% bisabolol, 0.3% 18β-glycyrrhetinic acid.

In addition, the patient developed clinical and biochemical features of adrenal insufficiency after the cream administration was stopped. An ACTH stimulation test (250 μg) was then performed, showing no response of cortisol and DHEAS. Replacement therapy with cortisone acetate (25 mg daily) was started, Cushing somatic features progressively resolved and the adrenal function partially recovered after three years.

The purpose of this study is to investigate whether and by which mechanism the chronic application of the herbal cream could have caused a clinical and biochemical pattern of iatrogenic hypercortisolism, as in the described clinical case. Cardiospermum halicacabum L. (family Sapindaceae) (CH) is an herbaceous climber widely found in tropical and subtropical Asia and Africa, largely used in traditional medicine for many purposes, particularly for its anti-inflammatory properties (7–11). In Western medicine, it is mainly used as hydro-alcoholic extract (mother tincture) in creams and ointments for the treatment of atopic dermatitis, eczema and psoriasis (12, 13).

Materials and Methods

Halicalm cream was purchased from Labo Phyto Tre S.r.l (Rovedo in Piano, Pordenone, Italy). Whole plant mother tincture of CH was purchased from Laboratoires Lehning SAS (Sainte-Barbe, France). HPLC grade acetonitrile, formic acid, betamethasone (BM), methylprednisolone (MP), budesonide (BD) were purchased from Sigma-Aldrich (Milan, Italy). Purified water from Milli-Q System (Milliporem Milford, MA, USA) was used to prepare mobile phase and standard solutions.

Nitrocellulose syringe filters (0.20 μm) were purchased from Phenomenex (Torrance, CA, USA). Stock standard solutions of BM, MP, BD were prepared by dissolving each compound at a concentration of 1 mg/mL in acetonitrile. Stock solutions were stored at 4°C for at most one month; further dilutions were prepared daily.

Table 1. Laboratory tests. Serum ACTH and ACTH stim test were performed just after, 3 months and 4 yr after cream discontinuation.

Test	Result			Reference limits
Na	137 mmol/liter			135–145
K	4.3 mmol/liter			3.3–5.1
serum cortisol	44 nmol/liter			138–690
Urine free cortisol-UFC	30 nmol/24 h			90–694
DHEAS	<0.4 umol/liter			0.9–11.7
ACTH	**basal**	**3 months later**	**4 yr later**	10–50
	<5 ng/liter	<5 ng/liter	45 ng/liter	
ACTH test (250 μg)	**basal**	**3 months later**	**4 yr later**	
serum cortisol t 0′	45 nmol/liter	87 nmol/liter	171 nmol/liter	
serum cortisol t 60′	55 nmol/liter	632 nmol/liter	221 nmol/liter	

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Dexamethasone, mifepristone and Corticotrophin Releasing Hormone (CRH) were purchased from Sigma-Aldrich (Saint Louis, MO, USA) and were diluted in charcoal-dextran stripped culture medium.

LC/HRMS analysis

The extraction of the compounds of interest from the cream was achieved by following a validated published procedure (14). 0.5 g of Halicalm cream, containing CH ethanol extract, were fortified with the internal standard (methyltestosterone) at the final concentration of 20 μg/g and diluted acetonitrile (2 mL). The mixture was heated up to 45°C, sonicated for 30 minutes and diluted with water/acetonitrile 50/50 v/v (5 mL). The solution was then filtered through a membrane (0.45 μm). LC-MS analysis was carried out on a Surveyor LC system (Thermo, San Jose, CA, USA) coupled with a high-accuracy - high resolution Orbitrap XL mass spectrometer (Thermo, Brema, Germany) equipped with an electro-spray ionization source (ESI) operating in positive ion mode. Other working parameters were set as follows: transfer capillary 300°C; ESI needle spray voltage +4.0 kV; sheath gas 30 au; auxiliary gas 5 au. Mass spectra were acquired at a resolution of 30 000.

Liquid chromatography was performed on a Surveyor binary pump (Thermo, Brema, Germany) equipped with an Atlantis T3 Waters (150 mm x 2,1 mm x 2,5 μm) kept at 40°C. The following mobile phases were used: A (0,1% formic acid in water), B (0,1% formic acid in acetonitrile) delivered in a linear gradient, t = 0–5 minutes 80% A, t = 20 minutes 50% A, t = 25 minutes 5% A, t = 27 minutes 5% A, t = 30 minutes 80% A. The flow was kept constant at 300 μL/min. The injection volume 20 μL full loop. Analytes were identified by searching the exact mass of the ion $[M+H]^+$.

