Mutational analysis of the GNAS1 gene in patients with chronic renal failure, secondary hyperparathyroidism and uglifying face appearances (Sagliker syndrome)

Osman Demirhan [1], Yahya Sagliker [2], Eylul Akbal [1], Mehmet Ali Erkoc [1], Nuray Paylar [2], Deniz Tastemir [3]

[1] Department of Medical Biology and Genetics, Faculty of Medicine, Cukurova University, Adana-Turkiye
[2] Sagliker Hypertension and Nephrology Unit, Adana-Turkiye
[3] Vocational Scool of Health Services, Adiyaman, Turkiye

Abstract

Sagliker syndrome (SS) seems to be related to chronic kidney disease (CKD), secondary hyperparathyroidism (SH) and uglifying face appearances. The etiology of SS is not known, and it is strongly thought that genetics may be the major factor in the etiology. The genetics importance of GNAS1 gene mutations on outcome in patients with SS is unclear, and no search has addressed GNAS1 mutations. Therefore, we conducted clinical and genetical studies including screening for mutations in the 13 exons of the GNAS1 gene in 23 subjects with SS. In 47.8% of the patients, 17 genetic abnormalities in GNAS1 were detected. Seven (58.3%) of 12 nucleotide alterations comprised novel missense mutations and three nonsense. Mismutations were in different manners. For 16 regions of the GNAS1 gene in which a missense and nonsense mutations, and heterozigot transversions (polymorphisms) were identified in 11 patients, and no mutated GNAS1 genomic in DNA of control subjects. There was nonsense mutations in 5 patients. Polymorphisms and other nonpathogenic mutations have been identified in 43.5% of the patients. There were also 6 heterozygous tranversion polymorphism in exons. Six were introngenic mutations. These results expand the spectrum of GNAS1 missense mutations associated with SS, and are consistent with an insufficiency of GNAS1 playing a role in the clinical phenotype of loss of function mutations and with a functional GNAS1 allele having a predominant role. At the same time, these findings may be helpful in conducting further molecular and biological studies on CKD, secondary hyperparathyroidism and uglifying face appearances.

Key words: *Sagliker syndrome, GNAS1 gene, mutations, polymorphism, chronic kidney disease, hyperparathyroidism, uglifying face appearances*

Corresponding Author: *Prof. Dr. Osman DEMİRHAN, Ph.D. Department of Medical Biology and Genetics, Faculty of Medicine, Çukurova University, 01330 Balcalı-Adana/Turkey*
Tel./Fax: *+90 3223387140* ***E-mail:*** *osdemir@cu.edu.tr*

Анализ мутаций гена GNAS1 у пацинетов с хронической почечной недостаточностью, вторичным гиперпаратиреозом и обезображивающей деформацией лицевого скелета (синдромом Сагликера)

Осман Демирхан [1], Яхья Сагликер [2], Элул Акбаль [1], Мехмет Али Эркоч [1], Нурэй Пайлар [2], Дениз Тастемир [3]

[1] Отдел медицинской биологии и генетики, Медицинский факльтет, Университет Чукурова, Адана, Турция

[2] Отделение гипертензии и нефрологии Сагликера, Адана, Турция

[3] Профессиональная школа здравоохранения Университета Адиаман, Адиаман, Турция

Резюме

Синдром Сагликера (СС) ассоциирован с хронической болезнью почек (ХБП), вторичным гиперпаратиреозом (ВГПТ) и обезображиванием лицевого скелета. Этиология СС остается неустановленной, однако имеются серьезные основния полагать, что в его основе лежат генетические факторы. Значимость мутаций гена GNAS1 для исходов СС не вполне ясна, поиск мутаций GNAS1 с этой целью не проводился. Мы выполнили клиническое и генетическое обследование, включающее скрининг мутаций в 13 экзонах гена GNAS1 у 23 пациентов с СС. У 47,8% пацинетов было выявлено 17 генетических аномалий GNAS1. Семь (58,3%) из 12 нуклеотидных нарушений представляют собой новые миссенс-мутации, и три – нонсенс-мутации. Мутации носили разнородный характер. У 11 пацинетов выявлены миссенс- и нонсенс-мутации и гетерозиготные трансверсии (полиморфизмы) в 16 регионах гена GNAS1, тогда как в контрольной группе мутаций GNAS1 не обнаружено. Нонсенс-мутации обнаружены у 5 пацинетов. Полиморфизмы и другие непатогенные мутации найдены у 43,5% больных. Обнаружены также 6 гетерозиготных трансверсий в экзонах. Шесть представляли собой интронные мутации. Эти результаты расширяют спектр миссенс-мутаций гена GNAS1, ассоциированных с СС, и согласуются с представлениями о том, что недостаточность GNAS1 играет роль в формировании клинического фенотипа, причем основное значение имеют мутации, ведущие к полной потере функции за счет дефицита функциональной аллели GNAS1. Кроме того, полученные данные могут быть полезны для проведения дальнейших молекулярных и биологических исследований у больных с ХБП, вторичным гиперпаратиреозом и обезображиванием лицевого скелета.

