The aryl hydrocarbon receptor-interacting protein gene is rarely mutated in sporadic GH-secreting adenomas

Short Title: Mutations of *AIP* in GH-secreting adenomas

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Key words: isolated familial somatotropinomas; GH-secreting adenomas; aryl hydrocarbon receptor-interacting protein

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Summary

Background

Recently, germ-line mutations of aryl hydrocarbon receptor-interacting protein (*AIP*) gene located on 11q13 were identified in patients with pituitary adenoma predisposition.

Aim/ Patients and Methods

We investigated the involvement of the *AIP* gene in one family with isolated familial somatotropinomas (IFS). To investigate the role of *AIP* in sporadic GH-secreting adenomas, we first analyzed somatic mutations in 40 tumours. Second, DNA from corresponding leukocytes was analyzed in tumours showing genetic changes of the *AIP* gene.

Results

Germ-line mutation of *AIP* was found in an IFS family. Biallelic inactivation of *AIP* by a combination of germ-line mutation and loss of heterozygosity was confirmed in two pituitary adenomas. Mutation analysis of the *AIP* gene in the 40 sporadic GH-secreting adenomas showed no mutations except for a missense mutation, suggesting that germ-line mutations in patients diagnosed with sporadic acromegaly or gigantism were rare. In a patient with gigantism, a missense mutation of V49M was identified at the germ-line level.

Conclusion

Based on these results, we conclude that the loss of function of *AIP* contributes to IFS, but not for most Japanese sporadic GH-secreting adenomas.

Introduction

Pituitary tumours are usually sporadic, but a significant minority presents as a component of multiple endocrine neoplasia type 1 (MEN1), Carney Complex (CNC), and isolated familial somatotropinomas (IFS).

IFS is defined as the occurrence of the at least two cases of acromegaly or gigantism in a single family in the absence MEN1 or CNC feature.[1] Recently, Vierimaa et al.[2] identified three germ-line mutations of the aryl hydrocarbon receptor-interacting protein (*AIP*) gene located on 11q13 in patients with pituitary adenoma predisposition (PAP). PAP was defined as having a very-low-penetrance susceptibility to GH-secreting adenoma and prolactinoma. Loss of heterozygosity (LOH) analysis in germ-line mutation positive GH-secreting adenomas, prolactinomas and mixed-type adenomas showed that both alleles of the *AIP* gene were inactivated. In a population-based study, germ-line mutations in the *AIP* gene were found in 16% of patients with sporadic GH-secreting pituitary adenomas. Furthermore, 40% of patients aged younger than 35 years had an *AIP* mutation. These results prompted us to analyze the germ-line mutation of the *AIP* gene in a Japanese IFS family and patients with sporadic acromegaly.

Patients and Methods

Case presentation of IFS

The clinical findings in one pedigree of IFS (family A) were previously reported.[3] Briefly, two of three brothers (II-2 and II-3 in Figure 1) and their uncle (I-3) presented acromegaly or gigantism. The LOH at 11q13 was detected in two adenomas (a GH-secreting adenoma from II-2 and a mixed GH cell and PRL cell adenoma from II-3), but germ-line mutations of *MEN1* gene were not detected.[4] Written informed consent was obtained from each patient, and the protocol was approved by the ethics committees of Toranomon Hospital and The University of Tokushima.

Sporadic GH-secreting adenomas patients

Tumour tissue samples were collected from 40 unselected patients with sporadic GH-secreting adenomas operated on at Toranomon Hospital, Tokyo. Clinical information on 40 patients with acromegaly or gigantism is described in Table 1. They had no family histories of pituitary adenomas. Blood samples were also obtained. Fully informed consent was obtained in accordance with institutional guidelines.

DNA extraction

Tumour and normal tissues were separated under a microscope by cutting them into small pieces using a razor blade. We microscopically confirmed that tumour tissues were scarcely contaminated with normal tissues. Small pieces of tumours and peripheral leukocytes were treated with proteinase K. Genomic DNA was obtained after phenol-chloroform extraction and ethanol precipitation.

Primer design of the *AIP* gene

The nucleotide sequences of primers for the *AIP1* gene accorded to the reports of Vierimaa et al..[2] Mutations of the *AIP* gene were screened for 5 overlapping PCR products with the corresponding primer sets covering the entire coding region and splice junctions.

PCR and sequence analysis

Genomic DNA was subjected to 30 cycles of PCR. PCR cycle involved a denaturation step at 95 °C for 30 s, annealing step at 65 °C for 30 s, and extension step at 72 °C for 60 s. Samples were subjected to direct sequencing in sense and antisense directions.

