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Int. J. Cancer: **97,** 357–360 (2002)
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Arnaldi 29

Publication of the International Union Against Cancer

MUTATIONAL ANALYSIS OF *StAR* GENE IN ADRENAL TUMORS

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Adrenal adenomas and carcinomas are mostly monoclonal, suggesting that a genetic alteration in a progenitor cell may contribute to their development. However, the molecular pathogenesis of these tumors still remains unclear. It has been already excluded that activating mutations of the ACTH receptor or of G protein stimulator alpha sub-units, affecting cAMP pathway, is involved in the tumorigenesis. Therefore, this work has been focused on post-transductional (ACTH) signal alterations and in particular on the mutational analysis of the Steroid Acute Regulatory protein (*StAR*) gene to verify whether somatic mutations or genomic polymorphisms of this gene may be correlated with adrenal tumorigenesis. Tissue DNA was extracted from 40 functional and non-functional adrenocortical tumors that were removed from patients aged between 17 and 72 years (mean 43 ± 4). Blood DNA was obtained from 24 patients (aged between 26 and 70 years) affected by adrenal tumors and from 100 healthy subjects without radiological and clinical evidence of adrenal masses, aged between 25–35 years (90 Caucasians and 10 Africans). The DNA was used as the template for the amplification of the *StAR* gene using the polymerase chain reaction. The amplified DNA of each exon of the *StAR* gene was purified and sequenced in automatic sequenciator. With the exception of exon 5 showing in codon 203 an homozygous missense mutation, the sequence of the other exons of the *StAR* gene resulted normal in all tumors studied. The same homozygous mutation (Asp203Ala) was observed in the sequence of exon 5 performed on genomic DNA of the 24 affected patients and in the control subjects. The homozigousity of the mutation observed in all patients (either in tissue or blood samples) and in control subjects, independently of their ethnic origin, led us to suggest that the Asp203Ala cannot be considered as mutation or as polymorphism, but that it must be considered as a mistake in the sequence entered in the Genbank, which needs to be modified accordingly. These data, and those up to now reported in the literature, allow us to suggest that mutations of the gene coding for the protein involved in the initial step of the steroidogenesis could not be considered as a possible cause for the development of adrenal tumors.
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Key words: *human mutation;* StAR *gene; adrenal neoplasia*

Seventy to ninety percent of adrenal tumors are benign. When these tumors do not hypersecrete hormones, their discovery occurs by chance during radiological examinations performed for other clinical problems.[1,2] A small percentage of adrenal tumors are malignant and only very few are diagnosed according to their hormonal hypersecretion, leading to their characteristic clinical manifestations.

Adrenal adenomas and carcinomas are mostly monoclonal,[3] suggesting that a genetic alteration in a progenitor cell may contribute to tumorigenesis. However, the molecular pathogenesis of these tumors remains unclear.[4] It has been already excluded that activating mutations of the ACTH receptors[4] or of G protein stimulator alpha subunits are involved in the tumorigenesis due to an impaired cAMP pathway.[5] Therefore our interest has been focused on the possible alterations localized in the post-trasductional signals and in particular on mutational analysis of the Steroid Acute Regulatory protein (*StAR*) gene, which represents a rate limiting step in the steroidogenesis process, to verify if somatic mutations or genomic polymorphism of this gene may be correlated with adrenal tumorigenesis. The rational basis of our study on the mutational analysis of this gene is based on the observation that an intrinsic alteration of steroidogenesis is described in most of the adrenal tumors,[6] even in the absence of clinical symptoms. The observation that patients affected by 21-OH lase deficiency develop adrenal masses, more often described in homozigous compared to heterozigous,[7–12] strongly supports the rationale of our study. Moreover recent studies reported the observation that subjects with Congenital Lipoid Adrenal Hyperplasia (CLAH) may develop testicular neoplasias and ovarian cysts.[13,14] This is the reasons that in these patients the pediatrician's therapeutic approach is mainly preventive, with a precise indication toward adrenalectomy.[15] The *StAR* mutations that have been described until now are all correlated with Congenital Lipoid Adrenal Hyperplasia (CLAH) and are represented by transitions and/or transversions and mostly localized in exon 5.[16–20] Moreover a genetic polymorphism (Asp203Ala) in exon 5 that is not correlated with any clinical manifestation has been already described.[21] In this article, we report the results of the mutational analysis of the *StAR* gene performed on 40 adrenal tumors, showing different histological types and the results of the study of the genomic polymorphism performed in the patients (when genomic DNA was available) and in 100 control subjects of various ethnic origins.