Cell culture, ELISA assay and Western blot analysis

The murine ACTH-secreting pituitary adenoma cells AtT-20/D16v-F2 were obtained from ATCC (American Type Culture Collection, Manassas, VA, USA) and cultured in DMEM (Life Technologies, Milano, Italy) with 1% penicillin/streptomycin (EuroClone, Milano, Italy) and 10% horse serum (EuroClone) (15).

ACTH 'Ultra Sensitive' lumELISA kit (Calbiotech, Spring Valley, CA, USA) was employed as previously described (16). The sensitivity was < 1 pg/mL at the 95% confidence limit (CL). Intra- and interassay variation coefficients were 6 and 8.7% respectively. ACTH levels were assessed in the conditioned culture medium of AtT-20/D16v-F2 cells treated for 48 hours with 1 μM dexamethasone, 2.5% CH tincture, 1 μM mifepristone alone or in combination. Dexamethasone was used as reference glucocorticoid (GR agonist), while mifepristone was used as reference GR antagonist. Results are expressed as mean value ± standard error percent relative light unit vs. control cells from three independent experiments in seven replicates.

Western blot analysis was performed to evaluate GR alpha (GR-α) protein expression in AtT-20/D16v-F2 cells. 30 μg of protein extract for each sample were fractionated on 10% SDS–PAGE and transferred by electrophoresis to nitrocellulose membranes (Protran, Dassel, Germany). Subsequently, the membranes were incubated at 4°C overnight with 1:1000 polyclonal rabbit anti-β-actin and 1:1000 antiglucocorticoid receptor antibody (both from Cell Signaling, Beverly, MA, USA). Secondary antibodies (1:2000 antirabbit and 1:5000 antimouse HRP-conjugated IgG, both from Dako Italia, Milano, Italy) were incubated for 1 hour at room temperature and binding was revealed using enhanced chemiluminescence Western blotting detection reagents (Pierce).

Transfection and Dual Glo luciferase assay

The GloResponse 9XGAL4UAS-luc2P HEK293 cell line (Promega, Milano, Italy) is generated by clonal selection of human embryonic kidney 293 (HEK293) cells, stably transfected with the pGL4.35[luc2P/9XGAL4UAS/Hygro] vector. This vector is specifically responsive to glucocorticoids. GloResponse 9XGAL4UAS-luc2P HEK293 cells were cultured in DMEM (Life Technologies) supplemented with 1% penicillin/streptomycin, 10% fetal bovine serum (EuroClone) and 200 μg/mL hygromycin B (σ- Aldrich, Milano, Italy). The cells were maintained at 37°C in a humidified atmosphere with 5% CO_2. GloResponse 9XGAL4UAS-luc2P HEK293 cells were seeded in 96-well culture plates at 1×10^4 cells/well, using phenol red-free DMEM (Life Thecnologies) containing 5% charcoal/dextran-treated FBS (EuroClone). The next day the cells were transfected with 100 ng pBIND-GR Vector (Promega) containing the GR ligand-binding domain, using Lipofectamine 3000 (Thermo Fisher Scientific) according to the manufacturer's instruction. One day after transfection, the cells were treated with 1 μM dexamethasone, 2.5% CH tincture, 1 μM mifepristone alone or in combination. Control cells were treated with vehicle solution (1.625% ethanol). After 24 and 36 hours, cells were assessed for firefly and renilla luciferase activity using the Dual-luciferase reporter assay (Promega) according to the manufacturer's instruction. Chemiluminescence was measured by EnVision Multilabel Reader (PerkinElmer, Waltham, MA, USA). Results are expressed as mean value ± standard error percent relative light unit vs. control cells from three independent experiments in four replicates.

STATISTICAL ANALYSIS

Paired or unpaired Student t test was used to assess individual differences between means. P value < 0.05 was considered significant.

Results

First we ruled out the presence of synthetic glucocorticoids in the cream containing CH extract, by means of a high accuracy-high resolution LC-MS method that excluded the presence of the analytes reported in Table 2. The limit of detection (LOD) of the technique was 1 μg/g of cream.

In order to verify a possible glucocorticoid-like effect of CH tincture, we tested whether CH tincture influences basal and CRH-stimulated ACTH secretion in AtT-20/D16v-F2 cells.

