

## ORIGINAL ARTICLE

# ARMC5 Mutations in Macronodular Adrenal Hyperplasia with Cushing's Syndrome

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## ABSTRACT

**BACKGROUND**

Corticotropin-independent macronodular adrenal hyperplasia may be an incidental finding or it may be identified during evaluation for Cushing's syndrome. Reports of familial cases and the involvement of both adrenal glands suggest a genetic origin of this condition.

**METHODS**

We genotyped blood and tumor DNA obtained from 33 patients with corticotropin-independent macronodular adrenal hyperplasia (12 men and 21 women who were 30 to 73 years of age), using single-nucleotide polymorphism arrays, microsatellite markers, and whole-genome and Sanger sequencing. The effects of armadillo repeat containing 5 (*ARMC5*) inactivation and overexpression were tested in cell-culture models.

**RESULTS**

The most frequent somatic chromosome alteration was loss of heterozygosity at 16p (in 8 of 33 patients for whom data were available [24%]). The most frequent mutation identified by means of whole-genome sequencing was in *ARMC5*, located at 16p11.2. *ARMC5* mutations were detected in tumors obtained from 18 of 33 patients (55%). In all cases, both alleles of *ARMC5* carried mutations: one germline and the other somatic. In 4 patients with a germline *ARMC5* mutation, different nodules from the affected adrenals harbored different secondary *ARMC5* alterations. Transcriptome-based classification of corticotropin-independent macronodular adrenal hyperplasia indicated that *ARMC5* mutations influenced gene expression, since all cases with mutations clustered together. *ARMC5* inactivation decreased steroidogenesis in vitro, and its overexpression altered cell survival.

**CONCLUSIONS**

Some cases of corticotropin-independent macronodular adrenal hyperplasia appear to be genetic, most often with inactivating mutations of *ARMC5*, a putative tumor-suppressor gene. Genetic testing for this condition, which often has a long and insidious prediagnostic course, might result in earlier identification and better management. (Funded by Agence Nationale de la Recherche and others.)

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**C**ORTICOTROPIN-INDEPENDENT MACRONODULAR adrenal hyperplasia can lead to excess cortisol secretion and Cushing's syndrome.<sup>1,2</sup> Adrenocortical nodules in corticotropin-independent macronodular adrenal hyperplasia are, by definition, larger than 10 mm in diameter and frequently reach 30 to 40 mm in diameter. The condition is typically diagnosed in patients with Cushing's syndrome who are between 40 and 60 years of age and who have suppressed levels of circulating corticotropin. Tumor growth and cortisol dysregulation appear to progress slowly in cases of corticotropin-independent macronodular adrenal hyperplasia, and the diagnosis is often made only after several years or decades of disease progression.<sup>3</sup> Milder forms are commonly detected in patients with incidentally discovered adrenal tumors or hyperplasia. Aberrant receptor expression leading to unexpected cortisol responses to ligands that stimulate G-protein-coupled receptors has been reported.<sup>4-7</sup> Treatment is most often surgical, but medical therapies targeting aberrant receptor expression have been effective in controlling excess cortisol in some cases.<sup>6</sup>

The bilateral nature of adrenal tumors in corticotropin-independent macronodular adrenal hyperplasia provides support for the hypothesis of a germline genetic predisposition. Reports of familial cases suggest the involvement of germline hereditary factors in the emergence of corticotropin-independent macronodular adrenal hyperplasia.<sup>1,8-11</sup> The familial occurrence of the disease might be underrecognized because of the variation in disease severity. Corticotropin-independent macronodular adrenal hyperplasia has been reported in a small subgroup of patients with familial multiple tumor syndromes.<sup>1,11-13</sup> In the McCune-Albright syndrome, mosaic mutations of the gene encoding G-protein subunit  $\alpha_s$  (GNAS) have been observed in young children with bilateral adrenocortical nodules.<sup>14</sup> However, the vast majority of cases are not part of any known multiple tumor syndrome, and the genetic basis of this condition has not been established.

## METHODS

### STUDY OVERSIGHT

The first two authors and the last two authors vouch for the completeness and accuracy of the data and analyses. Written informed consent for

the analysis of the tumor and leukocyte DNA was obtained from all patients. The study was approved by the institutional review board of Cochin Hospital.