Ключевые слова: *синдром Сагликера, ген GNAS1, мутации, полиморфизм, хроническая болезнь почек, гиперпаратиреоз, обезображивание лица*

Адрес для переписки: *Профессор Осман Демирхан, Отдел медицинской биологии и генетики, Медицинский факльтет, Университет Чукурова, 01330 Балкали, Адана, Турция*
Тел./Факс: *+90 3223387140* ***E-mail:*** *osdemir@cu.edu.tr*

Introduction

SS is a new disease entity, and were first described by Yahya Sagliker et al., and have named SS, with prominent features being bone deformities (Saglıker 2004). The exact etiology of SS is not known. The frequency of SS has been reported as approximately 0.5% of patients with chronic renal failure and secondary hyperparathyroidism, and mostly seen in poor and underdeveloped countries. There may be genetic predisposition in these patients to develop SS. SS is a very prominent feature in CKD including uglifying human face appearances, short stature, extremely severe maxillary and mandibulary changes, soft tissues in the mouth, teeth-dental abnormalities, finger tip changes, neurologic and audiological manifestations, and severe psychological problems (Saglıker 2004-Ozenli 2010). Some patients suffering from CKD develop SS. The incidence of CKD stage late III is around 8% in the world. It appears that patients with chronic renal failure may have a new syndrome of bone deformities that have long been neglected, ignored. The definite causes of this entity has not been clearly understood yet, however studies on risk factors that can lead to this syndrome are underway. In CKD patients, having bad prognosis, and turning into SS, it is plausible to think that they are genetically predisposed. The polimorphisms and mutations in the genes encoding protein elements of these molecular pathways may have some associations with progress of SS, and genetic studies need to be done in order to clarify this syndrome.

Like all G proteins, the gene for Gsα is guanine nucleotide-binding protein a stimulating activity polypeptide 1 (GNAS1); its chromosome maps to the chromosome 20q13 region (Levine 1999-Cohen 1999) and is associated with several human endocrine disorders, including Albright hereditary osteodystrophy (AHO). GNAS is a complex locus encoding multiple overlapping transcripts. AHO is characterized by short stature, subcutaneous calcification, and brachydactyly and is caused by mutations that inactivate Gsα (Patten 1990-Weinstein 1990). Activating and inactivating GNAS1 mutations produce opposite effects on endocrine function and bone development, presumably secondary to opposite effects on intracellular cAMP. In view of reports showing that progressive osseous heteroplasia is also caused by mutations in GNAS1 (Eddy 2000, shore 2002). Activating mutations lead to the activation of hormone signaling pathways in the absence of circulating hormone, while inactivating mutations lead to hormone resistance. Activating mutations inhibit osteoblast differentiation, leading to fibrous dysplasia (FD), while inactivating mutations promote osteoblast differentiation, leading to ectopic ossifications in Albright hereditary osteodystrophy (AHO) and Progressive osseous heteroplasia (POH). Estimates of the proportion of Pseudohypoparathyroidism type 1a (PHP1a) caused by GNAS mutations range form 60 to 90%. Causative mutations have been found throughout the 13 exon gene; however, about 35-50% of the mutations by incidence are a 4bp deletion in exon 7 (Yu 1995, Aldred 2000). However, not all patients with PHPIa and pseudopseudohypoparathyroidism were found to carry detectable GNAS1 mutations (Ahmed 1998, Weinstein 1998). Loss-of-function mutations characterize types Ia and Ib PHP, PPHP, AHO, and POH (OMIM 2005). But, there was no evidence of linkage to the GNAS1 gene in the patients affected with SS. Genetic studies are needed in order to clarify the pathogenesis of this syndrome.

Here, we describe the first mutational analysis of GNAS1 in a group of patients with SS. Exactly how GNAS insufficiency might lead to SS is unclear, although there are potential mechanisms to explore. This is the first report describing an important association between GNAS1 mutations and the patients with SS.