As shown in Figure 1, basal ACTH levels were significantly reduced in conditioned medium from AtT-20/D16v-F2 cells treated with either dexamethasone (-29%, $P < .01$) or CH tincture (-23%, $P < .01$) as compared to control untreated cells. GR antagonist mifepristone did not significantly affect ACTH levels, but significantly re-

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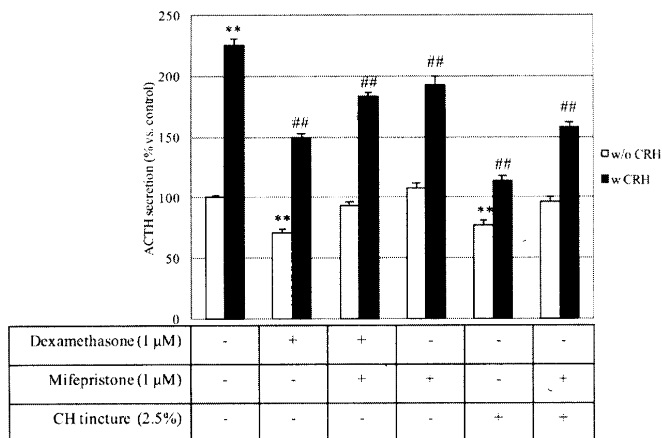

Dexamethasone (1 μM)	-	+	+	-	-	-
Mifepristone (1 μM)	-	-	+	+	-	+
CH tincture (2.5%)	-	-	-	-	+	+

Figure 1. Effects of CH tincture on basal and CRH-induced ACTH secretion AtT-20/D16v-F2 cells were incubated in 96-well plates for 48 hours in culture medium supplemented with 1 μM dexamethasone, 2.5% CH tincture, 1 μM mifepristone alone or in combination. Control cells were treated with vehicle solution. ACTH levels were determined as described in the Materials and methods section, before (white bars) and after treatment with 100 nM CRH (black bars). $** = P < .01$ vs control; $\#\# = P < .01$ vs CRH treated cells.

duced the inhibitory effect of dexamethasone and CH tincture on this parameter (+22% vs. dexamethasone and +19% vs. CH tincture, with $P < .01$ and $P < .05$, respectively).

CRH significantly stimulated ACTH secretion (+125% vs. control untreated cells, $P < .01$). Dexamethasone and CH tincture significantly reduced CRH-induced ACTH secretion (-33% and −49% vs. CRH treated cells, respectively, both with $P < .01$). Mifepristone significantly reduced CRH-induced ACTH secretion (-14% vs. CRH-treated cells, $P < .01$), and significantly reduced the inhibitory effect of dexamethasone and CH tincture on this parameter (+14% vs. CRH + dexamethasone and +19% vs. CRH + CH tincture, both with $P < .01$).

Briefly, CH tincture reduces basal and CRH-induced ACTH secretion in AtT-20/D16v-F2 cells supporting the hypothesis that CH tincture may have a glucocorticoid-like effect.

The effects of CH tincture on GR-α expression were then explored. As shown in Figure 2, GR-α levels decreased after treatment of AtT-20/D16v-F2 cells with either dexamethasone or CH tincture as compared to control. The GR antagonist mifepristone slightly reduced GR-α expression and counteracted the inhibitory effect of dexamethasone and CH tincture on this parameter. These results further support the hypothesis that CH tincture may have a glucocorticoid-like effect. Finally, to evaluate whether CH tincture directly binds to GR, we transfected GloResponse 9XGAL4UAS-luc2P HEK293 cells with pBIND-GR vector. Later, the cells were treated with 1 μM dexamethasone, 2.5% CH tincture, 1 μM mifepristone alone and in combination. After 24 and 36 hours, luciferase activity was measured using dual-luciferase reporter assay. As shown in Figure 3, after 24 hours dexamethasone significantly induced luciferase activity (+2521% vs. control, $P < .01$) which was not modified by CH tincture. Mifepristone reduced luciferase activity (-64% vs. control, $P < .01$) and counteracted the stimulatory effects of dexamethasone (-1785% vs. dexamethasone, $P < .01$). Moreover, mifepristone reduced luciferase activity also in the presence of CH tincture (-87% vs. control, $P < .01$). After 36 hours, both dexamethasone and CH tincture significantly induced luciferase activity (+9929% and +4829% vs. control, both with $P < .01$). Mifepristone significantly reduced luciferase activity (-51% vs. control, $P < .05$) and counteracted the stimulatory effects of both dexamethasone (-7345% vs. dexamethasone, $P < .01$) and CH tincture (-4019% vs. CH tincture, $P < .01$). These results demonstrate that the CH tincture directly and specifically binds GR, supporting its glucocorticoid-like activity.

