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An Apparent Homozygous Deletion in Maltase-Glucoamylase, a Lesson in the Evolution of SNP Arrays

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Abstract

Single nucleotide polymorphism (SNP) arrays possess clinical potential due to their high throughput capacity, sensitivity and versatility. We used such an array to perform a genome-wide SNP analysis of a patient with a multi-system undiagnosed disease involving peripheral neuropathies and food intolerances. The patient had a homozygous deletion within the gene encoding maltase-glucoamylase (MGAM), an intestinal starch digestion enzyme, predicting absence of enzyme activity and potential starch indigestion. We then performed validation testing using a functional MGAM analysis that involved starch ingestion followed by measuring blood glucose and insulin levels as well as hydrogen breath levels. Gastrointestinal tissue was also obtained via endoscopy and immunohistochemical staining for intestinal MGAM was performed. Our results strongly suggest the presence and functioning of MGAM which disproved deficiency predictions based on SNP array analysis findings, classifying the deletion as a functional polymorphism. This study highlights a current clinical limitation of SNP arrays, i.e., distinguishing deleterious genomic alterations from misleading functional polymorphisms. We conclude that novel findings from SNP arrays should be clinically validated and published.

Keywords

Single Nucleotide Polymorphism; Maltase-glucoamylase; Gene Array Analysis; Genetic Polymorphism; Genome-wide Association Study

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Introduction

Throughout history, laboratory advances have improved the practice of medicine. Recently, genetic discoveries and research developments in research tools have expanded the potential of genomic-based medicine. For example, single nucleotide polymorphism (SNP) arrays provide high throughput analysis comparing an individual's genetic information to a reference sequence. SNPs themselves are stable, polymorphic nucleotides (A, T, C or G) scattered throughout the human genome in both coding and non-coding (e.g., miRNA) regions. These heritable variations may constitute disease-causing mutations but may also be silent. With an estimated 3 million SNPs in the human genome, SNP research frequently employs microarray technology for analysis.

Detection methods for SNP arrays vary considerably, but most utilize small, pre-made chips containing up to 10^6 probes. Each probe consists of an individual target-SNP, flanked by its reference oligonucleotide sequence, immobilized to a microscopic bead. The SNPs are then collectively assayed. In the event of sample hybridization, a fluorescent signal indicates the presence of a target-SNP. These sensitive arrays can detect deletions, duplications and DNA methylation alterations.¹ Redundancy of SNP-probes provides determination of heterozygosity/homozygosity as well as aneuploidy. Microarray technology enables SNP analysis to meet clinical demands for genome- and population-wide efficiency.²

Over the last decade, extensive clinical application of SNP arrays has demonstrated its versatility and proficiency. SNPs are now used in genetic diagnosis and prenatal screening to identify copy number variations (CNV) and uniparental disomies (UPD).³⁻⁵ For drug therapy, they enable personalized medicine using pharmacogenomics.⁶ SNP technology has also been used in large cohort genome-wide association (GWA) studies, and has led to discoveries in genealogy and lineage tracing.⁷ It has identified allelic associations in both Mendelian diseases and sporadic genomic alterations.^{8, 9} Furthermore, SNP arrays have identified genetic components to many complex diseases like cancer and diabetes, as well as discoveries in undiagnosed diseases.¹⁰⁻¹² While this technology has demonstrated various successes, the clinical application of SNP arrays also appears to have limitations. We describe an example in which advanced SNP technology suggested a diagnosis of deficiency of MGAM (an intestinal brush border glycosyl hydrolase), but clinical rigor and functional testing suggested otherwise.

Methods

Patient

The patient was enrolled in an institutional review board (IRB) approved National Human Genome Research Institute (NHGRI) clinical protocol: 76-HG-238, "Diagnosis and Treatment of Patients with Inborn Errors of Metabolism and Other Genetic Disorders," where she provided signed, informed consent.

Analysis of SNPs

DNA was extracted from whole blood and analyzed as previously described.¹³ Illumina 1Mduo chips were run by the NHGRI Genomics Core and compared to 208 other samples run under identical conditions. The analysis software used was Illumina BeadStudio v1. and the reference genome was hg18, build36.

