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(54) Title: MARKERS FOR OBESITY AND METHODS OF USE THEREOF

(57) Abstract: Markers for obesity, particularly childhood obesity, are provided along with methods of use.

Markers for Obesity and Methods of Use Thereof

FIELD OF THE INVENTION

The present invention relates to the field of obesity. Specifically, the instant invention provides markers for obesity, particularly childhood obesity, and methods of use thereof.

BACKGROUND OF THE INVENTION

Several publications and patent documents are cited throughout the specification in order to describe the state of the art to which this invention pertains.

Obesity is a major health problem in modern societies, with increasing prevalence in Western societies, particularly in children (Troiano et al. (1998) Pediatrics, 101:497-504). Obesity and its associated phenotype, insulin resistance (Reaven, G.M. (1988) Diabetes, 37:1595-607; DeFronzo et al. (1991)

Diabetes Care, 14:173-94), is also considered a contributor to the major causes of death in the United States and is an important risk factor for type 2 diabetes (T2D), cardiovascular disease (CVD), hypertension and other chronic diseases (National Institutes of Health Consensus Development Conference Statement (1985) Ann. Intern. Med., 103:147-51). Approximately 70% of obese adolescents grow up to become obese adults (Nicklas et al. (2001) J. Am. Coll. Nutr., 20:599-608; Whitaker et al. (1997) N. Engl. J. Med., 337:869-73; Parsons et al. (1999) Int. J. Obes. Relat. Metab. Disord., 23(Suppl 8):S1-107). Indeed, obesity during adolescence has been shown to be associated with increased overall mortality in adults (Must, A. (2003) Nutr. Rev., 61:139-42).

Despite environmental changes over the last 30 years, in particular the unlimited supply of convenient, highly calorific foods together with a sedentary lifestyle, there is also strong evidence for a genetic component to the risk of obesity (Friedman, J.M. (2004) Nat. Med., 10:563-9; Lyon et al. (2005) Am. J. Clin. Nutr., 82:215S-217S). This is reflected in prevalence differences between racial groups (Knowler et al. (1990) Diabetes Metab. Rev., 6:1-27; Zimmet et al. (1990) Diabetes Metab. Rev., 6:91-124). In addition, the familial occurrences of obesity have been long noted with the concordance for fat mass among monozygotic (MZ) twins reported to be 70-90%, higher than the 35-45% concordance in dizygotic (DZ) twins (Stunkard et al. (1986) JAMA 256:51-4; Borjeson et al. (1976) Acta Paediatr. Scand., 65:279-87). Accordingly, the estimated heritability of BMI ranges from 30 to 70% (Hebebrand et al. (2003) Obes. Rev., 4:139-46; Farooqi et al. (2005) Int. J. Obes. (Lond), 29:1149-52; Bell et

al. (2005) Nat. Rev. Genet., 6:221-34; Schousboe et al. (2003) Twin Res., 6:409-21).

In the past three years, thirteen genetic loci have been implicated for BMI from the outcomes of genome wide association studies (GWA) studies primarily in adults. Insulin-induced gene 2 (*INSIG2*) was the first locus to be reported by this method to have a role in obesity (Herbert et al. (2006) Science 312:279-83), but replication attempts have yielded inconsistent outcomes (Loos et al. (2007) Science 315:187; Dina et al. (2007) Science 315:187; Rosskopf et al. (2007) Science 315:187; Lyon et al. (2007) PLoS Genet., 3:e61; Hotta et al. (2008) J. Hum. Genet., 53:857-62). The second reported locus, the fat mass- and obesity-associated gene (FTO; Frayling et al. (2007) Science 316:889-94) has been more robustly observed by others (Grant et al. (2008) PLoS ONE 3:e1746; Hinney et al. (2007) PLoS ONE 2:e1361; Dina et al. (2007) Nat. Genet., 39:724-6; Scuteri et al. (2007) PLoS Genet., 3:e115). Subsequent larger studies have uncovered eleven additional loci (Loos et al. (2008) Nat. Genet., 40:768-75; Thorleifsson et al. (2009) Nat. Genet., 41:18-24; Willer et al. (2009) Nat. Genet., 41:25-34). In addition, a copy number variation study of extreme syndromic obesity in children with developmental delay reported a handful of rare variants contributing to the trait (Bochukova et al. (2010) Nature 463:666-70).