In sporadic GH-secreting adenomas showing genetic changes, the DNA from corresponding leukocytes was analyzed.

Results

Germ-line mutation of the *AIP* gene in an IFS family

Direct sequencing of leukocyte DNA PCR products from an IFS family detected a mutation of c.286_287delGT on exon 3. The mutation resulted in a frameshift, leading to a change with proline 96 as the first affected amino acid and the new reading frame being open for 32 amino acids. The mutation was found not only in affected members (I-3, II-2, and II-3 in Figure 1), but also in unaffected members (I-2 and II-1). The mutation was not detected in an unaffected family member (I-1) or 50 normal Japanese individuals.

Biallelic inactivation of the *AIP* gene in two pituitary adenomas from family A

In addition to the germ-line mutation, loss of the wild-type allele was observed in the two pituitary adenomas. The co-presence of germ-line mutation and LOH showed the biallelic inactivation of the *AIP* gene in the tumours.

Mutations of the *AIP* gene in sporadic GH-secreting adenomas

Sequencing of genomic DNA from 40 sporadic GH-secreting adenomas revealed no somatic mutations of the *AIP* gene. However, we detected a polymorphism of c.516C>T on exon 4 (NCBI reference SNP ID number rs2276020) in 12 patients. Furthermore, three nucleotide changes of c.135C>T, c.145G>A, and c.1053G>C were found in the respective leukocyte DNA. A silent nucleotide change of c.135C>T on exon 2 was detected in one patient (No. 34 in Table 1). The c.135C>T was not detected in 191 normal Japanese individuals, suggesting a possibility of single nucleotide polymorphism (SNP) with low frequency. A silent nucleotide change of c.1053G>C on the 3'-noncoding region was

detected in two patients (No. 16 and 37 in Table 1). The c.1053G>C was detected in 6 alleles among 50 normal Japanese individuals, suggesting a SNP. A 28-year-old male with gigantism (No. 35 in Table 1) showed a missense mutation of c.145G>A (V49M) at the germ-line level. The c.145G>A was not detected in 191 normal Japanese individuals. He did not have an apparent family history of GH-secreting adenomas. In his GH-secreting adenoma, a wild-type allele of the *AIP* gene was retained.

Discussion

Most of the molecular mechanisms involved in the genesis of pituitary adenomas have not been uncovered. In IFS, LOH at 11q13 in pituitary adenomas was detected and no *MEN1* germ-line mutations were identified.[3,4,5] Based on these results, the disease locus for IFS appears to exist within the region of approximately 2.21 Mb on 11q13.3.[5] Recently, germ-line mutations of the *AIP* gene were identified in patients with PAP.[2] A nonsense mutation of Q14X in exon 1 was segregated with the GH-secreting adenoma phenotype in two Finnish PAP families. One pedigree with 3 patients of IFS in our study against the total number of only 46 reported pedigrees[1] afforded us an important opportunity to screen germ-line mutations of the *AIP* gene.

The mutation of c.286_287delGT was detected in three patients with acromegaly or gigantism and a healthy mother and brother (I-2 and II-1 in Figure 1) with normal serum levels of GH and IGF1. This is consistent with the result of haplotype analysis on 11q13 in family members.[3] IFS is inherited as an autosomal dominant disease with incomplete penetrance. Vierimaa et al.[2] reported that newly identified *AIP* mutations leading to PAP show low penetrance. A precise estimate of the age-related penetrance of *AIP* mutation requires information from a large number of families.

The co-presence of germ-line mutation and LOH at the *AIP* gene in two pituitary adenomas from one IFS family suggested that the loss of function of *AIP* contributed to IFS. Interestingly, the mixed GH and prolactin adenoma from II-3 showed somatic inactivation of *MEN1* with c.1682delA and LOH,[4] suggesting the inactivation of two tumour suppressor genes, *AIP* and *MEN1,* in the tumour.

AIP displays primary sequence homology with the FK506-binding protein family of

immunophilins.[6] AIP can interact with both Hsp90 and aryl hydrocarbon receptor (AhR), which leads to enhanced stability of the complex.[7] The AhR is a ligand-activated transcription factor that is a member of the basic helix-loop-helix Per/ARNT/Sim family. The AIP acts as a modulator to differentially regulate AhR activity possibly by regulating the levels of p23 incorporation in the AhR complex.[8] AIP is known to inhibit a phosphodiesterase,[9] linking AIP to cyclic AMP. AIP directly associates with survivin, which is a member of the inhibitor of apoptosis family.[10] The knock down of AIP destabilizes survivin levels. Thus, it has been thought that AIP has oncogenic activity; however, the function of AIP as a tumour suppressor protein has not been reported. Because AhR arrests cell cycle through activation of p27 and repression of cyclin E,[11] AIP may be related to cell cycle regulation. Molecular mechanisms of AIP as tumour suppressor proteins remain to be elucidated.

