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Highly elevated plasma γ-glutamyltransferase elevations: a trait caused by *GGT1* transmembrane mutations

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HUMAN MATERIAL

All probands and control persons provided written informed consent to participate in this study. The study was approved by the institutional review boards in Innsbruck, Austria, Bolzano, Italy, and Poprad-Veľká, Slovakia. Probands were evaluated clinically for signs of chronic disease, with emphasis on liver and renal function.

Dominant inheritance of GGTemia in the range of 2000-9600 U/L (ref. 9-55 U/L) was observed in two unrelated families of Italian and Slovakian origin, in whom 15 and 17 trait carriers were identified. The diagnosis of GGTemia was made based on isolated, highly elevated plasma GGT values in both presented families. GGTemia was neither associated with clinical complaints nor with additional indicators of liver disease. None of these individuals had suffered from any major medical problems, except one with alcohol abuse (Family 1, individual III-1). None were treated with hepatotoxic drugs or with drugs that could affect liver enzyme activities. Neuromuscular status was normal. Except for elevated plasma GGT activity, the results of the following tests were normal: plasma aspartate aminotransferase (AST), plasma alanine aminotransferase (ALT), alkaline phosphatase, cholinesterase, creatine kinase, lactate dehydrogenase, total bilirubin, conjugated bilirubin, and prothrombin time. Tests for viral (hepatitis A, B, and C viruses, cytomegalovirus, and Epstein-Barr virus), metabolic (α1-antitrypsin, ceruloplasmin, and urinary copper before and after penicillamine load), and autoimmune (antinuclear, anti-smooth muscle, antimitochondrial, and anti-liver-kidney microsomal and anti-transglutaminase antibodies) conditions were conducted in a subset of these individuals and revealed no abnormalities. Additionally, hemolytic, renal, and pancreatic causes of elevated GGT activity were excluded. Liver ultrasound was normal in 14 individuals with GGTemia. Full penetrance of the mutations underlying GGTemia in both families was indicated by family tree analyses.

SUPPLEMENTARY METHODS

Laboratory tests for diagnosis of GGTemia

Standard assay of all blood tests including plasma GGT were simultaneously performed according to the standard clinical laboratory procedures by automated analyzers (Beckman Synchron CX 9-PRO analyzer, Beckman Coulter, Vienna, Austria; Abbott Cell-Dyn Sapphire for blood cell count, Abbott Laboratories, Libertyville, IL, USA).

GGT fraction analyses

Samples for plasma GGT fraction analysis were available from 10 trait carriers from family 1. Total and fractional plasma GGT activities were determined[1,2] in plasma-ethylenediamine-tetra-acetic acid (EDTA)-anticoagulated samples using a fast protein liquid chromatography system (AKTA purifier; GE Healthcare Europe, Milan, Italy) equipped with a gel-filtration column (Superose 6 HR 10/300 GL; GE Healthcare Europe) and a fluorescence detector (Jasco FP-2020; Jasco Europe, Lecco, Italy). GGT activity was measured using gamma-glutamyl-7-amido-4-methylcoumarin (Nova Chimica, Milan, Italy) as substrate (0.03 mmol/L, final concentration) and glycylglycine (5.4 mmol/L, final concentration) as acceptor of the transpeptidation reaction. The fluorescence detector operating at excitation/emission wavelengths of 380/440 nm detected the amino- 4-methylcoumarin signal; the intensity of the fluorescence signal was expressed in arbitrary fluorescence units. Area under curve (AUC) is proportional to GGT activity, and fractional GGT activity was quantified as described.[1,2]

Plasma samples from high-GGT carriers were analyzed twice: once undiluted to quantify b- and m-GGT and once diluted 100-fold to allow a more accurate quantification of f-GGT. s-GGT activity was estimated as the difference between total GGT values and the sum of b-, m- and f-GGT, except for subject III-1 from family 1, where the direct quantification of s-GGT was possible. Fractional GGT analysis of cell culture media was performed as described for plasma samples, but the injected volume was 0.1 mL instead of 0.025 mL.