SUMMARY OF THE INVENTION

In accordance with the present invention, methods of diagnosing an increased risk for obesity in a patient are provided. In a particular embodiment, the methods comprise assessing the presence of at least one copy number variation in a biological sample from the

patient. In one embodiment, the copy number variation is in a gene selected from the group consisting of EDIL3, S1PR5, FOXP2, KIF2B, ARL15, and DNAJC15. In one embodiment, the copy number variation is in a gene selected from the group consisting of EDIL3, S1PR5, FOXP2, TBCA, ABCB5, ZPLD1, KIF2B, ARL15, and EPHA6. The copy number variation may be a deletion or duplication.

BRIEF DESCRIPTIONS OF THE DRAWING

Figures 1A-1D provide graphs depicting the distributions of the number of copy number variations (CNVs) called per individual of each cohort.

Figure 2 provides examples of CNV observance based on B-allele frequency (BAF) and Log R Ratio (LRR). Distributions of the number of CNVs called per individual of each cohort. LRR is the normalized intensity and BAF is the normalized genotype data. BAF and LRR values are based on the deviation of each sample on each SNP from the cluster center. LRR tends around 0 meaning expected diploid intensity signal. If LRR is elevated, more DNA is being bound indicating there is a duplication. Conversely, LRR decreased signals indicate less DNA is bound, consistent with deletion. In tandem, BAF has three normal diploid modes 0=AA, 0.5=AB, and 1=BB. If 0.5 values are not observed in a contiguous region of SNPs, deletion is probable. If 0.5 values are not observed but 0.33 and 0.66 values are observed, duplication is probable attesting to AAB and ABB genotypes. ROH: a copy number neutral run of homozygosity. The homozygosity is evident in BAF but the LRR does not deviate substantially from zero resulting in a ROH call rather than deletion call.

Figures 3A-3F show loci being directly impacted by deletions or duplications using the UCSC Genome Browser

with Build 36 (March 2006) of the human genome. Figure 3A-3C show deletions directly impacting *EDIL3*, *S1PR5*, and *FOXP2*, respectively. Figures 3D-3F show duplications impacting *KIF2B*, *DNAJC15*, and *ARL15* respectively.

Figure 4 provides graphs of the quantitative PCR validation of CNVs at the indicated loci. Error bars denote the standard deviation of quadruple runs.

Figure 5 is an analysis flow diagram for statistical informatics of CNV calls from PennCNV (532,898 SNPs utilized on the Illumina® array).

DETAILED DESCRIPTION OF THE INVENTION

The prevalence of obesity in children and adults in the United States has increased dramatically over the past decade with 30% of the U.S. adult population having BMI above 30 and 5% above 40. A number of genetic determinants of adult obesity have already been established through genome wide association studies and studies of syndromic obesity in childhood. In an attempt to comprehensively identify CNVs conferring susceptibility to regular childhood obesity ($\geq 95^{th}$ percentile of BMI), a whole-genome CNV study was performed on a cohort of 1,080 childhood obesity cases and 2,500 lean controls ($<50^{th}$ percentile of BMI) of European ancestry who were genotyped with 550,000 SNP markers. Positive findings were evaluated in an independent cohort of 1,479 childhood obesity cases and 1,575 lean controls of African ancestry. 35 CNV loci were identified that were unique in the European American cases, 15 (42.9%) of which also replicated exclusively in African American cases (7 duplications and 8 deletions). 17 CNV loci were identified that were unique to at least three EA cases that both were not

previously reported in the public domain and were validated using quantitative PCR. Eight of these loci (47.1%) also replicated exclusively in AA cases (6 deletions and 2 duplications). Replicated deleted loci included *EDIL3* (EGF-like repeats- and discoidin I-like domains-containing protein 3), *S1PR5* (endothelial differentiation, sphingolipid), *FOXP2* (forkhead box P2), TBCA (tubulin folding cofactor A), *ABCB5* (ATP-binding cassette, sub-family B (MDR/TAP), member 5), and *ZPLD1* (zona pellucida-like domain containing 1) while replicated duplications at loci included *KIF2B* (kinesin family member 2B), *AGR3* (breast cancer membrane protein 11 precursor), ARL15 (ADP-ribosylation factor-like 15), and *DNAJC15* (DnaJ homolog, subfamily C, member 15), particularly KIF2B and ARL15. Evidence for a deletion at the EPHA6-UNQ6114 locus was also observed when the AA cohort was investigated as a discovery set. All variants were experimentally validated using quantitative PCR. These variants target genes involved in neurological function, which is an important mediator in the pathogenesis of obesity. These results indicate that CNVs contribute to the genetic susceptibility of obesity in multiple ethnicities.