Functional Analysis of MGAM

MGAM, an intestinal brush border glycosyl hydrolase, is one of two membrane bound digestive enzymes contributing to the final glucose-releasing stage of starch digestion.¹⁴ The other is sucrase-isomaltase (SI). Presently, there are no described enzyme-specific substrates for MGAM.¹⁵ However, mutual substrates of both MGAM and SI include alpha-limit dextrins (LDx), consisting of maltose, maltotriose and branched oligosaccharides. These substrates are generated after alpha-amylase (salivary and pancreatic) digestion of amylopectin, a highly branched polysaccharide found within starch.^{14, 16}

After ingestion of 50 grams of amylopectin in patients with functioning MGAM and SI, the intestinal absorption of free glucose monomers produced by MGAM and SI digestion can be assessed directly by measuring blood glucose levels and indirectly by measuring the blood insulin response. Studies of glucose and insulin responses in normal healthy adults have been described utilizing 50g of carbohydrate.¹⁷ However, in patients with poorly functioning or absent MGAM and SI, consistent with intestinal malabsorption, amylopectin would be digested by colonic bacteria. The colonic bacterial digestion of amylopectin leads to production of hydrogen gas which can be measured via breath hydrogen measurements. This is similar to testing for small intestinal bacterial overgrowth (SIBO). Peak breath hydrogen levels greater than 20 parts per million above the fasting baseline value indicate carbohydrate malabsorption.¹⁶

Quezada-Calvillo et al (2007) described a coupled carbohydrate digestive mechanism characterized by MGAM priming, while SI sustains activity in carbohydrate digestion. To overcome the predicted MGAM priming deficiency in our patient, starch digestion was induced using sacrosidase (Sucraid™), an enzyme supplement used to treat Sucrase-Isomaltase deficiency.^{15, 18} Acarbose, an alpha-glucosidase inhibitor commonly used to treat type II diabetes, lowers both MGAM and SI activity,^{19, 20} but MGAM shows greater sensitivity to acarbose inhibition, so a relatively low-dose was administered.²⁰

Lab Studies and Protocols

Timed breath hydrogen levels were evaluated using the Easy Sampler™ Small Intestinal Bacterial Overgrowth (SIBO) Breath Test (QuinTron Instrument Co, Milwaukee, WI). Blood glucose and insulin levels were obtained at identical time points to further assess for MGAM activity. The following protocol was adapted from *Guidelines for Breath Testing* (QuinTron Instrument Co) and performed using *SIBO Easy Sampler Instructions* (QuinTron Instrument Co). Throughout the testing period, the patient's diet and activities were limited to those described in *Preparations for the Breath Test* (QuinTron Instrument Co).

Endogenous MGAM activity was assayed by amylopectin starch challenging without enzyme supplementation or enzyme knockdown. Enzyme supplementation using sacrosidase (Sucraid™) was then performed to assess the predicted MGAM priming deficiency by comparing measured values with those of endogenous MGAM testing. Supplementation testing also assayed the deficiency's potential for being overcome using sacrosidase. Enzyme knockdown using acarbose was performed to further prove the predicted MGAM deficiency by comparing measured values with levels under endogenous MGAM conditions. Measurements acquired throughout testing included blood glucose and insulin levels to directly measure starch digestion as well as breath hydrogen levels to indirectly determine malabsorption.

Three days of testing were performed. Each day of testing began with a baseline measurement of breath hydrogen, blood glucose and insulin at time "0" followed by repeat measurements at 20-minute intervals for 3 hours. On day 1 of testing, baseline measurements of endogenous MGAM activity was performed with oral administration of

unmodified waxy cornstarch (50g dissolved in 250mL of H₂O), which is essentially pure amylopectin. On day 2, induced digestion of amylopectin testing was performed, administering sacrosidase (1mL Sucraid™ diluted in 50mL H₂O) immediately prior to starch ingestion as well as 5 minutes after initiation of the testing. On day 3, MGAM knockdown testing was performed, administering acarbose (50mg) both the night prior and 1 hour before starch ingestion.

Endoscopy and Biopsy

To evaluate for the existence of MGAM and SI in small bowel tissue, a standard esophagogastroduodenoscopy (EGD) was performed and duodenal biopsies were obtained.

Immunohistochemistry

Immunohistochemical analysis of MGAM & SI was performed on the patient's duodenal tissue. After formalin fixation and paraffin embedding, sections were cut 4µm thick, deparaffinized in xylene and hydrated with graded ethanol. Sections then underwent heat-induced epitope retrieval (HIER) in 10mM citrate buffer (pH 6.0) for 4 min in a pressure cooker. Phosphate-buffered saline (PBS) washes (5 min, 3×) were performed between subsequent steps. After blocking for 10 min with Endogenous Enzyme Block (Dako, Glostrup, Denmark), samples were incubated with Anti-MGAM or Anti-SI produced in rabbit (Sigma Prestige Antibodies, St. Louis, MO, USA) for 1 hour diluted 1:250 in PBS. Sections were then incubated for 10 min with Envision System HRP-Labeled Polymer (Dako, Glostrup, Denmark). Finally, sections were treated with diaminobenzidine (DAB) substrate (Dako, Glostrup, Denmark) for 5 min and counterstained for 10 min using Methyl Green (Vector Laboratories, Burlingame, CA). Normal small bowel was used as a positive control, and for a negative control; Anti-C3 (Sigma Prestige Antibodies), a marker not found on the gut luminal membrane, was substituted as a primary antibody while maintaining identical incubations and dilution.