Amino acids and leukotrienes in plasma, and ammonia and glutathione concentrations in blood or in urine

Plasma amino acid levels were determined by the ion exchange chromatography with ninhydrin reaction, on an automatic analyzer with or without addition of 10% trichloroacetic acid to the samples for protein precipitation and GGT inactivation. Glutathione in blood and in urine was analyzed by using the EnzyChromTM GSH/GSSG Assay Kit (EGTT-100) (Bioassay Systems, Hayward, USA). Cysteinyl leukotrienes in plasma and LTE_4 in urine were determined by enzyme immunoassays (LTD_4 BlueGene, LTC_4 Neogen, Glutathione and LTE_4 Cayman Chemical).

Statistical Analysis

Student's unpaired t test for non-Gaussian parameters was performed between patients with GGTemia and controls for leukotriene and glutathione determinations.

DNA and RNA isolation, complementary DNA (cDNA) synthesis, linkage analyses, whole-genome sequencing (WGS), Sanger verification, GGT1 cDNA expression

In order to map the disease locus in families 1 and 2, DNA samples from a subset of 26 individuals with and without GGTemia from the two families **(Figure 1A)** were genotyped with genome-wide high-resolution single nucleotide polymorphism arrays (HumanCytoSNP-12v2 BeadChip SNP array, Illumina), according to the manufacturer's instructions. Raw SNP call data were processed with the Genotyping Analysis Module of GenomeStudio 1.6.3 (Illumina). Copy-number variants and segments of loss-of-heterozygosity (LOH) were called and visualized using Nexus software and the SNPFASST segmentation algorithm (BioDiscovery Inc.). Multipoint likelihood-of-the-odds (LOD) scores were obtained with the

Merlin program[3] under the hypothesis of an autosomal-dominant, fully penetrant mutation.

One microgram of purified DNA from one patient of each family (III-IV, family 1, IV-6 in family 2, **Fig. 1**) was sent for WGS on an Illumina HiSeq4000 sequencer at GATC-Biotech (Konstanz, Germany). Paired-end reads were aligned to the hg19 human reference genome with Burrows-Wheeler transformation [4]. Polymerase chain reaction (PCR) duplicates were removed with PICARD (http://picard.sourceforge.net) and single nucleotide substitutions, and small indels were called with GATK's UnifiedGenotyper software with a number of variant quality filters in place (Supplemental Methods) and that included a "Depth of Coverage" filter of ≤ 20 and a "quality by read depth" filter of < 2 applied for single nucleotide variants. Local realignment of regions with misalignments due to indels was performed using GATK. Annotation and categorization of sequence variants was performed using snpEff. Final average depths of coverage were 34.17 x and 28.97 x, respectively, for the proband´s samples.

GGT1 mutations identified by WGS were verified and tested for co-segregation by Sanger sequencing using flanking intronic primers, GGT1_6f 5´-CCAGGGAATGTCTGAGGCTA and GGT1_6r 5´-AAACAGTGCAGTTTATTCCAAAGAG, amplifying a 460-bp fragment containing exon 6 of GGT1. The relative expression of mutant and wild-type *GGT1* alleles was determined by partial GGT1 gene sequencing in cDNA following RNA extraction from peripheral leukocytes using the QIAamp RNA Blood Mini Kit (Qiagen, Hilden, Germany), and cDNA synthesis using the SuperScript ® III First-Strand Synthesis System for RT-PCR (Thermofisher, Vienna, Austria). All sequencing analyses were performed using ABI PRISM 3130XL Genetic Analyzers and the BigDye Terminator v.1.1 Cycle Sequencing Kit (Applied Biosystems, ThermoFisher Scientific, Vienna, Austria). The *GGT1* variant designation is based on NCBI transcript reference sequence NM_013421.2 (corresponding to Ensembl transcript ENST00000400380.5) and genomic reference sequence NG_008111.1 (corresponding to Ensembl gene ENSG00000100031). The variants reported in this manuscript were submitted to the Leiden Open Variation Database

(https://databases.lovd.nl/shared/genes/GGT1) with IDs
https://databases.lovd.nl/shared/individuals/00263434 and
https://databases.lovd.nl/shared/individuals/00263435.