The most prominent molecular lesions are point mutations in the *GNAS1*, which occurs in up to 50 per cent of GH-secreting adenomas.[12, 13] Inactivation of *MEN1* and *PRKAR1A* in sporadic pituitary adenomas, including GH-secreting adenomas, was reported to be infrequent events.[13, 14, 15, 16] Thus, molecular pathogenesis of GH-secreting adenomas is largely unknown.

The population-based study in patients with 45 GH-secreting adenomas, including 4 patients from 2 Finnish PAP families, found germ-line mutations in the *AIP* gene; 6 patients had Q14X and one patient had IVS3-1G>A.[2] Furthermore, Q14X was found in 2 of 10 unselected Finnish sporadic acromegaly.[2] Based on these results, a role of the *AIP* gene in sporadic GH-secreting pituitary adenomas remains an important question. Mutation analysis of the *AIP* gene showed no somatic mutations in the 40 GH-secreting

adenomas. In one patient with gigantism, a missense mutation of V49M was identified. Although a wild-type allele of the *AIP* gene was retained in the GH-secreting adenoma, it cannot be completely denied that lack of LOH was due to contaminated normal tissues. Whether V49M has a function as a tumour suppressor remains to be elucidated. Although methylation-dependent inactivation or mutations in the promoter, introns or untranslated regions of the *AIP* gene were not excluded in our study, our results suggest that somatic inactivation of the *AIP* gene was not a common etiology of sporadic GH-secreting adenomas. Furthermore, germ-line mutations of the *AIP* gene were found to be rare in Japanese patients diagnosed with sporadic GH-secreting adenomas compared to the results in Finland.[2]

In the present study, a germ-line *AIP* mutation was detected in one IFS family and a GH-secreting adenoma and mixed GH and prolactin adenoma in the family showed null mutation of *AIP*; however, *AIP* somatic mutations or germ-line mutations, except for one missense mutation, were not detected in the 40 Japanese sporadic GH-secreting adenomas. We conclude that *AIP* does not play a significant role in the tumourigenesis of sporadic GH-secreting adenomas.

Acknowledgements

This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, a grant from the Foundation for Growth Science, and a Novo Nordisk growth award.

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Figure legends

Figure 1. Pedigree of family A. Family members are indicated by generation (Roman numerals) and individuals (Arabic numerals). Individuals are represented as male (squares) and female (circles). The proband is indicated by an arrow. Closed symbols denote affected members. Open symbols denote unaffected members. Sequencing of the *AIP* gene showed a wild-type sequence (wt) or the presence of a mutation (mut).

Table 1 Cinical data of patients with sporadic GH-secreting adenomas

Patient No.	Sex	Age at diagnosis	G H (μg/l)	PRL (μg/l)	Hardy's classification
1	male	48	32.5		II-0
2	male	71	38.2		I-0
3	male	56	24		I-0
4	male	32	14		I-0
5	female	79	13		I-0
6	female	54	795.2		III-C
7	female	20	134.6		IV-B
8	female	33	33.7	21.8	III-B
9	female	31	2.2		III-B
10	female	29	135.2		IV-B
11	male	40	34.2		III-B
12	female	41	29.1		IV-B
13	female	26	16		III-C
14	male	34	22		III-0
15	female	26	23		II-0
16	female	28	78		III-C
17	female	65	4.7		I-0
18	female	36	46	26.1	III-B
19	male	42	11		III-0
20	male	46	47		III-A
21	male	38	80		III-A
22	male	73	9		I-0
23	male	25	78.5		II-A
24	male	43	22		III-A
25	male	59	67		II-A
26	female	30	38		IV-B
27	male	63	17		III-A
28	male	28	60	38.3	III-A
29	male	42	112		III-0
30	female	45	5.7		II-0
31	female	64	22		II-0
32	male	48	43		III-0
33	male	56	35.9		II-A
34	female	67	104	42.4	III-0
35	male	28	80	24.8	III-0
36	male	63	7.8		I-0
37	male	51	7.1		I-0
38	male	47	370		III-0
39	male	38	28	28	III-0
40	female	70	25		III-A

Fig.1