### STUDY PATIENTS

A total of 33 patients (21 women and 12 men; age range, 30 to 73 years) who had undergone surgery for corticotropin-independent macronodular adrenal hyperplasia were included in the study. They had various levels of corticotropin-independent hypercortisolism and underwent computed tomographic (CT) imaging and adrenalectomy.<sup>5,15</sup> The diagnosis of corticotropin-independent macronodular adrenal hyperplasia was confirmed histologically. Detailed clinical phenotypes are described in Table 1 and in Table S1 in the Supplementary Appendix, available with the full text of this article at NEJM.org. Thirty-one patients were unrelated; 2 were siblings. The tumor samples were obtained prospectively by the Corticomedullosurrénale Tumeur Endocrine Network tumor bank.<sup>16</sup>

### GENOTYPING AND SEQUENCING

Genomic DNA was isolated as previously described from 41 adrenal nodules and 25 leukocyte samples obtained from the 33 patients.<sup>17,18</sup> The DNA from 34 nodules and 18 leukocyte samples was hybridized to single-nucleotide polymorphism (SNP) arrays (Affymetrix SNP 6.0). Somatic loss of heterozygosity and copy-number variants were identified as previously described<sup>19</sup> (see the Methods section and Fig. S1 in the Supplementary Appendix). Loss of heterozygosity was further investigated in 7 nodules and their paired leukocytes by means of microsatellite analysis.

DNA from five adrenal nodules and their paired leukocyte samples were sequenced by Complete Genomics<sup>20</sup> with the use of the Cancer Sequencing Service pipeline, version 2.0.2.26. Subsequent filtering is detailed in the Methods section and Figure S2 in the Supplementary Appendix. Sanger sequencing of armadillo repeat containing 5 (ARM5) is described in the Methods section in the Supplementary Appendix.

The transcriptomes of 10 adrenal samples from patients with corticotropin-independent macronodular adrenal hyperplasia were studied as previously described<sup>21</sup> (see the Methods section in the Supplementary Appendix).

**Table 1. Characteristics of the Patients.\***

Patient No.	Sex	Age yr	Corticotropin Level† pg/ml	Plasma Cortisol Level after 1 mg of Dexamethasone‡ µg/dl	Urinary Cortisol Level§ µg/24 hr	Cushing's Syndrome	Adrenal Weight		
							Right	Left	Total
<b>Patients with mutated ARMCS5</b>									
1	F	34	<5	23.6	373	Clinical	40	26	66
2	F	41	<5	NA	NA	Clinical	30	94	124
3	M	64	6	27.2	866	Clinical	39	118	157
4	F	40	<5	18.0	233	Clinical	NA	NA	NA
5	F	30	<5	26.1	458	Clinical	52	67	119
6	F	48	<5	27.4	118	Clinical	34	50	84
7	F	51	<10	22.4	248	Clinical	45	64	109
8	M	45	<10	36.7	1463	Clinical	48	71	119
9	F	70	<5	16.0	122	Clinical	21	60	81
10	M	41	<5	29.0	394	Clinical	65	121	186
11	M	46	<5	18.0	204	Clinical	34	46	80
12	M	55	<5	3.6	135	Subclinical	42	30	72
13	M	63	8	22.9	302	Clinical	35	50	85
14	M	53	<5	25.1	293	Clinical	65	77	142
15	M	56	<5	NA	107	Clinical	56	110	166
16	M	51	<5	NA	NA	Clinical	NA	NA	NA
17	F	52	<5	8.9	37	Subclinical	24	29	53
18	F	73	<5	15.0	NA	Subclinical	21	34	55
Total¶	9 F, 9 M	51 (27–74)	6 (<5 to 9)	21.3 (4.7 to 38)	357 (0 to 1089)	Clinical, 15; subclinical, 3	41 (13 to 69)	65 (3 to 128)	106 (25 to 188)
<b>Patients with nonmutated ARMCS5</b>									
Total	12 F, 3 M	52 (31 to 74)	8 (<5 to 18)	14.6 (0 to 32.5)	362 (0 to 1466)	Clinical, 11; subclinical, 3	27 (0 to 75)	38 (0 to 88)	55 (0 to 144)

\* NA denotes not available.

† The normal corticotropin level is 10 to 60 pg per milliliter.

‡ The normal plasma cortisol level is <1.8 µg per deciliter.

§ The normal urinary cortisol level is <90 µg per 24 hours.

¶ Quantitative variables are expressed as means with 95% confidence intervals, and qualitative variables are expressed as numbers of patients.

|| P<0.05.

**CELL CULTURE AND TRANSFECTION**

Human HeLa cells and adrenocortical cancer cells (H295R) were cultured, transfected, and stimulated with forskolin as previously described.<sup>22,23</sup> The small interfering RNA (siRNA) targeting ARMCS5 and the control siRNA used are described in the Methods section in the Supplementary Appendix. ARMCS5 expression vector containing a FLAG tag (Origen RC226267) was used for mutagenesis with Agilent Technologies kit 200521.