Definitions

The following definitions are provided to facilitate an understanding of the present invention:

As used herein, the term "obesity" generally refers to a condition in which there is an excess of body fat in a subject. "Obesity" may refer to a condition whereby a subject has a Body Mass Index (BMI; body weight per height in meters squared (kg/m^2)) greater than or equal to 30.0 kg/m^2. An "obese subject" is a subject with a Body Mass Index (BMI) greater than or equal to

30.0 kg/m^2. An "overweight subject" is a subject with a BMI of 25.0 up to 30.0 kg/m^2.

The term "copy number", as used herein, refers to the number of copies of a particular region in the genomic DNA of a sample. If a genomic region has a "copy number variation", the genomic region has a copy number that is different than that of the average copy number of the remainder of the genome (e.g., 2 copies in humans).

As used herein, a "biological sample" refers to a sample of biological material obtained from a subject, preferably a human subject, including a tissue, a tissue sample, a cell sample, a tumor sample, and a biological fluid (e.g., blood, urine, or amniotic fluid). In a particular embodiment, the biological sample is blood.

As used herein, "diagnose" refers to detecting and identifying a disease or disorder in a subject. The term may also encompass assessing or evaluating the disease or disorder status (progression, regression, stabilization, response to treatment, etc.) in a patient known to have the disease or disorder.

As used herein, the term "prognosis" refers to providing information regarding the impact of the presence of a disease or disorder (e.g., as determined by the diagnostic methods of the present invention) on a subject's future health (e.g., expected morbidity or mortality, the likelihood of getting diabetes, and the risk of cardiovascular disease). In other words, the term "prognosis" refers to providing a prediction of the probable course and outcome of a disease/disorder or the likelihood of recovery from the disease/disorder.

The term "treat" as used herein refers to any type of treatment that imparts a benefit to a patient afflicted with a disease or disorder, including

improvement in the condition of the patient (e.g., in one or more symptoms), delay in the progression of the condition, etc.

The term "probe" as used herein refers to an oligonucleotide, polynucleotide or nucleic acid, either RNA or DNA, whether occurring naturally as in a purified restriction enzyme digest or produced synthetically, which is capable of annealing with or specifically hybridizing to a nucleic acid with sequences complementary to the probe. A probe may be either single-stranded or double-stranded. The exact length of the probe will depend upon many factors, including temperature, source of probe and use of the method. For example, for diagnostic applications, depending on the complexity of the target sequence, the oligonucleotide probe typically contains about 10-100, about 10-50, about 15-30, about 15-25, about 20-50, or more nucleotides, although it may contain fewer nucleotides. The probes herein may be selected to be complementary to different strands of a particular target nucleic acid sequence. This means that the probes must be sufficiently complementary so as to be able to "specifically hybridize" or anneal with their respective target strands under a set of pre-determined conditions. Therefore, the probe sequence need not reflect the exact complementary sequence of the target, although they may. For example, a non-complementary nucleotide fragment may be attached to the 5′ or 3′ end of the probe, with the remainder of the probe sequence being complementary to the target strand. Alternatively, non-complementary bases or longer sequences can be interspersed into the probe, provided that the probe sequence has sufficient complementarity with the sequence of the target nucleic acid to anneal therewith specifically.