Subjects and Methods

We have analyzed the clinical and molecular genetic features of a group of patients with SS. The GNAS1 gene mutations that have been incriminated in the pathogenesis of hereditary bone dysplasias (BD), such as McCune-Albright syndrome and likely achondroplasias etc. Although, our patients were not looking like any of them but they could be in between.

Patients

Clinical symptoms

We evaluated the patients with SS, and they are associated with CKD, secondary hyperparathyroidism (SH), hypocalcemia, hyperphosphatemia, irregularly scattered innocent tumoral accumulations in skull and face bones, unique and serious facial deformities which is unrecognizably uglifying human face appearance, short stature, extremely severe destructional nasal, maxillary and mandibular changes, severe type II malocclusion on cephalometric changes of maxilla and mandibula-maxillary protrusion, soft and pathologycally demonstrated innocent tissues in the mouth, irregularly located teeth and dental abnormalities, innocent soft tissue accumulations in the upper oral cavities, type two maloclusion of the maxillary bones (frontal forward and upward malformation of the upper jaws), curved finger tip changes, x or o type knee deformities and walking abnormalities, crippled walkings in particularly left legs, abnormal scapulas, neurologic manifestations, audiological findings, hearing abnormalities and severe psychological problems (Figure 1).

Biochemical symptoms

There was no striking results for vitamin D deficiency and calcitonin levels thought to be responsible for bone deformities. They all had high phosphate, low calcium, high ALP levels and high PTH values. We did not find any abnormalities in hemoglobin electrophoresis including HbA1, HbA2, HbS, HbF and G6PD levels for any kind hemoglobinopathies and G6PD deficiencies. We didn't find any striking differences in growth hormone

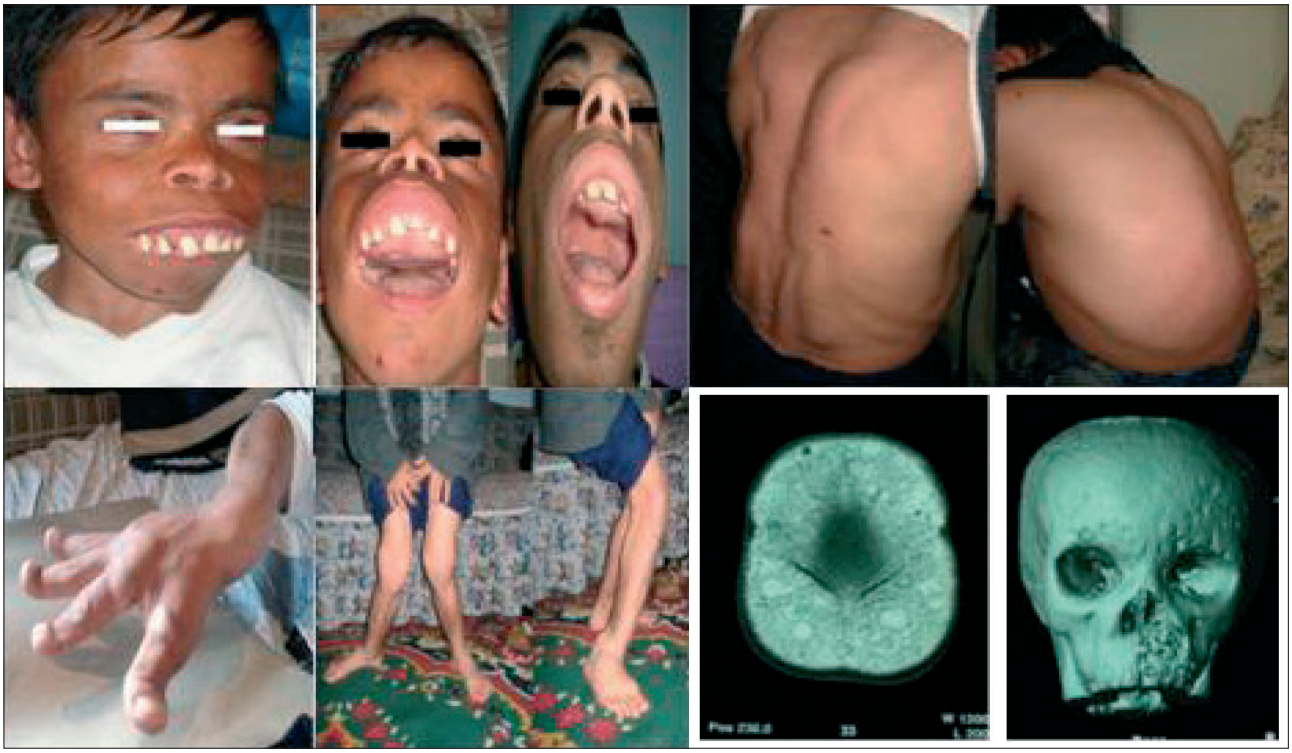

Figure 1. Clinical and radiological findings in the patients with Sagliker syndrome.