Expression analysis for GGT genes were performed using three specific TaqMan® Gene Expression Assays (Hs00269779_m1 for GGT5, Hs02387913_g1 for GGT2 and GGT3P, and Hs00359124_g1 for *GGT1*) and two custom TaqMan MGB probes designed to target all GGT transcripts on chromosome 22 except GGT5: one probe corresponding to the mRNA for the light chain genes of the GGT protein and another corresponding to the heavy chain genes. The human TATA-box binding protein (Applied Biosystems, Part No. 4326322E) and the human GAPDH (Applied Biosystems, Part No. 4326317E) probes were used as endogenous expression controls. Standard amplification conditions (Applied Biosystems) were applied using the TaqMan Gene Expression or Power SYBR Green PCR Master Mix.

Cell culture, cloning and transient transfection of wild-type and mutated GGT1 plasmids

The *GGT1* point mutation identified in family 1 was introduced into the full-length cDNA of human GGT[5] contained in a pcDNA3 plasmid (Invitrogen). Mutagenesis was performed using the GeneArt® Site-Directed Mutagenesis System (Invitrogen) according to manufacturer's protocol, a liposome/DNA ratio of 0.120mL/0.007mg was employed. Mutated and wild-type plasmids were amplified in DH5α-T1 competent E.coli and purified using the EndoFree Plasmid Kit (Quiagen) and transfected with the Lipotaxi mammalian transfection kit (Stratagene) into a melanoma cell clone (Me 665/2/c21) expressing only traces of GGT activity.[6] Cells were grown in RPMI 1640 medium, supplemented with 10% (v/v) foetal calf plasma, 2mM L-glutamine (L-Gln) under water-saturated atmosphere with 5% CO2, at 37 °C. Cell monolayers and medium aliquots were collected 48h after transfections; cell monolayers

were harvested with hypotonic lysis buffer (10 mMTris–HCl, pH 7.8) and cells were disrupted by a tight-fitting glass–glass Dounce homogeniser. All samples were stored at –20°C until analysis. The experiment was performed in triplicate.

Spectrophotometrical and cytochemical analysis of GGT

Determination of GGT activity in cell homogenates and medium aliquots was performed using the gamma-glutamyl-4-anilide as substrate and glycylglycine as reaction acceptor.[7] Total protein content was determined by the method of Bradford (Bio-Rad Protein Assay).

For cytochemical staining, cell monolayers were air-dried overnight at room temperature. After fixing for 15s in phosphate-buffered acetone-formalin, dishes were washed twice in ice-cold double-distilled water to remove the fixative. GGT activity was revealed by using gamma-glutamyl-4-methoxy-2-naphtylamide as a substrate and Fast Garnet GBC as a chromogen.[8]

Western blot analysis.

Aliquots from diluted plasma samples from Family 1 were separated by 7.5% SDS-PAGE using a 15 cm long gel. Plasma samples from controls were diluted 5-fold (final dilution, 50 µl corresponding to 0.2 – 0.3 mU of GGT activity), while plasma samples from GGTemia carriers were diluted 60-fold (final dilution, 20 µl corresponding to 0.6 – 3 mU of GGT activity). Lysates and media from transfected cells were collected in hypotonic cell lysis buffer (10 mM Tris–HCl, pH 7.8) and aliquots corresponding to 0.6 mU of GGT activity were analyzed.