Discussion

Although the laboratory pattern of our clinical case was evocative of an iatrogenic hypercortisolism, the patient denied any glucocorticoid consumption in the last months, only reporting chronic application of a phytocosmetic cream containing CH extract. Supported by the development of adrenal insufficiency after cream withdrawal, the following step was to rule out a possible sophistication of the cream with synthetic glucocorticoids. Several reports of herbal remedy adulterations with these drugs have already been described before in some cases of suspected hypercortisolism, also after administration of apparently innocuous and above suspicion products of the so-called "traditional medicine" (17–22). In our case, however, high-accuracy high resolution LC-HRMS analysis allowed us to exclude the presence of known synthetic glucocorticoids in the cream, with a reliable limit of detection.

Our research then focused on the components of the phytocosmetic product, particularly on Cardiospermum

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Table 2. Exact mass of the glucocorticoids screened in Halicalm cream

Analyte	Exact mass MH$^+$	Analyte	Exact mass MH$^+$
Beclomethasone	521.23 006	Fluticasone	445.16 549
Betamethasone	393.20 718	Fluticasone proprionate	501.19 171
Budesonide	431.24 281	Hydrocortisone	363.21 664
Cortisone	361.20 095	Meprednisone	373.20 095
Cortisone Acetate	403.21 152	Methylprednisolone	375.21 660
Deflazacort	422.22 241	Paramethasone	393.20 718
Dexamethasone	393.20 718	Prednisolone	631.20 095
Flunisolide	435.21 774	Triamcinolone	395.18 644
Fluocortolone	377.21 226	Triamcinolone acetonide	435.21 774

halicacabum, a plant whose anti-inflammatory properties are well known in Asiatic and African traditional medicine. The extract of this plant is mainly used in Western medicine for the topical treatment of several dermatological pathologies, where it is considered as a "natural cortisone". Previous studies have confirmed anti-inflammatory, antipyretic and analgesic property of CH in animal models (23) and in vitro. Some authors demonstrated that CH ethanol extract suppresses the production of TNF-α and nitric oxide by human peripheral blood mononuclear cells (24). Another study documented that CH ethanol extract in mouse macrophage cell lines dose-dependently inhibits mRNA expression of COX-2, iNOS, TNF-α, and COX-2 protein expression; these effects could probably be mediated by blocking NF-kB activation (9). However, to date a specific glucocorticoid-like action of CH and, in particular, the influence of CH on HPA axis have not been described.

Glucocorticoids exert a renowned negative feedback on the pituitary through the interaction with the GR, which acts as a nuclear transcription factor. In ACTH-secreting cells glucorticoids bind to GR leading to the transcriptional repression (so-called "transrepression") of proopiomelanocortin (POMC) gene and then to the inhibition of ACTH synthesis (25, 26). In our settings, CH was capable of inhibiting basal and CRH-induced ACTH secretion, in a similar fashion to dexamethasone. These data confirm the hypothesis that CH has a specific glucocorticoid-like activity, since it is antagonized by the GR antagonist mifepristone.

Activation of GR by its cognate ligand is involved in the receptor downregulation in most cell lines and animal tissues. This effect of "homologous down-regulation" of GR seems to take place both at a transcriptional and post-translational level (eg, GR protein turnover) (27–30), but the question is still open to debate, particularly concerning the different cell and tissue-specific differences observed in several studies. Our Western blot results show that CH tincture reduces GR-α protein expression in AtT-20/D16v-F2 cells, similarly to the reference glucocorticoid

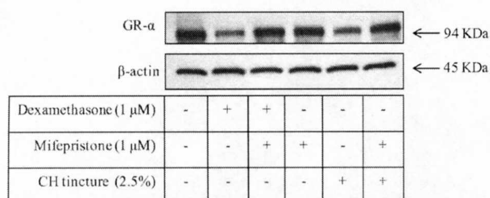

Figure 2. Effects of CH tincture on GR-α expression AtT-20/D16v-F2 cells were incubated in culture medium for 48 hours in the presence of 1 μM dexamethasone, 2.5% CH tincture, 1 μM mifepristone alone or in combination. Control cells were treated with vehicle solution. GR-α levels were assessed by Western blot as described in the Materials and methods section. β-actin is shown as a loading control.