levels that we could accuse for pathogenesis of shortness. Eventhough the shortest patients had the highest levels of growth hormons. We didn't find any thyroid function defects in T3, T4 and TSH levels which might be taken responsible for growing deficiency. We did not find any sex hormone abnormalities in measuring FSH, LH and total testosteron levels to produce early cicatrization of epyphisis syndromes. As expected we have shown osteoporosis, thought to be caused by SS in all patients and cephalometric changes in type II malocclusions on the skull.

GNAS1 mutation analysis

We have analyzed the molecular features of the patients with SS in easily from our near region.

Blood samples were collected from 23 patients and 23 healthy controls to investigate their GNAS1 gene mutations after their parents had given written informed consent, according to the Ethics Committee of Medical School of Cukurova University. Venous blood samples were transferred to tubes with 10 ml EDTA and then were stored at 4°C. The DNA isolation of the blood samples collected from both groups of cases was performed by a precipitation method in which a saturated saline solution was used. Genomic DNA was isolated from peripheral leukocytes by standard procedures. Approximately 5 ml of blood was taken for both groups for DNA isolation. DNA extracted according to standart high salt method and then stored -20°C before using. Genomic DNA was isolated from peripheral leukocytes by standard procedures. Our study was conducted in two stages. All exons analysis was performed in two stages. In the first step, 1, 4, 5, 7, 10 and 13 exons were analyzed, and the other exons in the second step. All 13 exons of GNAS1 gene was amplified by PCR using the specific primers pairs and fragment size (bp) and at specific annealing temperatures shown in Table 1.

All exons of GNAS1 gene were screened. First PCR was performed in a final volume of 25 ml containing 12.5 µl AmpliTaq Gold® PCR master mix (2X), 2 µl of 2 pmol/µl of reverse and 2 µl of 2 pmol/µl forward primers, 3 µl of 40-413 ng/µl DNA and 5.5 µl water. Then, for the purification of PCR product, ExoSap Purification was performed using cycle conditions protocol of 37°C for 30 min and 80°C for 15 min. Then, Second PCR (Bigdye reaction) was performed in a final volume of 10 µl containing Big Dye Cycle Sequencıng v3.1 Kit 2 µl, 5xSequencing Buffer 2µl, Forward primer or rewerse primer 2 µl, PCR product 2 µl and water 2 µl. PCR conditions were 96°C for 1 min (activation), 96°C for 10 s, 50°C for 5 s, 60°C for 4 min (25 cycle amplification). Then, obtained PCR products were purified using Sephadex Purification protocol. Finally, sephadex purified PCR products were loaded to ABI 3130 machine for capillar electrophoresis and then results were evaluated using seqscape software.

Statistical analysis; genotype frequencies of patients as well as healthy control subjects were found to be in Hardy-Weinberg equilibrium, as tested by the X^2 test. Genotype and allele frequencies were compared by Fisher's exact test using the Statistica SPSS 17.0 statistics program (SPSS Inc., Chicago, IL).