**WESTERN BLOTTING, IMMUNOSTAINING, AND MESSENGER RNA ANALYSIS**

Preparations of whole-cell or tissue lysates, Western blotting, immunohistochemical analysis, and immunofluorescence were performed as previously described<sup>23,24</sup> (see the Methods section in the Supplementary Appendix).

Total RNA was extracted from the cell lines, and the expression levels of target genes were determined by means of real-time polymerase

chain reaction (PCR) as previously described<sup>23</sup> (see the Methods section in the Supplementary Appendix). Cortisol concentrations in culture medium were assayed as previously described.<sup>15</sup>

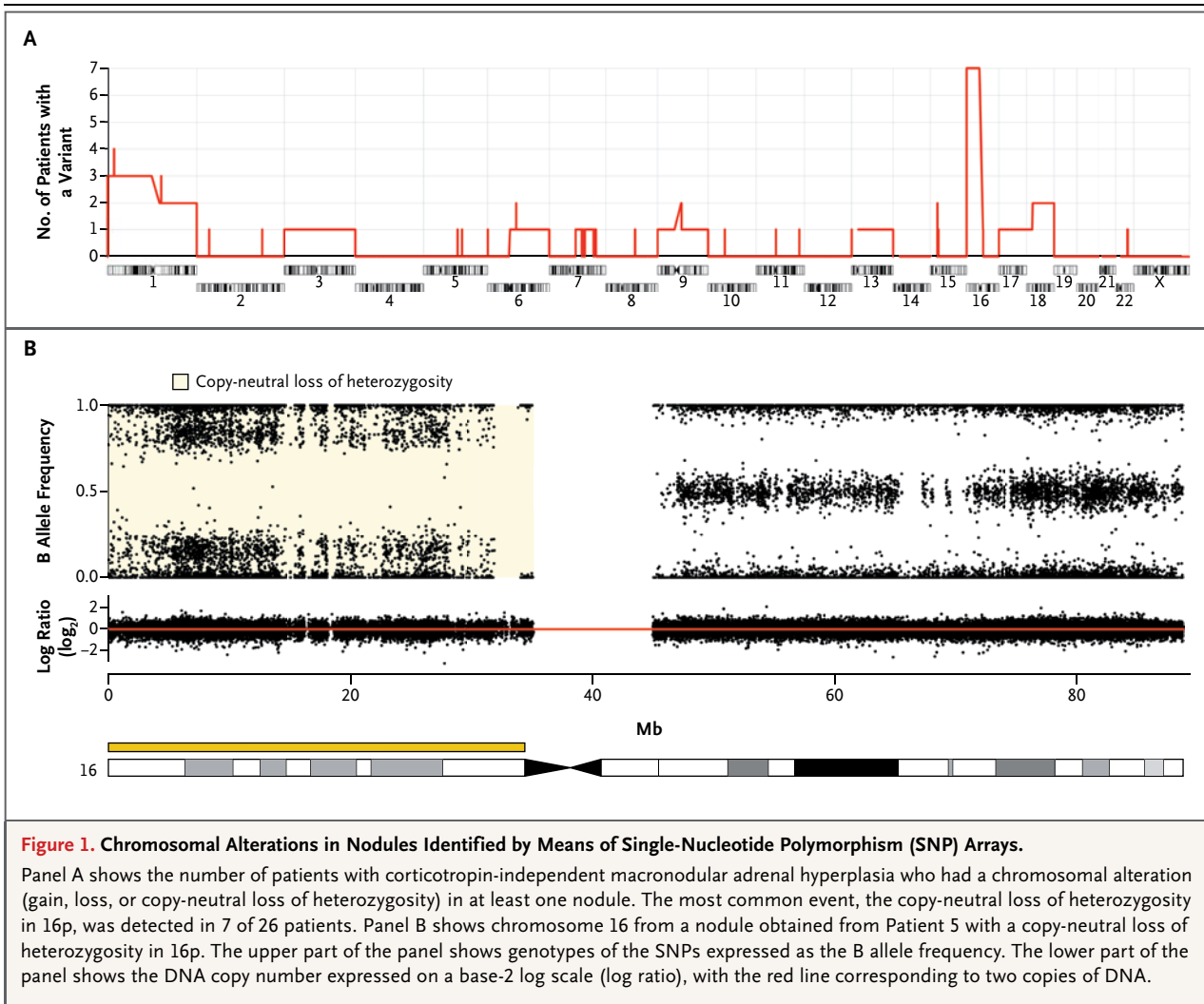
## RESULTS

### GENOMEWIDE GENOTYPING AND SEQUENCING

To search for gene alterations with the potential to cause corticotropin-independent macronodular adrenal hyperplasia, we used SNP arrays for genome-wide screening of chromosomal alterations in 34 tumor specimens obtained from 26 patients with corticotropin-independent macronodular adrenal hyperplasia who had undergone surgery. Recurrent somatic chromosomal alterations in nodules from the patients were rare (Fig. S3 and Tables S2, S3, and S4 in the Supplementary Ap-