The term "primer" as used herein refers to an oligonucleotide, either RNA or DNA, either single-stranded or double-stranded, either derived from a biological system, generated by restriction enzyme digestion, or produced synthetically which, when placed in the proper environment, is able to functionally act as an initiator of template-dependent nucleic acid synthesis. When presented with an appropriate nucleic acid template, suitable nucleoside triphosphate precursors of nucleic acids, a polymerase enzyme, suitable cofactors and conditions such as appropriate temperature and pH, the primer may be extended at its 3' terminus by the addition of nucleotides by the action of a polymerase or similar activity to yield a primer extension product. The primer may vary in length depending on the particular conditions and requirement of the application. For example, in diagnostic applications, the oligonucleotide primer is typically about 10-25 or more nucleotides in length. The primer must be of sufficient complementarity to the desired template to prime the synthesis of the desired extension product, that is, to be able to anneal with the desired template strand in a manner sufficient to provide the 3' hydroxyl moiety of the primer in appropriate juxtaposition for use in the initiation of synthesis by a polymerase or similar enzyme. It is not required that the primer sequence represent an exact complement of the desired template. For example, a non-complementary nucleotide sequence may be attached to the 5′ end of an otherwise complementary primer. Alternatively, non-complementary bases may be interspersed within the oligonucleotide primer sequence, provided that the primer sequence has sufficient complementarity with the sequence of the desired template strand to functionally

provide a template-primer complex for the synthesis of the extension product.

Polymerase chain reaction (PCR) has been described in U.S. Patents 4,683,195, 4,800,195, and 4,965,188.

With respect to single stranded nucleic acids, particularly oligonucleotides, the term "specifically hybridizing" refers to the association between two single-stranded nucleotide molecules of sufficiently complementary sequence to permit such hybridization under pre-determined conditions generally used in the art (sometimes termed "substantially complementary"). In particular, the term refers to hybridization of an oligonucleotide with a substantially complementary sequence contained within a single-stranded DNA molecule of the invention, to the substantial exclusion of hybridization of the oligonucleotide with single-stranded nucleic acids of non-complementary sequence. Appropriate conditions enabling specific hybridization of single stranded nucleic acid molecules of varying complementarity are well known in the art.

For instance, one common formula for calculating the stringency conditions required to achieve hybridization between nucleic acid molecules of a specified sequence homology is set forth below (Sambrook et al., 1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press):

$$Tm = 81.5°C + 16.6\text{Log}\,[Na+] + 0.41(\%\ G+C) - 0.63\ (\%\ \text{formamide}) - 600/\#bp\ \text{in duplex}$$

As an illustration of the above formula, using [Na+] = [0.368] and 50% formamide, with GC content of

42% and an average probe size of 200 bases, the Tm is 57°C. The Tm of a DNA duplex decreases by 1 - 1.5°C with every 1% decrease in homology. Thus, targets with greater than about 75% sequence identity would be observed using a hybridization temperature of 42°C.

The stringency of the hybridization and wash depend primarily on the salt concentration and temperature of the solutions. In general, to maximize the rate of annealing of the probe with its target, the hybridization is usually carried out at salt and temperature conditions that are 20-25°C below the calculated Tm of the hybrid. Wash conditions should be as stringent as possible for the degree of identity of the probe for the target. In general, wash conditions are selected to be approximately 12 20°C below the Tm of the hybrid. In regards to the nucleic acids of the current invention, a moderate stringency hybridization is defined as hybridization in 6X SSC, 5X Denhardt's solution, 0.5% SDS and 100 µg/ml denatured salmon sperm DNA at 42°C, and washed in 2X SSC and 0.5% SDS at 55°C for 15 minutes. A high stringency hybridization is defined as hybridization in 6X SSC, 5X Denhardt's solution, 0.5% SDS and 100 µg/ml denatured salmon sperm DNA at 42°C, and washed in 1X SSC and 0.5% SDS at 65°C for 15 minutes. A very high stringency hybridization is defined as hybridization in 6X SSC, 5X Denhardt's solution, 0.5% SDS and 100 µg/ml denatured salmon sperm DNA at 42°C, and washed in 0.1X SSC and 0.5% SDS at 65°C for 15 minutes.

The term "isolated" may refer to a compound or complex that has been sufficiently separated from other compounds with which it would naturally be associated. "Isolated" is not meant to exclude artificial or synthetic mixtures with other compounds or materials, or

the presence of impurities that do not interfere with fundamental activity or ensuing assays, and that may be present, for example, due to incomplete purification, or the addition of stabilizers.

As used herein, an "instructional material" includes a publication, a recording, a diagram, or any other medium of expression which can be used to communicate the usefulness of the composition of the invention for performing a method of the invention.

The phrase "solid support" refers to any solid surface including, without limitation, any chip (for example, silica-based, glass, or gold chip), glass slide, membrane, plate, bead, solid particle (for example, agarose, sepharose™, polystyrene or magnetic bead), column (or column material), test tube, or microtiter dish.