Table 1. Primers and annealing temperatures used to amplify GNAS1, exons 1–13

Exon	Fragment size (bp)	Annealing Temp (°C)	Upstream primer	Downstream primer
1	141	59	5'-gcgctccttgccgaggagccgagcc-3'	5'-gcgagagcaagagagagacactgagc-3'
2	73	57	5'-tgtaaaacgattgccc-3'	5'-caggaaacagttccca-3'
3	45	52	5'-tgtaaaacgatggatg-3'	5'-caggaaacagtggtcc-3'
4	381	60	5'-taatttgcaactatgtttattcagc-3'	5'-gaagtcaaagtcaggcacgttcatc-3'
5	381	63	5'-gcagtactcctaactgacatggtgc-3'	5'-gacaggtgagctaagatgtagaagg-3'
6	98	55	5'-tgtaaaacgacgtgaa-3'	5'-caggaaacagagtggg-3'
7	345	62	5'-tggcaaattgatgtgagcgctgtg-3'	5'-agtctggagtagtttggaaagag-3'
8	74	51	5'-tgtaaaacgaaactgt-3'	5'-caggaaacagacagca-3'
9	59	51	5'-tgtaaaacgaaactgt-3'	5'-caggaaacagacagca-3'
10	357	60	5'-tgcttcgctgccgtgtcctgacttctg-3'	5'-agccatctacaagaagggaggccgtg-3'
11	131	60	5'-tgtaaaacgacgccca-3'	5'-caggaaacagctctcc-3'
12	68	52	5'- tgtaaaacgaaaccat-3'	5'-caggaaacagtctcca-3'
13	322	72	5'-ctggcgagggtgtcactgacaag-3'	5'-gccctatggtgggtgattaactgc-3'

Results

GNAS1 Mutations

The GNAS1 on chromosome 20 is a complex locus. More recently, addittional exons have been identified and it has become apparent that GNAS1 is a complex locus encoding multiple overlapping transcripts (Hayward 1998).

In our study, in 47.8% of our patients (11/23), 17 different genetic lessions in GNAS1 were detected. In seven patients (58.3%) of 12 nucleotides there were novel missense mutations in GNAS1. In our study, for 16 regions of the GNAS1 gene in which a missense and nonsense mutations, and heterozigot transversions (polymorphisms) were identified in 11 patients, and no mutated GNAS1 genomic in DNA of control subjects. There was nonsense mutations in 5 patients. Polymorphisms and other nonpathogenic mutations have been identified in 43.5% (Weinstein 1990) of the patients. Some had uninformative for some introngenic GNAS1 polymorphisms (introns; 65626, 70387, 70817), and some heterozigot transversions were found in intron regions of exon 5, 10 and 12. One, six, one, five, one and three of these alterations were found in exons 1, 4, 5, 10, 11 and 12 respectively. In seven patients (58.3%) of

Table 2. The GNAS1 mutations and polymorphisms of patients with SS

Exon	Patient	Codon	Nucleotid sequences	Amino acid changes
1	P1	284	AGC	ACC Missense mutation (Ser→Thr)
4	P5	760	ATC	ACC Missense mutation (Ile→Thr)
4	P15	750	ATT	ACT Missense mutation (Ile →Thr)
4	P12	750	ATT	ACT Missense mutation (Ile →Thr)
4	P13	747	GAG	GGG Missense mutation (Glu→Gly)
4	P23	769	TTC	TGC Missense mutation (Phe →Cys)
4	P6	765	CCC	CCA Nonsense mutation (Pro)
5	P12	65626 (intron)	T	C Heterozygous transverion (Polymorphism)
10	P8	865	GCG	GCC Nonsense mutation (Ala)
10	P20	885	ATG	ATA Missense mutation (Met→Ile)
10	P22	854	GGT	GCG Nonsense mutation (Gly)
10	P13	Intron	C	T Heterozygous transverion (Polymorphism)
10	P1	878	GCC	GCA Nonsense mutation (Ala)
11	P15	70387 (intron)	C	T Heterozygous transverion (Polymorphism)
12	P15	70817 (intron)	T	C Heterozygous transverion (Polymorphism)
12	P13	70817 (intron)	T	C Heterozygous transverion (Polymorphism)
12	P10	70817 (intron)	T	C Heterozygous transverion (Polymorphism)

12 nucleotides with novel mutations of GNAS1, in the 284 codon of exon 1 (AGC→ACC transversion) showed a different missense mutation leading to the substitution of serionin by threonin in P1. For P5, P12, P13, P15 and P23, mutations were revealed in exon 4 of GNAS1: P5 had a ATC→ACC heterozigot transversion mutation in codon 760 resulting in a substitution of izolecine by threonin (Fig. 2). P12 showed a transversion of timin (ATT→ACT) in codon 750 of exon 4 of GNAS1 resulting in a substitution of izolecine by threonin. Resulting in missense mutations of exon 4, GAG→GGG homozigot transversion resulting in glutamic to glycine substitution at codon 747 was identified in P13. In the 750 codon of exon 4 (TTA→ACT transversion) showed a different missense mutation leading to the substitution of izolecine by threonin in P15. One different novel mutations was detected in 769 codon of exon 4 (phenilalanin to cytosine) identified in P23. P20 showed a single base substitution from mthionin to izolecine in codon 885 of exon 10. Nonsense mutations in 5 patients (P1, P6, P8, P13, P22): exon 4 and 10 exons, so this alteration does not have any effect on gene product. It described