pendix), except at 16p (Fig. 1). A copy-neutral loss of heterozygosity was identified in 10 of 34 tumor specimens (29%) obtained from 7 of the 26 patients (27%). In addition, somatic loss of heterozygosity in 16p11.1 was detected with the use of microsatellite markers in 1 of 7 other patients (Table S5 in the Supplementary Appendix); thus, loss of heterozygosity was detected at 16p in 8 of 33 patients (24%) with corticotropin-independent macronodular adrenal hyperplasia.

Whole-genome sequencing in five paired tumor and leukocyte DNA samples identified somatic mutations affecting the coding sequence of 85 genes and structural variants affecting the coding sequence of 12 genes (Tables S6, S7, and S8 in the Supplementary Appendix). Only 1 gene, *ARMC5*, which maps to 16p11.2, was modified in more than one tumor sample; tumor samples



obtained from three patients had a mutation. The somatic mutations of *ARMCS* included two frameshift mutations and one missense mutation. These mutations were confirmed by means of Sanger sequencing.

Direct sequencing of tumor DNA identified *ARMCS* mutations in 18 of the 33 patients (55%). A total of 26 tumor specimens obtained from these 18 patients were analyzed. All tumors tested had 2 genetic alterations in the *ARMCS* locus: 2 mutations in 16 specimens, 1 mutation with loss of heterozygosity at 16p (loss of the *ARMCS* nonmutated allele) in 9 specimens, and 1 mutation plus a microdeletion (1.3 Mb) in 1 specimen (Fig. 2A). The 28 mutations identified included 6 nonsense, 10 frameshift, 8 missense, and 4 more complex mutations (Fig. S4 in the Supplementary Appendix). None of these mutations were detected in the 186 control leukocyte DNA samples that were sequenced in the laboratory or in several thousand other controls from the exome variant server, hosted by the National Heart, Lung, and Blood Institute (<http://evs.gs.washington.edu/EVS>), with the exception of the p.R267X mutation, which was detected in 1 of 6297 controls (Table S9 in the Supplementary Appendix). Western blot analysis showed that the level of *ARMCS* protein was decreased in the majority of patients with corticotropin-independent macronodular adrenal hyperplasia who had an *ARMCS* mutation, and especially in those with nonsense mutations leading to a premature stop codon combined with a somatic loss of the nonmutated allele (Fig. 2B).

*ARMCS* was analyzed in leukocyte DNA obtained from 14 of the 18 patients with *ARMCS* mutations. A germline *ARMCS* alteration was detected in all 14 patients: a mutation in 13 patients and a microdeletion (1.3 Mb) in 1 patient (Fig. 2A, and Fig. S5 in the Supplementary Appendix). This observation suggests that *ARMCS* might be a tumor-suppressor gene that leads to the development of a tumor when a primary inactivating alteration on one allele in the germline is present and a somatic secondary event affecting the second allele occurs. This could be directly observed by sequencing for two nodules obtained from 2 patients with two *ARMCS* mutations close enough to be amplified together; the sequences of the PCR products from these two nodules showed that the two mutations were present on two different alleles (Fig. S6 in the Supplementary Appendix).