MATERIAL AND METHODS

Patients and tumor specimens

Tumor tissue was obtained at adrenalectomy. Tumors were frozen in liquid nitrogen immediately after surgical removal and stored at −80°C until analyzed. Diagnosis was established by clinical and hystological criteria. Forty adrenocortical tumors were studied: 3 non-functional adenomas, 2 non-functional carcinomas, 2 aldosterone-producing adenomas, 14 cortisol-producing adenomas, 13 cortisol-producing carcinomas, 4 androgen-producing carcinomas and 2 macronodular cortisol-producing hyperplasias. The tumors were obtained from patients aged between 17 and 72 years (mean 43 ± 4); 94% of these were females and most affected by functional neoplasia (Table I). This casuistry reflects the incidence of adrenal neoplastic disease for age and gender.[22–25]

DNA extraction

The DNA from the tumors was extracted by using the Qiagen tissue kit (Qiagen, Hildesheim, Germany). Blood DNA obtained from 24 of the patients affected by adrenal tumors (aged between 26 and 70 years) was extracted using the Qiagen blood kit (Qiagen,

Grant sponsor: Il Endocrinologia, Dipartimento di Fisiopatologia Medica Università di Roman "La Sapienza;" Grant sponsor: Istituto Superiore di Santià.

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Received 18 April 2001; Revised 11 June 2001; Accepted 18 June 2001

DOI 10.1002/ijc.1604

TABLE I – CLINICAL DATA AND PATHOLOGICAL FEATURES OF 40 PATIENTS WITH ADRENAL TUMORS

Tumor number	Age	Sex	Maximum diameter (mm)	Clinical and pathological features
1	55	F	30	Non-functional adenoma
2	52	F	40	Non-functional adenoma
3	41	F	40	Non-functional adenoma
4	23	F	90	Adrenocortical carcinoma (non functional)
5	34	M	35	Adrenocortical carcinoma (non functional)
6	57	F	20	Aldosterone producing adenoma
7	39	M	30	Aldosterone producing adenoma
8	48	F	45	Cortisol producing adenoma
9	29	F	40	Cortisol producing adenoma
10	50	M	30	Cortisol producing adenoma
11	65	F	40	Cortisol producing adenoma
12	26	F	30	Cortisol producing adenoma
13	55	F	40	Cortisol producing adenoma
14	46	F	30	Cortisol producing adenoma
15	63	F	45	Cortisol producing adenoma
16	25	F	30	Cortisol producing adenoma
17	40	F	30	Cortisol producing adenoma
18	39	F	30	Cortisol producing adenoma
19	46	F	30	Cortisol producing adenoma
20	34	F	30	Cortisol producing adenoma
21	57	F	50	Cortisol producing adenoma
22	33	M	30	Adrenocortical carcinoma (cortisol producing)
23	70	F	45	Adrenocortical carcinoma (cortisol producing)
24	69	F	90	Adrenocortical carcinoma (cortisol producing)
25	42	F	120	Adrenocortical carcinoma (cortisol producing)
26	54	M	150	Adrenocortical carcinoma (cortisol producing)
27	43	M	110	Adrenocortical carcinoma (cortisol producing)
28	50	F	160	Adrenocortical carcinoma (cortisol producing)
29	59	F	110	Adrenocortical carcinoma (cortisol producing)
30	26	F	150	Adrenocortical carcinoma (cortisol producing)
31	25	F	80	Adrenocortical carcinoma (cortisol producing)
32	56	F	160	Adrenocortical carcinoma (cortisol producing)
33	18	F	50	Adrenocortical carcinoma (cortisol producing)
34	50	F	40	Adrenocortical carcinoma (cortisol producing)
35	50	F	70	Adrenocortical carcinoma (androgen producing)
36	17	F	60	Adrenocortical carcinoma (androgen producing)
37	27	F	100	Adrenocortical carcinoma (androgen producing)
38	27	F	35	Adrenocortical carcinoma (androgen producing)
39	54	F	48	Macronodular hyperplasia (cortisol producing)
40	50	F	27	Macronodular hyperplasia (cortisol producing)