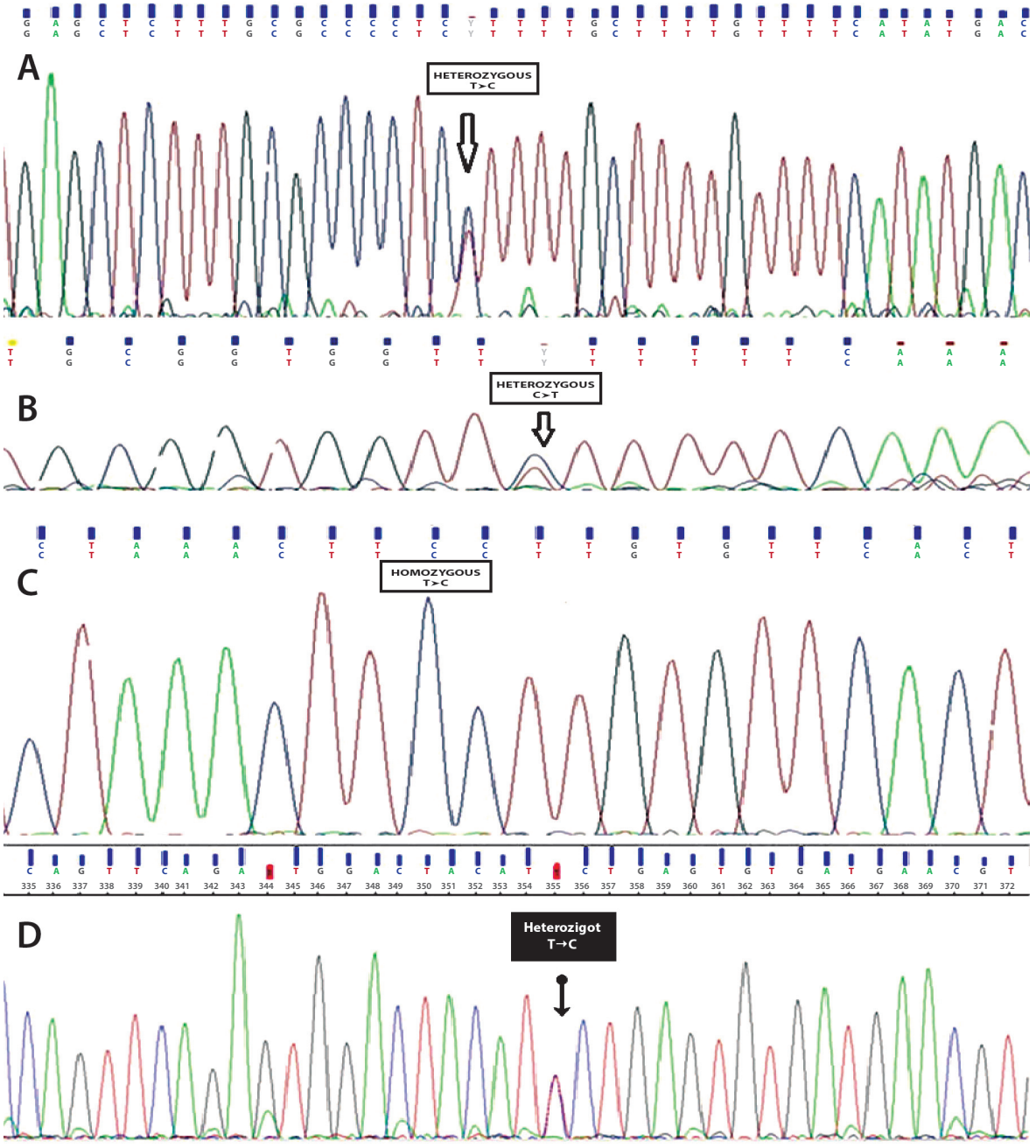

Figure 2. Sequencing analysis of some exons in patients with Saglıker syndrome; DNA sequence data showing a heterozygous T→C polymorphism in nucleotid 70817 (intron) of exon 12 of patient 15 (A), a heterozygous C→T polymorphism in nucleotid 70387 (intron 11) of exon 11 of patient 15 (B), a homozygous T→C polymorphism in nucleotid 65626 (intron) of exon 5 of patient 12 (C), a heterozygous T→C (ATC→ACC) mutation in codon 760 of exon 4 of patient 5 (D).