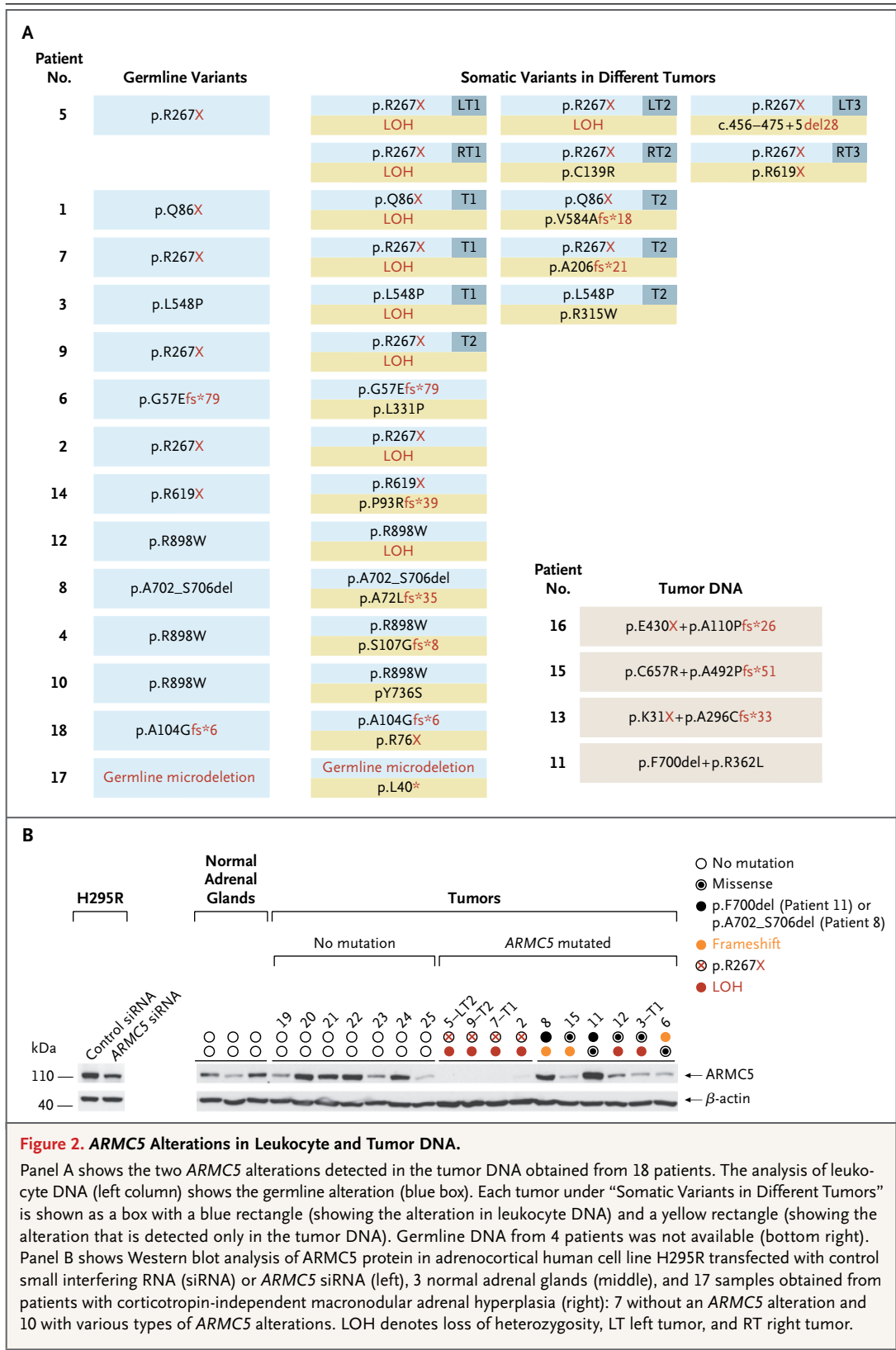
In four patients with an *ARMCS* germline

mutation, two or more nodules from one or both adrenal glands were analyzed. In each case, the same germline *ARMCS* mutation was associated with different, nodule-specific, somatic *ARMCS* alterations (Fig. 2A and 3). This observation suggests that in addition to the germline inactivating mutation, there were different somatic *ARMCS* second hits in the two adrenals of each patient, one for each nodule. In contrast, in the inter-nodular regions in four other patients with corticotropin-independent macronodular adrenal hyperplasia, no somatic second hit could be detected; only the germline *ARMCS* mutation was observed (Fig. S7 in the Supplementary Appendix).

Familial screening for *ARMCS* mutations was subsequently performed in 11 first-degree relatives of 7 index patients. None of these relatives had known Cushing's syndrome or any known adrenal tumor. A germline *ARMCS* alteration was detected in 6 of the 11 relatives, who were younger siblings or children of the index patients. Subsequent CT scanning revealed adrenal nodular hyperplasia with bilateral nodules ranging from 8 mm to 2.5 cm in 5 of these 6 relatives. Abnormal responses to dexamethasone were observed in 3 of the 6 relatives, and an increased 24-hour urinary cortisol excretion was detected in 1. There were no abnormalities in adrenal secretion or CT studies in any of the 5 relatives with nonmutated *ARMCS* alleles.

#### TRANSCRIPTOME ANALYSIS

The function of *ARMCS* is not known. To assess the functional consequences of *ARMCS* inactivation, we analyzed the transcriptome of 10 tumor specimens obtained from patients with corticotropin-independent macronodular adrenal hyperplasia (5 with *ARMCS* mutations and 5 with nonmutated *ARMCS*). Sorting of these tumors without awareness of their mutation status (unsupervised hierarchical clustering) resulted in two distinct groups: one containing the 5 mutated tumors and the other containing the 5 nonmutated tumors ( $P=0.008$  by Fisher's exact test) (Fig. S8 in the Supplementary Appendix). Several hundred genes are differentially expressed between the two groups. The transcriptome of mutated tumors was significantly enriched in genes related to RNA processing (Tables S10 and S11 in the Supplementary Appendix). This clearly indicates that *ARMCS* mutations have a large effect on gene expression and identify a subgroup of patients with corticotropin-independent macronodular adrenal hyperplasia.