As used herein, the term "array" refers to an ordered arrangement of hybridizable array elements (e.g., proteins, nucleic acids, antibodies, etc.). The array elements are arranged so that there are at least one or more different array elements on a solid support. In a particular embodiment, the array elements comprise oligonucleotide probes.

"Pharmaceutically acceptable" indicates approval by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans.

A "carrier" refers to, for example, a diluent, adjuvant, excipient, auxilliary agent or vehicle with which an active agent of the present invention is administered. Pharmaceutically acceptable carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic

origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water or aqueous saline solutions and aqueous dextrose and glycerol solutions are preferably employed as carriers, particularly for injectable solutions. Suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E.W. Martin (e.g., Remington's Pharmaceutical Sciences, 18th Ed. (1990, Mack Publishing Co., Easton, PA 18042)) and "Remington: The Science and Practice of Pharmacy" (Ed. Troy; Lippincott Williams & Wilkins, Baltimore, MD).

Diagnostic Methods, Kits, and Screening Methods

The instant invention provides methods of diagnosing and/or determining the susceptibility to/risk for obesity in a subject (e.g., mammal, human). The methods may further comprise providing a prognosis for related health problems. If a subject is diagnosed as being at risk for obesity, the subject's diet may be altered to avoid/lessen/treat obesity; the subject may be administered therapeutic agents to prevent/treat obesity; and/or the subject may be provided an exercise regimen.

In one embodiment, the method comprises determining the genomic copy number of at least one loci provided in Table 3, 4, or 7 in a biological sample obtained from a patient. In another embodiment, the method comprises determining the copy number of at least one gene selected from the group consisting of EPHA6, EDIL3, S1PR5, FOXP2, TBCA, ABCB5, ZPLD1, DERA, PNLIPRP1, PRR20A, BC015432, CDS2, CACNA2D4, LRTM2, CENTD1, KIF2B, BC073935, CR611653, BRDT, DNAJC15, AGR3, DFNB31, COL27A1, AKNA, ATP6V1G1, ORM1, ORM2, C9orf91, ARL15, PKD1L2, BCMO1, MICB, COMT, SLCO1A2, NXPH1, EGFL11,

NAALADL2, GPR98, PCDH20, BC048997, AK091626, APOB, and RPAP3 in a biological sample obtained from a patient.

In another embodiment, the gene is selected from the group consisting of S1PR5, FOXP2, TBCA, ABCB5, PNLIPRP1, BC015432, CDS2, CACNA2D4, LRTM2, CENTD1, BC073935, CR611653, DNAJC15, AGR3, DFNB31, COL27A1, AKNA, ATP6V1G1, ORM1, ORM2, C9orf91, ARL15, PKD1L2, BCMO1, MICB, COMT, NXPH1, GPR98, BC048997, and AK091626.

In yet another embodiment, the gene is selected from the group consisting of EDIL3, S1PR5, FOXP2, TBCA, ABCB5, ZPLD1, KIF2B, BC073935, CR611653, BRDT, DNAJC15, AGR3, DFNB31, COL27A1, AKNA, ATP6V1G1, ORM1, ORM2, C9orf91, and ARL15.

In still another embodiment, the gene is selected from the group consisting of EDIL3, S1PR5, FOXP2, KIF2B, ARL15, and DNAJC15. In still another embodiment, the gene is selected from the group consisting of EDIL3, S1PR5, FOXP2, TBCA, ABCB5, ZPLD1, KIF2B, and ARL15.

In a particular embodiment, when the gene is selected from the group consisting of EPHA6, EDIL3, S1PR5, FOXP2, TBCA, ABCB5, ZPLD1, DERA, PNLIPRP1, PRR20A, BC015432, CDS2, CACNA2D4, LRTM2, and CENTD1, the copy number variation indicative of obesity is a deletion.

In another embodiment, when the gene is selected from the group consisting of KIF2B, BC073935, CR611653, BRDT, DNAJC15, AGR3, DFNB31, COL27A1, AKNA, ATP6V1G1, ORM1, ORM2, C9orf91, ARL15, PKD1L2, BCMO1, MICB, COMT, SLCO1A2, NXPH1, EGFL11, NAALADL2, GPR98, PCDH20, BC048997, AK091626, APOB, and RPAP3, the copy number variation indicative of obesity is a duplication.