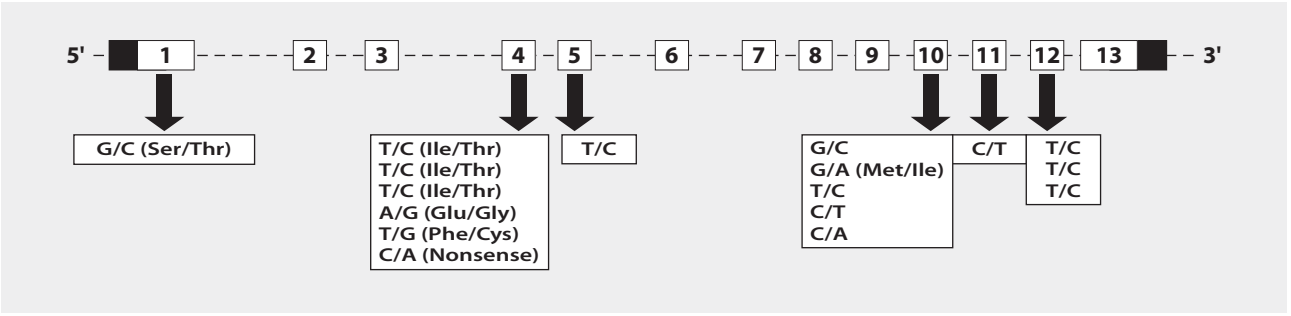

Figure 3. Schematic representation of GNAS1 with the location of 17 nucleotide changes detected in patients with SS. Exons are represented by rectangles.

four nonsense mutations of exon 10 (GCC→GCA, codon 878; GGT→GGC, codon 854; GCG→GCC, codon 865; in intron region) and one nonsense mutation of exon 4 (CCC→CCA, codon 765). Polymorphisms and other non-pathogenic mutations have been identified in 43.5% (Weinstein 1990) of the patients. P10, P12, P13 and P15 have uninformative for a introngenic GNAS1 polymorphism (introns; 65626, 70387 and 70817) and was also found the C→T and T→C heterozigot transversion in intron regions of exon 5, 10 and 12. Those novel genetical changes and missense mutations might be extremly important in the pathogenesis of SS. The nucleotide changes were not detectable in exon 2, 3, 6, 7, 8, 9 and 13. In other six patients, no molecular abnormality was found in the GNAS1 gene (Table 2, Figure 2, Figure 3).

Discussion

Here we describe the first mutational analysis of GNAS1 in Turkish patients affected with SS; chronic kidney disease (CKD), secondary hyperparathyroidism and uglifying face appearances. No all patients with CKD developed SS and the incidence of SS is around 0.5% in CKD patients. If we only consider there is 8 billion population in the world and 640 million have late stage III CKD and 3.200.000 patients have SS, then we do understand that we are facing a very serious and disasterous medical problem in the world. Heterozygous inactivating mutations of GNAS1 result in a condition known as Mc-Cune Albright-Hereditary Osteodistrophy and possibly in achondroplasias. This suggests that there had to be some additional factors predisposing to the genesis and probably related to SS. The cause and genetic etiology of this turning is not known. Therefore, the differential imprinting of separate protein products of GNAS1 therefore may contribute to the variation of clinical manifestation in SS.

By direct DNA sequencing, we identified seven point missence mutations within exons 1, 4 and 10, thus expanding the spectrum of GNAS1 mutations associated with SS. However, no genetic alterations were found in exons 2, 3, 6, 7, 8, 9 and 13. The prevalence of all GNAS1 mutations in our patients was high (52.2%), and different genetic lessions were observed to be the most frequent exon 4 (26%) and 10 (21.7%), followed by those of the exon 1 (4.3%). Those novel genetical changes and missense mutations might be extremly important in the pathogenesis of SS. Indeed, a prevalence of GNAS1 mutations of about 50% in patients with PHP Ia has been reported (OMIM 2005). In the patients, these mutations are associated with a partial decrease in GNAS1 mRNA expression, suggesting that the premature codon leads to abnormal RNA processing, as determined for mutations in other genes.