Gels were blotted onto nitrocellulose membranes, that were incubated overnight with an anti-GGT antiserum (1:5000 in PBS, containing 0.01% Tween-20 and 2.5% (w/v) non-fat dry milk proteins) directed against the C-terminal 20 amino-acids of human GGT1 heavy chain [9]. Visualization of protein bands was obtained using a peroxidase conjugated anti-rabbit IgG

antibody and an enhanced chemiluminescence detection kit (Roche). Bands were evaluated by densitometric analysis with a Bio-Rad ChemiDoc apparatus equipped with the QuantityOne software. GGT concentrations were too low in cell culture media to perform western blot analysis of released GGT.

Samples from c21-Bz cells were concentrated with 10,000 NMWL Ultrafree®-MC centrifugal filter units (Millipore) in order to reduce the samples volume. Proteins were Separated by 7.5 % SDS-PAGE and gel was blotted onto nitrocellulose membranes.

In silico analysis of identified protein variants

Effects of variants on protein function were assessed by the PolyPhen-2 (Polymorphism Phenotyping, version 2) tool,[10] Provean (Protein Variation Effect Analyzer) software,[11] the SIFT (Sorting Intolerant from Tolerant) algorithm,[12] and the ConSurf server, and the TMHMM prediction (Kyte and Doolittle, sliding window = 15, https://web.expasy.org/) and signal peptide prediction (http://www.cbs.dtu.dk/services/SignalP/) servers.

Public databases dbSNP, the Exome Sequencing Project (ESP), and the Exome Aggregation consortium (ExAC), and the Genome Aggregation Database (gnomAD) were searched to determine the allele frequencies of identified GGTemia in control populations. (e.g., https://gnomad.broadinstitute.org/gene/ENSG00000100031)

Data availability

Sequencing data (fragments of BAM files containing identified variants) will be deposited upon request in a public repository.

SUPPLEMENTARY FIGURE S1.

Trait locus mapping and *GGT1* mutation detection.

A genome-wide linkage study mapped the trait locus to a 3.75-Mb region on chromosome

22q11.23. Linkage plots of families 1 and 2, respectively, generated by the MERLIN program and representing the parametric LOD score values on the y-axis in relation to genetic position on the x-axis are shown. The human chromosome 22 from p-ter (left) to q-ter (right) is shown on each x-axis, and the genetic distance is given in cM. The overlapping, combined maximal linkage interval was flanked by markers *rs4822940* and *rs2295102* (21458625 – 25209753 bp (GRCh37.p13); 21104336 – 24813786 bp (GRCh38.p2)), and contained 21 annotated consensus coding sequence protein set genes (not shown). **(B)** Two distinct GGT1 variants, NM_013421.2:c.44G>T, p.Leu15Arg and NM_013421.2:c.28_54del, p.Leu10_Val18del were identified in families 1 and 2 by WGS, and **(C)** were confirmed by Sanger sequencing.
WGS covered all exons and regulatory elements of GGT-encoding genes; we add GGTemia to the traits for which the causal genes have been identified by WGS.

SUPPLEMENTARY TABLE

Total and fractional plasma GGT activity values (U/L) in family 1 (Figure 1A).

ID	Gender	Total GGT	b-GGT	m-GGT	s-GGT	f-GGT
Trait carriers						
IV-6	F	2820*	1.5	5.0*	73*	2740*
III-6	F	2870*	1.5	6.5*	78*	2784*
IV-2	F	2980*	0.7	4.4*	85*	2890*
IV-9	F	4330*	2.0	9.9*	83*	4235*
V-1	M	1920*	0.9	4.2*	77*	1838*
V-5	M	2480*	0.5	3.3	46*	2430*
IV-5	M	5200*	1.7	7.8*	87*	5103*
III-4	M	7070*	3.6	15.4*	193*	6857*
III-5	M	9100*	5.8	25.1*	232*	8837*
III-1	M	9630*	59*	51*	305*	9209*
Normal subjects						
III-2	F	20	3.3	1.0	3.0	12.6
IV-3	M	21	1.7	1.1	7.4	10.8
III-7	M	19	1.6	1.5	6.4	9.5
IV-8	M	31	3.8	3.6	12.2	11.4

*Over the upper reference limit[13]. M: males, F: females.