The NCBI GeneID Nos. and GenBank Accession Nos. (which provide nucleotide and amino acid sequences) of the above genes are:

EDIL3 (EGF-like repeats and discoidin I-like domains 3): GeneID: 10085

S1PR5 (sphingosine-1-phosphate receptor 5; also known as EDG8): GeneID: 53637

FOXP2 (forkhead box P2): GeneID: 93986

TBCA (tubulin folding cofactor A): GeneID: 6902

ABCB5 (ATP-binding cassette, sub-family B (MDR/TAP), member 5): GeneID: 340273

ZPLD1 (zona pellucida-like domain containing 1): GeneID: 131368

DERA (2-deoxyribose-5-phosphate aldolase homolog): GeneID: 51071

PNLIPRP1 (pancreatic lipase-related protein 1): GeneID: 5407

PRR20A (proline rich 20A; also known as FLJ40296): GeneID: 122183

BC015432: GenBank Accession No. BC015432.1

CDS2 (CDP-diacylglycerol synthase (phosphatidate cytidylyltransferase) 2): GeneID: 8760

CACNA2D4 (calcium channel, voltage-dependent, alpha 2/delta subunit 4): GeneID: 93589

LRTM2 (leucine-rich repeats and transmembrane domains 2): GeneID: 654429

CENTD1 (centaurin, delta 1; also known as ARAP2): GeneID: 116984

KIF2B (kinesin family member 2B): GeneID: 84643

BC073935: GenBank Accession No. BC073935.1

CR611653: GenBank Accession No. CR611653.1

BRDT (bromodomain, testis-specific): GeneID: 676

DNAJC15 (DnaJ (Hsp40) homolog, subfamily C, member 15): GeneID: 29103

AGR3 (anterior gradient homolog 3): GeneID: 155465

DFNB31 (deafness, autosomal recessive 31): GeneID: 25861

COL27A1 (collagen, type XXVII, alpha 1): GeneID: 85301

AKNA (AT-hook transcription factor): GeneID: 80709

ATP6V1G1 (ATPase, H+ transporting, lysosomal 13kDa, V1 subunit G1): GeneID: 9550

ORM1 (orosomucoid 1): GeneID: 5004

ORM2 (orosomucoid 2): GeneID: 5005

C9orf91 (chromosome 9 open reading frame 91): GeneID: 203197

ARL15 (ADP-ribosylation factor-like 15): GeneID: 54622

BCMO1 (beta-carotene 15,15'-monooxygenase 1; also known as BCDO): GeneID: 53630

PKD1L2 (polycystic kidney disease 1-like 2): GeneID: 114780

MICB (MHC class I polypeptide-related sequence B): GeneID: 4277

COMT (catechol-O-methyltransferase): GeneID: 1312

SLCO1A2 (solute carrier organic anion transporter family, member 1A2): GeneID: 6579

NXPH1 (neurexophilin 1): GeneID: 30010

EGFL11 (EGF-like-domain, multiple 11; also known as EYS): GeneID: 346007

NAALADL2 (N-acetylated alpha-linked acidic dipeptidase-like 2): GeneID: 254827

GPR98 (G protein-coupled receptor 98; also known as KIAA0686): GeneID: 84059

PCDH20 (protocadherin 20): GeneID: 64881

BC048997: GenBank Accession No. BC048997.1

AK091626: GenBank Accession No. AK091626.1

APOB (apolipoprotein B): GeneID: 338

RPAP3 (RNA polymerase II associated protein 3): GeneID: 79657

EPHA6 (ephrin receptor A6; UNQ6114; MIM 600066): Gene ID: 285220

Copy number variation can be detected by any method known in the art. For example, copy number variation may be detected by PCR (e.g., qPCR), fluorescent in situ hybridization (FISH), comparative genomic hybridization, array comparative genomic hybridization, and virtual karyotyping.

Probes and primers which specifically hybridize at least one of the genes of the instant invention are also encompassed by the instant invention. The probes and primers may be used to determine the copy number of the genes. The probes may be immobilized on a solid support. Indeed, arrays comprising at least one probe for at least one, at least two, at least six, or all of the genes of the instant invention are also encompassed by the instant invention. In a particular embodiment, the array comprises at least one probe for each gene in the above-identified groups.

The probes, primers, and/or arrays of the instant invention may be incorporated into a kit. The kit may further comprise instruction material, buffers, and/or containers.