TABLE II – SEQUENCE OF OLIGONUCLEOTIDES THAT WERE USED FOR AMPLIFICATION OF EXONS OF THE *StAR* GENE

Exon I	
Sense	5′-AGG CTG CAG CTG CGG GAC TCA GAG G-3′
Antisense	5′-TCG CCT CCT TCC CGC AGC GCT CAC-3′
Exon II	
Sense	5′-AAC AAG GGT TAT TCC CTT CTG CAG-3′
Antisense	5′-GAG CCC AGA AGC CTC AGC ACT TAC-3′
Exon III	
Sense	5′-GTC TCT CCT CGG CTG TGT ATC CAG-3′
Antisense	5′-CAC AGG CTT CTC CCC GAC ACT TAC-3′
Exon IV	
Sense	5′-TCT GGG GGC TCC TTT CTC TGA CAG-3′
Antisense	5′-CAC CCG CAC CTG GAC TTT GGT CAC-3′
Exon V	
Sense	5′-TTC TGG TTC CCC ATG GCC TGG TAG-3′
Antisense	5′-GTT TGG AGC CTG CTG CCC GTA TTA C-3′
Exon VI	
Sense	5′-GAC TTG ACT TGC TCC ATT TGC CAG-3′
Antisense	5′-AGG TCC CCC TCC CAT GCC CTT CAC-3′
Exon VII	
Sense	5′-AAA TTC TCC TAC CTC CTA CTG CAG-3′
Antisense	5′-CCA GTG CAG CTG GGC ACA GTT GG-3′

Hildesheim, Germany). Blood DNA obtained from 100 healthy subjects, aged between 25 and 35 years, were used as controls. These subjects, 90 Caucasians and 10 Africans, according to data from ethnic studies, which demonstrated the prevalence of adrenal neoplastic disease in white subjects compared to blacks,[26–28] had no radiological and clinical evidence of adrenal masses. The study was approved by local Ethical Committee.

PCR and sequencing analysis

The DNA was used as the template for the amplification of the intronless *StAR* gene by the polymerase chain reaction (PCR) (PCR System 9700 Perkin Elmer). The pair of flanking primers used (Life Technologies, GIBCO-BRL, Gaithersburg, MD) is reported in Table I. The PCR protocol comprised 5 min melting of

the strands at 94°C, then 30 sec of denaturation at 94°C, 30 sec of annealing at 60°C and 30 sec of extension at 72°C, for 35 cycles. The final extension was performed for 10 min at 72°C, the reaction taking place in 20 μl volume containing Taq DNA polymerase (Promega Corp, Milan, Italy) with $MgCl_2$ at 2.5 mM. The expected size bands on agarose gel electrophoresis were as follows: 203 bp for exon 1, 114 bp for exon 2, 126 bp for exon 3, 159 bp for exon 4, 185 bp for exon 5, 94 bp for exon 6, and 113 bp for exon 7. The amplified DNA of each exon of *StAR* gene was purified by filtration through a Amicon membrane (Micropure-0.22 Separator, Amicon, Inc., Beverly, MA) and sequenced in automated DNA sequencer (Perkin-Elmer Cetus) with Dye-Deoxy® Rhodamine Terminator Cycle Sequencing kit (PE/ABI), using unlabeled PCR primers as sequencing primers, according to the manufacturer's protocol. The base calling was determined automatically by ABI PRISM Sequencing Analysis 3.0 software.

RESULTS

Each exon of the *StAR* gene was successfully amplified by PCR and shown to have the expected size bands on agarose gel electrophoresis. With the exception of exon 5 that showed in codon 203 an homozygous missense mutation with the substitution of Asp with Ala respect to the wild-type sequence (GenBank accession NM_000349 (gi:4507250) (Fig. 1), the automatic sequence of other exons of the *StAR* gene revealed a normal sequence in all tumors studied.

The same homozygous mutation (Asp203Ala) was observed in the sequence of exon 5 performed on genomic DNA of the 24 patients whose DNA was available.

To clarify if this mutation may be correlated with a silent polymorphism presented by patients developing adrenal tumors, exon 5 of the *StAR* gene from genomic DNA of control subjects was sequenced and in 100% of the cases, the same homozygous mutation (Asp203Ala) was observed.

DISCUSSION

This paper reports the results of the *StAR* gene mutational analysis performed on 40 adrenal tumors with different histological characteristics and in a group of 100 healthy subjects. In all cases an homozigous missense apparent mutation Asp203Ala (exon 5) of the *StAR* gene was shown. To our knowledge this is the first report in the literature that shows the results of mutational analysis of the *StAR* gene in adrenal tumors. Previous papers have demonstrated comparable expression of the *StAR* mRNA in normal and neoplastic adrenal tissues.[29–32] These results, however, do not permit excluding that *StAR* gene mutation may be involved in adrenal tumorigenesis.