SUPPLEMENTARY REFERENCES

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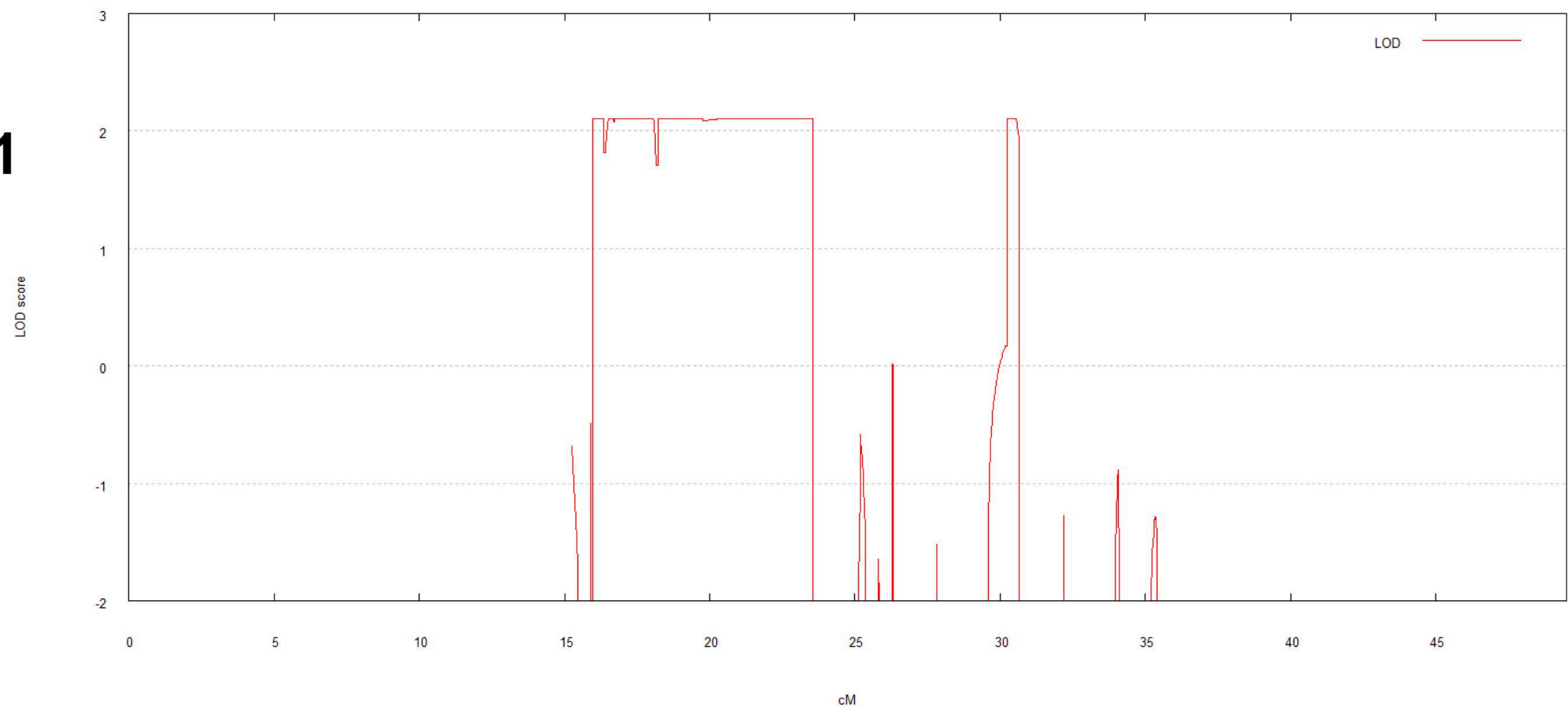
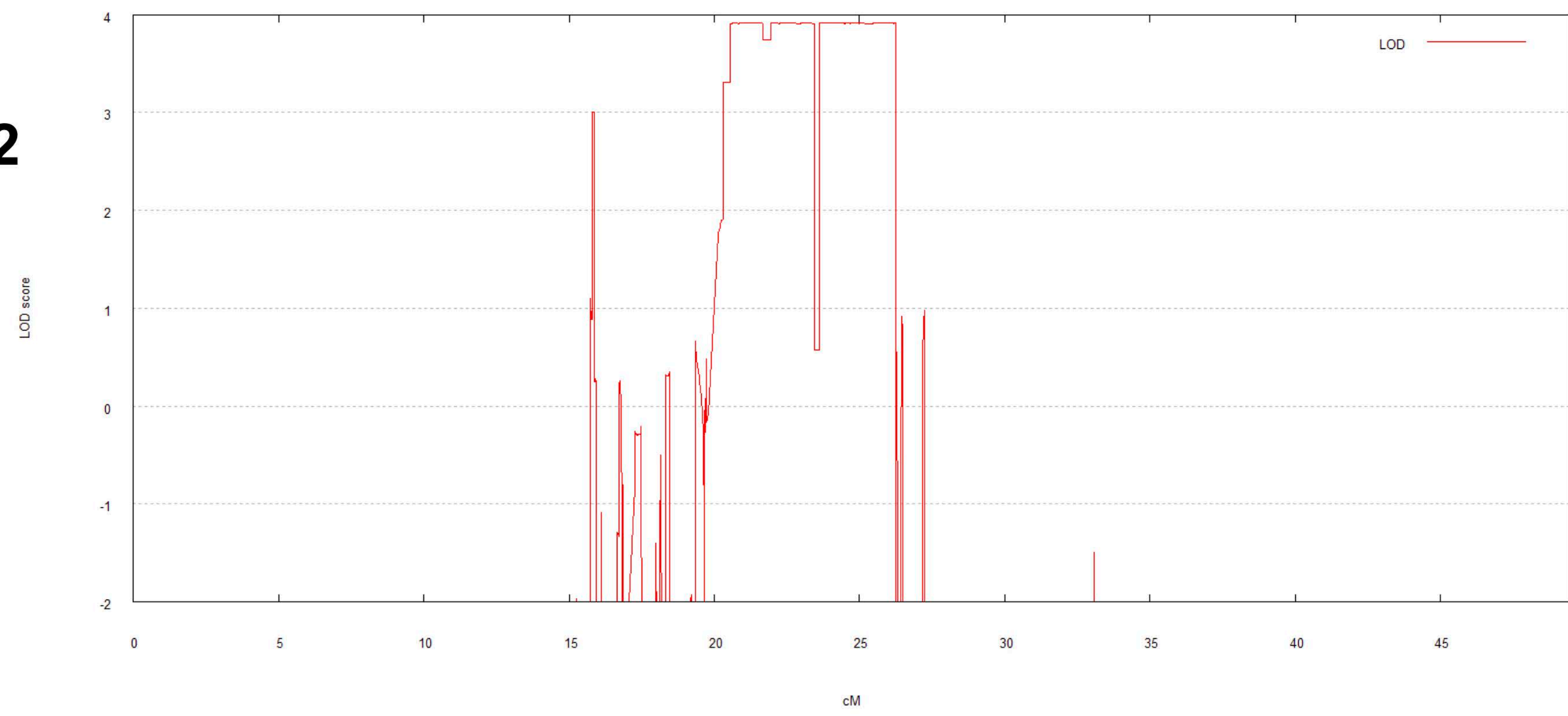
A