**IMMUNOHISTOCHEMICAL ANALYSIS AND CELL CULTURE AND TRANSFECTION**

Immunohistochemical analysis of adrenal samples and two cell lines (H295R and HeLa) showed that ARMCS is mostly located in the cytoplasm (Fig. S9 in the Supplementary Appendix). In patients with corticotropin-independent macronodular adrenal hyperplasia and an ARMCS nonsense mutation, a decreased level of ARMCS and steroidogenic enzyme CYP11A1 immunostaining were observed, findings that are consistent with the reduced expression of steroidogenic enzymes in the transcriptome (Table S11 in the Supplementary Appendix).

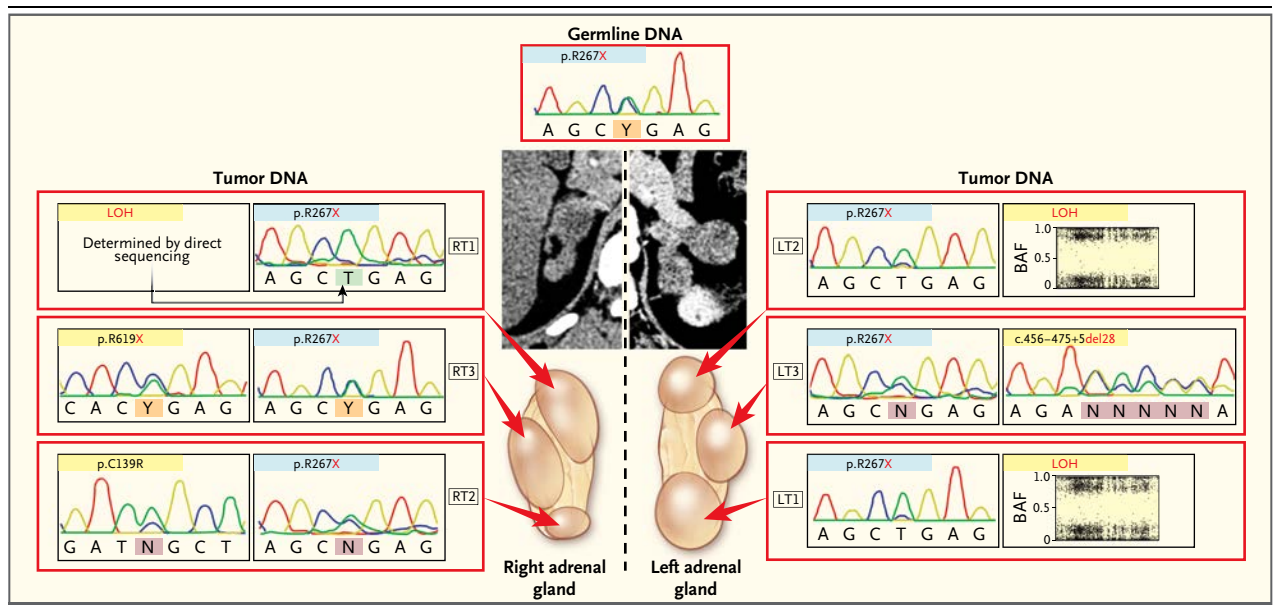
We investigated the role of ARMCS in steroidogenesis in vitro. ARMCS inactivation by siRNA in the H295R cell line reduced messenger RNA (mRNA) levels of genes encoding steroidogenic enzymes CYP17A1 and CYP21A2 and reduced mRNA levels of the gene encoding adrenal transcription factor NR5A1 (also called steroidogenic factor 1) and the melanocortin 2 receptor MC2R (Fig. 4A). In contrast, ARMCS inactivation had no effect on the mRNA level of the gene encoding transcription factor NROB1 (DAX-1). Cortisol synthesis was reduced in H295R cells after ARMCS inactivation (Fig. 4B).