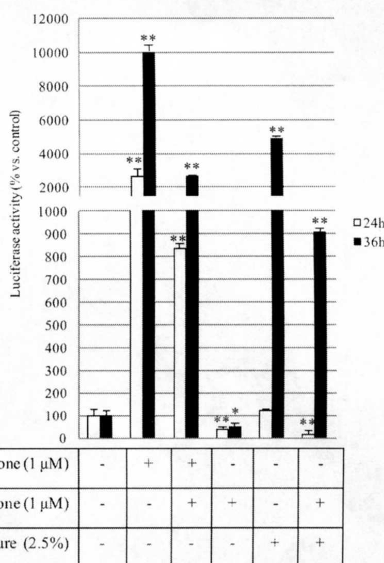

Figure 3. CH tincture directly binds GR-α GloResponse 9XGAL4UAS-luc2P HEK293 cells were incubated in 96-well plates for 24 (white bars) or 36 hours (black bars) in culture medium supplemented with 1 μM dexamethasone, 2.5% CH tincture, 1 μM mifepristone alone or in combination. Control cells were treated with vehicle solution. $^*P < .05$ and $^{**}P < .01$ vs control.

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dexamethasone, and furthermore that mifepristone antagonizes this effect for both dexamethasone and CH tincture. In addition, the direct and specific interaction of CH tincture with GR is evident in HEK cells engineered to show the induction of GR-DNA binding by exogenous stimuli. In these settings, DNA binding by GR was induced by dexamethasone after both 24 and 36 hours, while CH tincture induced this effect only after 36 hours. Once again these data confirm a glucocorticoid activity of CH, which, on the other hand, appears to be less potent in comparison to the employed reference glucocorticoid dexamethasone. In addition, the specificity of these effects has been confirmed by the evidence that GR-DNA interaction induced by both dexamethasone and CH are blocked by the GR antagonist mifepristone.

Overall, the data described in this study allow us to sustain that CH extract acts upon the HPA axis with a glucocorticoid-like mechanism, by binding to the GR. To date this is the first description of a iatrogenic hypercortisolism caused by a herbal drug.

However it is unrealistic to affirm that we are talking about a true "natural cortisone" with the same efficacy of synthetic glucocorticoids. It is very unlikely, indeed, that glucocorticoid effects of CH have not been brought up before, given the widespread and ancient use of this natural remedy in traditional Asian and African medicine and its increasing utilization in Western herbal medicine. Most probably, the inveterate application of the cream by our patient was decisive for the development of hypercortisolism with HPA axis suppression. As the famous physician Paracelso wrote in the XV century: "Dosis sola facit ut venenum non fit" (Only the dose distinguishes a drug from a poison).

Another aspect worthy of consideration is the possibility of a particular individual susceptibility to glucocorticoids in our patient. At present, this aspect represents a growing field of interest in clinical research, as variability in glucocorticoid sensitivity, particularly mediated by GR polymorphisms (31), seems to have implications in many clinical aspects (eg, response to glucocorticoid therapy, different phenotypes in Cushing syndrome and in several chronical diseases).

It is important to emphasize that the patient's adrenal function did not fully recover; we do not know whether the patient's adrenal function was sufficient before she started the cream use, but nothing suggests a pre-existing adrenal insufficiency. Even after ACTH normalization, cortisol secretion remained insufficient. We suppose that some components of Cardiospermum halicabum extract could have had a direct toxic effect on the adrenal glands and it might have prevented the full recovery of adrenal function, although we are not able to prove it. This toxic effect could be elicited by chronic application of the cream.

Nonetheless, the clinical case described in this study points out that herbal drugs must not be underestimated concerning their possible dangerous effects, and that it is necessary to improve knowledge about their pharmacodynamic and pharmacokinetic properties. This should become mandatory in a period where phytotherapy use is growing in Western society without a corresponding progress in pharmacognosy (ie, the branch of pharmacology dealing with herbal drugs). It is worthy to remember that research about herbal drugs is made intrinsically difficult for the peculiar characteristics of these remedies, compounds of multiple active principles that are overall responsible for the therapeutic profile. Another source of variability is represented by the different methods of cultivation and collection, and above all the different manufacturing leading to the final plant extract. All these aspects make it difficult to obtain a standardization of herbal drugs comparable to conventional drugs.

It should also be considered that this issue concerns not only scientific and safety aspects, but also involves legal consequences. Herbal drug marketing is indeed not constrained by the same strict regulation as for conventional drugs, which involves preclinical and clinical trials.

Finally, coming back to our clinical case, it will be interesting to find which element or combination of elements contained in CH tincture could have played a role to determine the glucocorticoid-like effect. However, for the reasons discussed above, it will be a very intriguing challenge.

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