Family 1



Family 2

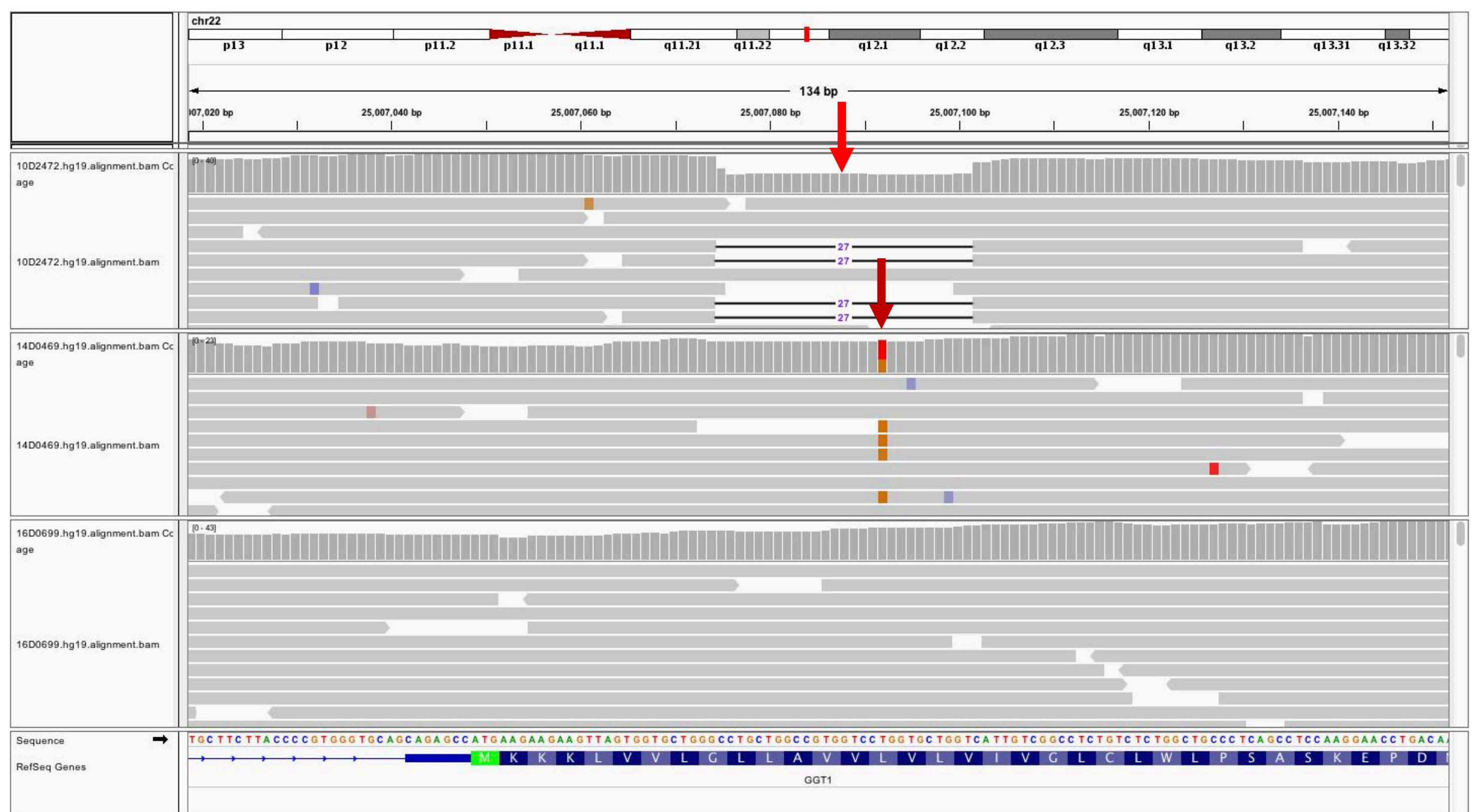


B

Family 2

Family 1

Control



C

Family 2

Family 1

Control