Results

Case Report

A 51-year-old Caucasian female was evaluated at the National Institutes of Health (NIH) Clinical Center under the Undiagnosed Diseases Program (UDP) for evaluation of peripheral neuropathy and a persistent taste abnormality of unknown etiology. Her symptoms began after a "viral-like" illness in December 2003 and progressively worsened. Her symptoms included extreme chronic fatigue, headaches, joint pain and stiffness, multifocal paresthesias with fasciculations, multiple food intolerances, persistent salty taste and multifocal stinging sensations. Over a period of years, her fatigue and abnormal taste sensations progressively worsened and were exacerbated by certain types of foods, causing a "stinging and burning" sensation in her mouth, crampy abdominal pain, headache, diarrhea and palpitations. Utilizing an elimination diet, she was able to decrease the number of symptomatic episodes. Her strict diet consisted of plain pasta, steamed broccoli, bananas, butter and coffee. Occasionally, she could tolerate tilapia fish, a plain hamburger, homemade vanilla ice cream or a baked potato. Notably, three out of her four siblings were diagnosed with peripheral neuropathy of uncertain etiology.

Prior to her admission to the NIH Clinical Center, she underwent multiple endoscopic evaluations, magnetic resonance imaging of the brain and axial skeleton, two electromyogram and nerve conduction studies, various subspecialty consultations, spinal x-rays, and a cardiac stress test, all of which were unremarkable.

While at the NIH, the patient's laboratory values within normal limits. Consultations by specialists in Internal Medicine, Neurology, Psychiatry, Ophthalmology, and Allergy and Immunology ruled out Sjogren's syndrome, rheumatoid arthritis, celiac sprue, inflammatory bowel and known digestive enzyme deficiencies. No sensory neuropathy was detected using standard nerve conduction studies, and brain imaging was normal.

As part of the evaluation for patients admitted to the NIH Clinical Center under the UDP, the patient underwent whole genome SNP array analysis. She displayed a homozygous deletion of 23,105kb (13 SNPs) within the region of chromosome 7q34 (genome bp number 141,412,174 to 141,435,188). The interpretation of this data at the time the SNP chip was run was that there appeared to be a disruption of approximately one-third of the coding region of the gene encoding maltase-glucoamylase(MGAM). This interpretation was based on the mapped locations of the (#SNPs deleted here) to the hg18 reference. As we show later, this interpretation changed during the course of the enzyme validation testing due to a remapping of these SNPs and a revision of the human genome sequence in hg19 that no longer makes this deletion appear to be present. The deletion was predicted to result in a total absence in MGAM activity and possible severe impairment of starch digestion; further testing for MGAM activity was performed.

SNP Array Analysis

The patient's SNP results, as seen in the Genome Viewer GUI, appeared to show a homozygous deletion of 23,105kb (13 SNPs) within the region of chromosome 7q34 (chr7:141,412,174 -141,435,188, hg18). Based on the analysis at the time the SNP array was performed, which utilized reference genome hg18, build36, the original indel was mapped to the MGAM locus. The deleted SNPs appeared to disrupt approximately one-third of the coding region of the gene locus for MGAM. The deletion was predicted to result in total absence of MGAM activity and severe impairment of starch digestion.

Functional Analysis of MGAM

During baseline amylopectin digestion, an increase in blood glucose and insulin levels was measured, peaking at 140 mg/dL and 20.7 mcU/mL respectively, similar to previously published results in normal healthy individuals, suggesting normal endogenous amylopectin digestion and absorption (Figure 1).¹⁷ The breath hydrogen levels remained constant at 2 ppm (within normal limits) and below the 20 ppm threshold for sugar malabsorption (Figure 1). Compared to endogenous enzyme activity, induced digestion of amylopectin with sacrosidase (Sucraid™) showed a more rapid increase in blood glucose and insulin levels, peaking at 134 mg/dL and 15.2 mcU/mL respectively, consistent with the kinetic profile of enzyme supplementation (Figure 1). The relatively similar hydrogen breath levels, with a peak level of 5 ppm, as well as peak blood glucose and insulin levels of endogenous and induced digestion suggest proficient endogenous digestion, which was unable to be enhanced with sacrosidase (Sucraid™) supplementation (Figure 1). MGAM knock down analysis showed a slower, more gradual increase in blood glucose and insulin levels compared to endogenous activity with lower peak levels of 118 mg/dL and 10.3 mcU/mL respectively, consistent with the kinetic profile of partial enzyme inhibition (Figure 1). The MGAM knock down hydrogen breath levels, with a peak level of 5 ppm, were relatively similar to those of endogenous activity as well as induced digestion of amylopectin; all were within normal limits and below the threshold for sugar malabsorption (Figure 1).