In the present study, mutations observed in SS patients are distributed throughout the gene. The 5 missence mutations in exon 4 were found to be the most frequent in our patients, involving 71.4% of missence mutations described. Amino acid substitutions predominate (codons 760, 750x2, 747 and 769), but nonsense mutation (codon 765) in exon 4 that lead to altered translation initiation, aberrant mRNA splicing or partial alterations in GNAS1 mRNA structure. Mutations observed in AHO patients are distributed throughout the gene. The majority of other mutations are unique to individual families, but some clustering is apparent, particularly in exons 1, 4, 5, 10 and 13. In previous studies, some different inactivating missense and frameshift mutations in the GNAS1 gene have been identified the sporadic de novo and missense familial comment in codons 275T>A; 275-312+8del43; 296T>C; 301-302delGA and 308T>C at exon 4 of the patients with AHO and PHP or PPHP (Ahmed 1998-Riminucci 1999). Using these studies, we have confirmed that there is a close correlation between the nucleotid substitutions of exon 4 and SS patients, and the nucleotid substitutions in codons 760, 750, 747 and 769 at exon 4 play a role in the pathogenesis of SS. Consequently, these results show that in SS patients, missence mutations can be used as reliable indicators of disease. The second novel missence, nonsence mutations and heterozygous transverion were a nucleotid transition identified in exon 10 (one missence, 3 nonsence mutations, one heterozygous transverion) (P1, P8, P13, P20 and P22), where a single base substitution from methionin to izolecine in codon 885 of one patients (P20). Of the some previously reported mutations, missense and frameshift mutations in exon 10 have been shown to be associated

with AHO, PHP1 and PPHP (Oude 1994, Warner 1997). Our results confirm a significant association between the codon 885 of exon 10 and SS.

In previous studies, inactivating missense and frameshift mutations in exon 1 of GNAS1 gene have been identified the sporadic de novo and missense familial comment in codn 1A>G, codon 1A>G, codon 115delG, codon119-139+17del38 and codon 124C>T at exon 1 of the patients with AHO and PHP or PPHP (Patten 1990, Warner 1997, Fischer 1998). The heterozygous alteration is predicted to be responsible for a reduced Gsa activity as demonstrated for the other previously reported deletions in exon 1 (Schwindinger 1992, Yu 1999). This novel mutation was associated with PHP. Mantovani et al. (Mantonavi 2000) identified two novel frameshift mutations within exons 1 and 11, thus expanding the spectrum of GNAS1 mutations associated with PHP1 and PPHP. We also have identified one pathogenic changes in codon 284 of exon 1 (AGC→ACC transition, ser→thr). This mutation can been shown to prevent the generation of a normal full length Gsa protein, resulting in a partial deficiency of Gsa activity. At the same time, this mutation is the third mutation affecting exon 1, confirming that this exon is subject to undergoing genetic alterations (Schwindinger 1992, Yu 1999) and should therefore be included in the analysis of GNAS1 mutations. SS can be difficult to diagnose for a mutations in exon 1 of GNAS1 can give rise to a classical SS phenotype indistinguishable from that observed with mutations elsewhere in the gene. Since this exon is unique to the Gsa transcripts. However, investigation of possible interactions between these overlapping sense and antisense transcripts may shed light on the molecular basis of the strong parent-of-origin effect associated with mutations of GNAS1. Lastly, we were found no genetic alterations in exons 2, 3, 6, 7, 8, 9 and 13 of GNAS1 gene in SS. As previously described, the identified mutations were scattered throughout the GNAS1 gene, occurring in exons 5 and 7 of the patients with AHO and PHP or PPHP (Patten 1990, Eddy 2000, Yu 1999, Warner 1997, Wilson 1994).

Conclusions

Our findings strongly suggest that the spectrum of GNAS1 missense mutations associated with SS disorder, and are consistent with missence mutations of GNAS1 playing a role in the clinical phenotype of loss of function mutations in SS; uglifying human face appearance in severe and late secondary hyperparathyroidism. Our findings expand the spectrum of phenotypic variability attributable to mutations in the GNAS1 gene. Also, this gene can be valuable tools to confirm the diagnosis of SS. Such a finding might help to resolve some of the complexity associated with the regulation of imprinting of GNAS1 and its involvement in multiple human bone disease states. Further investigation into imprinting effects and the regulation and function of the multiple transcripts of the GNAS1 locus will be required to understand the genotypic complexity and phenotypic variability associated with mutations at this locus. Identification of such individuals has important prognostic implications for genetic counseling. Therefore, GNAS1 molecular analysis should be performed in patients with isolated SS phenotype, although other pathogenic mechanisms are involved in a majority of cases. At the same time, such a finding might help to resolve some of the complexity associated with the regulation of GNAS1 and its involvement in multiple human disease states.

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