We next studied the role of ARMCS in cell survival. In transient transfection experiments,

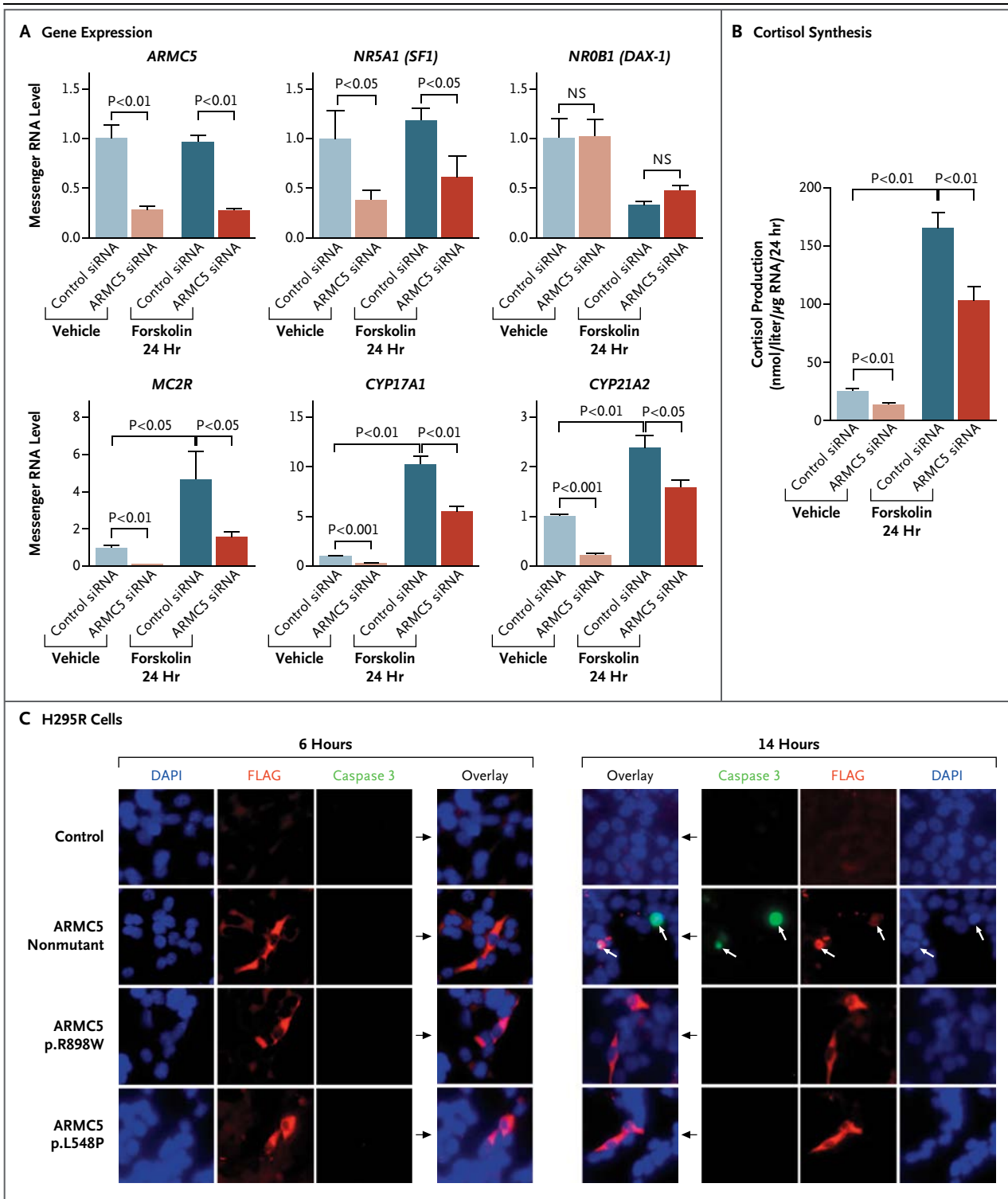
the expression of nonmutated ARMCS in H295R and HeLa cells resulted in cell death. No viable transfected cells could be detected after 14 hours, as shown by costaining of cleaved caspase 3 and ARMCS-FLAG (Fig. 4C, and Fig. S10 in the Supplementary Appendix). Moreover, apoptosis was confirmed by the cleavage of two markers of apoptosis: poly(ADP-ribose) polymerase and lamin A and C (Fig. S11 in the Supplementary Appendix). In contrast, this effect was not observed with the two germline missense mutations p.R898W and p.L548P (Fig. 4C, and Fig. S10 and S11 in the Supplementary Appendix).

**DISCUSSION**

The detection of ARMCS mutations in more than half the patients in this series who underwent surgery for corticotropin-independent macronodular adrenal hyperplasia suggests a common genetic cause of the condition. A combined genomic approach showed that ARMCS genetic alterations conform to the two-hit model for a likely tumor-suppressor gene. Indeed, patients with a germline ARMCS-inactivating mutation had nodule-specific secondary somatic mutations inactivating ARMCS. The occurrence of various somatic mutations within a single adrenal suggests polyclonal



**Figure 3. Analysis of Multiple Nodules Obtained from the Same Patient.** Computed tomographic scans and the various nodules present on both adrenal glands in Patient 5 are shown. Each nodule showed the germline defect (blue). A second alteration (yellow) differed between the two adrenals.



tumorigenesis related to a predisposition arising from a single germline mutation. The consistent occurrence of *ARMCS5* mutations in these adrenal nodules clearly suggests a major role of *ARMCS5* inactivation in their development.