Immunohistochemical Analysis

Duodenal tissue stained positive along the intestinal brush border for both MGAM & SI. Negative control staining demonstrated no positive intestinal brush border staining. (Figure 2)

Discussion

During an unrevealing workup for complaints of chronic fatigue, abnormal taste sensations and neuropathic pain, our patient was found to have a homozygous deletion of 23,105kb (13 SNPs) within the region of chromosome 7q34 on whole genome sequencing of SNPs. At the time of this evaluation, the original indel was mapped in hg18 reference genome to the MGAM locus as it was assigned by the Human genome consortium in 2008. This deletion suggested the total absence of MGAM activity and possible severe impairment of starch digestion. Given the lack of explanation and diagnosis for our patient's symptoms, the possibility of MGAM deficiency was sought through testing of functional analysis and immunohistochemistry for MGAM.

The combined results from the functional analysis and immunohistochemical evaluation suggest proficient endogenous MGAM activity. However, a limitation of the current study is the lack of a normal control subject. Our results disproved the MGAM deficiency predicted by the SNP array findings and demonstrated that the deletion is a functional polymorphism, highlighting a current clinical limitation of SNP array-based genome-wide analysis. Specifically, interpretation of vast amounts of information from SNP arrays may not result in clinically relevant genomic-based diagnosis and patient care related decisions. This makes SNP array results potentially misleading, in part because of incomplete understanding of the human genome and the role of SNPs.

The human genome sequence is more than 99% identical across populations, but little clinical relevance has been attached to the remaining variations that SNP arrays detect. Furthering our understanding of genomic variations across populations, for example, in context of a pan-genome, may enable deleterious alterations to be distinguished from functional polymorphisms. This can be accomplished by publishing validation results and updating human genome references.²⁰ Specifically, in reference to the MGAM deletion finding, the database of genomic variants more recently shows many examples of deletions within this same region (Human Genome 18, Build 36.1). Also, a contiguous track, new to Human Genome 19, demonstrates an alternative sequence in the region, which reflects the fact that the deletion does not translate into abnormal function. Additionally, with update to the current build 37, hg19, the location of MGAM has been subsequently changed.

In conclusion, SNP arrays have demonstrated their value as a powerful clinical tool, enabling the practice of genomic-based approaches in medicine. While we have identified a problem with hg18 that has subsequently been corrected in hg 19, it is likely that other similar problems are still present and will in turn be corrected with future versions. Therefore, due to the current incomplete knowledge of the genome, novel findings from SNP arrays should be clinically validated and published. Furthermore, findings should be published and/or catalogued in a freely accessible form such as those seen in examples like the Human Variome Project, Ensembl, National Center for Biotechnology Information (NCBI), Human Genome Mutation Database (HGMD) and dbSNP.^{21–25}

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Abbreviations

SNP	Single Nucleotide Polymorphism
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MGAM	Maltase-glucoamylase
DNA	Deoxyribonucleic Acid
CNV	Copy Number Variation
GWA	Genome-wide Association
UPD	Uniparental Disomy
NIH	National Institutes of Health
UDP	Undiagnosed Diseases Program
NHGRI	National Human Genome Research Institute
SI	Sucrase-isomaltase
LDx	Alpha-limit Dextrins
SIBO	Small Intestinal Bacterial Overgrowth
EGD	Esophagogastroduodenoscopy
HIER	Heat-induced Epitope Retrieval
PBS	Phosphate-buffered Saline
DAB	Diamino-benzidine

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Highlights

- GWAS identified a homozygous deletion encoding maltase-glucoamylase (MGAM).
- We designed a novel technique for functional testing of MGAM in humans.
- We designed a novel technique for staining of MGAM in human tissue.
- SNP array results did not result in clinically relevant genomic-based diagnosis.
- Novel findings from SNP arrays should be clinically validated and published.

Figure 1.

Functional MGAM testing (a) Blood glucose levels show digestion and intestinal absorption of amylopectin (b) Blood insulin levels indirectly show digestion and intestinal absorption of amylopectin (c) Hydrogen breath levels for each assay were within normal limits and below the threshold for intestinal malabsorption.

Figure 2. Duodenum tissue analyzed using Anti-MGAM (a) Anti-SI (b) antibodies shows positive staining along the intestinal brush border. Negative control Anti-C3 (c) shows no staining of the brush border.