The *StAR* gene mutations, until now described, are mostly localized in exon 5 and all identified in patients affected by CLAH.[16,33] Otherwise the possibility that an altered steroidogenesis, even in the cases not associated with clinical hormonal abnormalities, may be involved in adrenal tumorigenesis has been

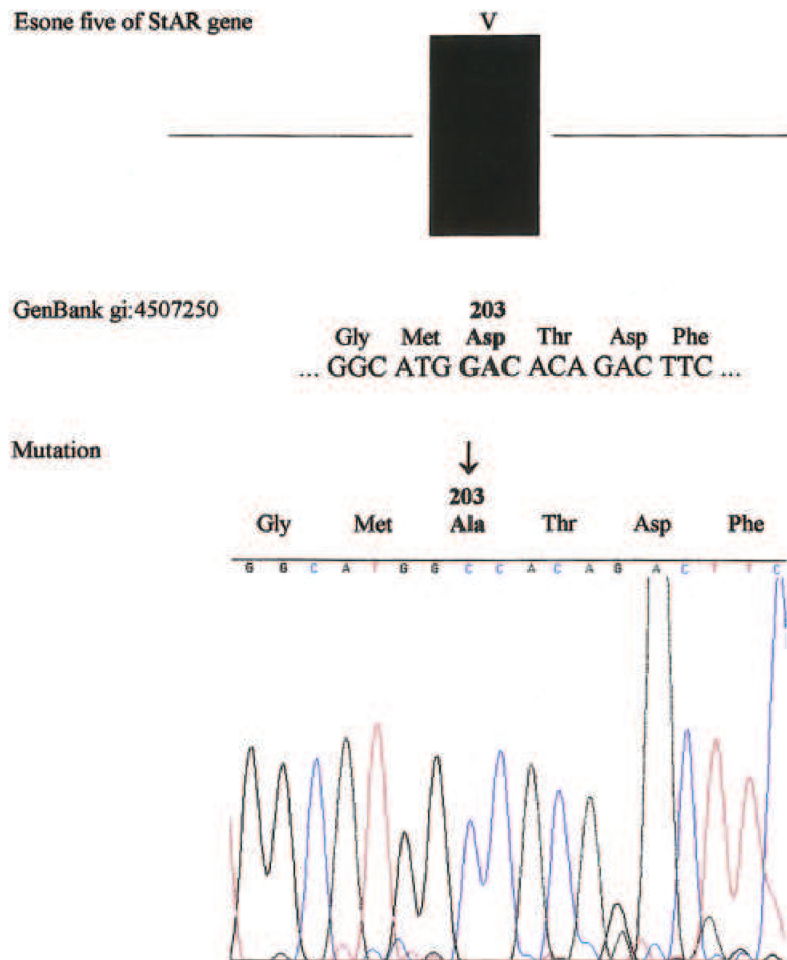

FIGURE 1 – Schematic representation of exon 5 of the *StAR* gene. Comparison of the partial sequence encoding the normal and mutated *StAR* gene. The arrow indicates the codon site of a single point mutation.

already advanced.[34–36] However, up to now no clear evidence for enzyme gene mutation along adrenal steroid pathways has been shown, even for the 21 hydroxylase gene[37] whose mutation was strongly suggested by the high response of 17-hydroxyprogesterone after ACTH stimulation shown in 30–70% of the patients affected by adrenal incidentalomas.[38,39]

The observation that the *StAR* gene apparent mutation Asp203Ala was found in all subjects studied, both in controls and in those affected by adrenal tumor, led us to exclude that this apparent sequence change may be involved in adrenal tumorigenesis.[36] It is difficult to accept this apparent mutation as polymorphysm because if this is the case, it would be expected to find in some people the same sequence comparable to that entered in the Genbank, or at least some heterozygous subject. A most recent entry in the Genbank of 19 March 1999 (accession NM_ 000349; gi:4507250) for *StAR* gene sequence reported the GAC at the codon 203. The possibility that this apparent mutation may be a polymorphysm that predisposes to the development of adrenal tumors could be postulated. Considering however that the prevalence of the adrenal tumors (including incidentalomas) cannot be expected to be higher than 10%[6] in the general population, we should expect the mutation in the same percentage of the cases in our controls, at least in homozygousity. Therefore the observation of the homozigousity of the apparent mutation in the 100% of patients and controls independently of their ethnic origin lead us to suggest that Asp203Ala has to be considered only as a mistake of the sequence entered in the Genbank, which could be modified accordingly.

On the basis of this body of evidence, we suggest that mutations of the gene coding for the protein involved in the initial step of the steroidogenesis cannot be considered involved in the development of functional or non-functional adrenal tumors, as well as excluding the involvement of gene mutations in the mechanisms of the ACTH signal induction.

ACKNOWLEDGEMENT

This work was performed within a collaboration between Il Endocrinologia, Dipartimento di Fisiopatologia Medica Università di Roman "La Sapienza" and Istituto Superiore di Santià.

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