In addition to the above, the instant invention encompasses methods for screening (including high throughput screening) for therapeutic agents for treating, inhibiting, and/or preventing obesity. Since the CNVs identified herein have been associated with the etiology of obesity, methods for identifying agents that modulate the activity of the genes and their encoded products (e.g., restore to normal levels) will result in the generation of efficacious therapeutic agents (e.g., nucleic acids (e.g., DNA, cDNA, RNA, siRNA, antisense, etc.), proteins, polypeptides, ligands, antibodies, small molecules, etc.) for the treatment of this condition. In a particular embodiment, the agents to be

tested were developed by rational drug design. As an example, for CNVs wherein there is a deletion of a gene and concomitant loss in activity in the gene and/or gene product (e.g., those provided in the above GeneID references), therapeutic agents which increase activity levels (e.g., to wild-type levels) would be desirable. Additionally, for CNVs wherein a gene is duplicated and there is a concomitant increase in activity in the gene and/or gene product, therapeutic agents which decrease activity levels (e.g., to wild-type levels) would be desirable.

In a particular embodiment, the screening methods comprise contacting cells comprising at least one CNV of the instant invention with a compound and assessing whether an activity of the gene and/or gene product affected by the CNV is modulated (e.g., closer to wild-type), thereby identifying a therapeutic agent. In a particular embodiment, the cells are obtained from a subject. In yet another embodiment, the CNVs are generated in a cell in vitro. Methods for modulating the CN of a gene or locus in a cell are well known in the art. In still another embodiment, the screening methods may be performed on an animal (e.g., a transgenic animal (e.g., mouse)) comprising at least one CNV of the instant invention.

The elucidation of the role played by the CNVs described herein in cellular metabolism facilitates the development of pharmaceutical compositions useful for treatment and diagnosis of obesity. These compositions may comprise, in addition to one of the above substances, a pharmaceutically acceptable carrier (e.g., excipient, carrier, buffer, stabilizer or other materials well known to those skilled in the art). Such materials should be non-toxic and should not interfere

with the efficacy of the active ingredient. Whether it is a polypeptide, antibody, peptide, nucleic acid molecule, small molecule or other pharmaceutically useful compound according to the present invention that is to be given to an individual, administration is preferably in a "prophylactically effective amount" or a "therapeutically effective amount" (as the case may be, although prophylaxis may be considered therapy), this being sufficient to show benefit to the individual.

In yet another embodiment, the instant invention encompasses pharmacogenomics. More specifically, the instant invention encompasses methods of screening a subject to predict the efficacy of a therapeutic agent or to select the most effective therapeutic agent for treatment. In a particular embodiment, the method comprises obtaining a biological sample from a patient and determining the presence or absence of at least one CNV of the instant invention. The subject is then administered the therapeutic agent if it is demonstrated that the administration of the therapeutic agent to a subject with the CNV is efficacious and without unacceptable side effects.

The following example provides illustrative methods of practicing the instant invention, and is not intended to limit the scope of the invention in any way.

EXAMPLE

METHODS

Study Subjects

All subjects were consecutively recruited from the Greater Philadelphia area from 2006 to 2009 at the Children's Hospital of Philadelphia. The study

consisted of 1,080 Caucasian obese children (BMI ≥ 95th percentile), 2,500 Caucasian lean controls (BMI < 50th percentile), 1,479 AA obese children and 1,575 AA lean controls that met strictly established data quality thresholds for CNVs. All of these participants had their blood drawn in to a 6 ml EDTA blood collection tube and were subsequently DNA extracted for genotyping. BMI ≥ 95th percentile was defined using the Center for Disease control (CDC) z-score = 1.645 (www.cdc.gov/nchs/about/major/nhanes/growthcharts/datafiles.htm). All subjects were biologically unrelated (based on available SNP data (pairwise IDB was used to filter out individuals showing relatedness above 0.25) and were aged between 2 and 18 years old (Table 1). All subjects were between -3 and +3 standard deviations of CDC corrected BMI (i.e., outliers were excluded to avoid the consequences of potential measurement error or Mendelian causes of extreme obesity; the latter category therefore specifically excluded individuals that fall in to the category previously reported for CNV discovery (Bochukova et al. (2010) Nature 463:666-70; Walters et al. (2010) Nature 463:671-5)). Self-reported ethnicity was confirmed by multidimensional scaling methodologies. Global ancestry was determined using Eigenstrat to ensure a singular cluster of ethnic ancestry in the European and African populations. This study was approved by the Institutional Review Board of the Children's Hospital of Philadelphia. Parental informed consent was given for each study participant for both the blood collection and subsequent genotyping.