Inactivation of *ARMCS5* was associated with a slow process of apparent dedifferentiation of adrenocortical cells and growth of bilateral masses in the patients with corticotropin-independent macronodular adrenal hyperplasia, along



**Figure 4 (facing page). Analysis of the Role of ARMC5.**

Panels A and B show the effects of *ARMC5* inactivation by siRNA on the expression of *ARMC5*, *NR5A1*, *NROB1*, *MC2R*, *CYP17A1*, and *CYP21A2* and on basal and forskolin-stimulated cortisol synthesis in adrenocortical H295R cells. NS denotes not significant. Panel C shows immunofluorescence staining of transfected H295R cells with different *ARMC5*-FLAG constructs (a nonmutated construct and two missense mutants: p.R898W and p.L548P). After 6 hours, the cells expressing the *ARMC5* constructs had normal morphologic features. After 14 hours, the cells expressing the nonmutant *ARMC5* construct (white arrows) were apoptotic (coexpression of the cleaved caspase 3 [green stain] and altered cell morphology with condensed nuclei), and the cells expressing the missense *ARMC5* constructs (p.R898W and p.L548P) were not apoptotic. Red staining shows FLAG antibodies (*ARMC5* constructs), and blue staining shows 4',6-diamidino-2-phenylindole (DAPI) (cell nuclei).

with reduced expression of steroidogenic enzymes and *MC2R* and abnormal cortisol production; these findings are consistent with previous expression-profile studies.<sup>25,26</sup> In our patients, the weight of the adrenals in patients in whom *ARMC5* was mutated was 4 to 24 times that of a normal adrenal. It is therefore likely that, despite the reduced secretory capacity of each cell, the overall production of cortisol was increased because of the large adrenal mass. This phenomenon may largely explain why a severe form of clinical Cushing's syndrome develops only in patients with very large adrenal nodules and why the disease often has an insidious course.

Most patients with an *ARMC5* mutation who were evaluated for aberrant membrane-receptor expression had positive upright and metoclopramide tests.<sup>27</sup> In contrast, the three patients in this cohort and three additional index patients with a food-dependent Cushing's syndrome, which is characterized by aberrant adrenal sensitivity to gastric inhibitory polypeptide,<sup>4,7</sup> did not have any *ARMC5* mutations (Table S1 in the Supplementary Appendix). Thus, *ARMC5* inactivation may be associated with a particular type of aberrant receptor expression. Furthermore, *ARMC5* mutations were associated with a specific transcriptome profile in the adrenal nodules.

As noted above, the function of *ARMC5* is unknown. It encodes a protein that contains an armadillo repeat domain, suggesting that protein-protein interactions may be important for its function.  $\beta$ -catenin, which also contains armadillo repeats, is another gene that is frequently mutated in various cancers, including adrenocortical tumors.<sup>28</sup>

The present study also shows that corticotropin-independent macronodular adrenal hyperplasia is frequently genetic. A limitation of previous studies that were based on clinical screening may be the substantial variation in disease severity both within a given pedigree and among index case patients. For example, the siblings of two of the

index patients in the present series had a severe form of adrenal Cushing's syndrome, but most relatives of the seven other index patients with an *ARMC5* mutation who agreed to be evaluated had adrenal nodules either without Cushing's syndrome or with only subclinical evidence of it. The slow progression of the disease and the advanced age at diagnosis make it difficult to study older generations in the families of index patients. In the current study, we were unable to evaluate any older relatives of the patients in whom mutations were identified. We speculate that mortality among some of the older relatives who were not examined may have been affected by cardiometabolic consequences of undiagnosed Cushing's syndrome. An alternative hypothesis is that secondary genetic or environmental factors might modulate the effects of germline *ARMC5* mutations. Such genetic secondary factors have been observed in another form of bilateral adrenal tumors leading to Cushing's syndrome (primary pigmented nodular adrenocortical disease) in patients with Carney complex due to *PRKAR1A* mutations.<sup>29</sup>

In conclusion, we identified *ARMC5* mutations in a substantial proportion of patients with corticotropin-independent macronodular adrenal hyperplasia. In affected patients, recovery from the effects of excess cortisol after definitive therapy for Cushing's syndrome is often only partial because of years of exposure to increased cortisol levels. This study provides information that may be helpful in developing diagnostic tests that will lead to earlier diagnosis and management of this disease.

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