Cohort	Age mean (SD)	Gender (Male Frequency)
Caucasian Obese	10.3 (4.47)	0.57
Caucasian Lean	9.10 (5.21)	0.54
African Obese	10.4 (4.72)	0.48
African Lean	7.11 (5.48)	0.52

Table 1: Gender and age distribution in the cohorts studied.

Illumina® Infinium assay for CNV Discovery

High-throughput, genome-wide SNP genotyping was performed using the Infinium™ II HumanHap550 BeadChip technology (Illumina®; San Diego, CA), at the Center for Applied Genomics at the Children's Hospital of Philadelphia. The genotype data content together with the intensity data provided by the genotyping array provides high confidence for CNV calls. Importantly, the simultaneous analysis of intensity data and genotype data in the same experimental setting establishes a highly accurate definition for normal diploid states and any deviation thereof. To call CNVs, the PennCNV algorithm (www.openbioinformatics.org/penncnv/) was used, which combines multiple sources of information, including Log R Ratio (LRR) and B Allele Frequency (BAF) at each SNP marker, along with SNP spacing, a trained hidden Markov model, and population frequency of the B allele to generate CNV calls. Each sample had CNVs called blinded to case status. Positive findings were evaluated in an independent replication cohort of similar size of African ancestry. A flow diagram of the CNV filtering and testing steps is provided in Figure 5.

CNV quality control

Quality Control (QC) measures was calculated on the HumanHap550 GWAS data based on statistical distributions to exclude poor quality DNA samples and false positive CNVs. The first threshold is the percentage of attempted SNPs which were successfully genotyped. Only samples with call rate > 98% were included. The genome wide intensity signal must have as little noise as possible. Only samples with the standard deviation (SD)

of normalized intensity (LRR) < 0.30 were included. All samples must have clear Caucasian or African ethnicity based on Multiple Dimension Scaling (MDS) scoring and all other samples were excluded. Wave artifacts roughly correlating with GC content resulting from hybridization bias of low full length DNA quantity are known to interfere with accurate inference of copy number variations (Diskin et al. (2008) Nucleic Acids Res., 36:e126). Only samples where the GC wave factor of LRR to wave model ranged between -0.1<X<0.1 were accepted. If the count of CNV calls made by PennCNV exceeds 100 (Figure 1), the DNA quality is usually poor. Thus, only samples with CNV call count < 100 were included. Any duplicate samples (such as monozygotic twins) had one sample excluded.

Statistical analysis of CNVs

CNV frequency between cases and controls was evaluated at each SNP using Fisher's exact test. Only loci that were significant between cases and controls ($p<0.05$) where cases in the European American discovery cohort had the same variation, replicated in African Americans or were not observed in any of the control subjects, and validated with an independent method were considered. Statistical local minimums are reported to narrow the association in reference to a region of nominal significance including SNPs residing within 1 Mb of each other. Resulting significant CNVRs were excluded if they met any of the following criteria: i) residing on telomere or centromere proximal cytobands; ii) arising in a "peninsula" of common CNV arising from variation in boundary truncation of CNV calling; iii) genomic regions with extremes in GC content which produces hybridization bias; or iv) samples contributing

to multiple CNVRs. DAVID (Database for Annotation, Visualization, and Integrated Discovery)(Dennis et al. (2003) Genome Biol., 4:P3; Huang da et al. (2007) Genome Biol., 8:R183) was used to assess the significance of functional annotation clustering of independently associated CNV results into InterPro categories (the top result is UniProt Category: Vision P=0.0069 (CACNA2D4, GPR98, DFNB31). To adjust for number of tests performed, correction of 16 deletion and 19 duplication CNVRs was made based on significance in the European American cohort.

CNV Filtering Steps

Multiple CNV filtering steps have been performed as part of the analysis. Firstly, it is important to note that of the 532,898 SNPs on the Illumina array, 8,672 (1.627%) showed deletion and 6,763 (1.269%) showed duplication in at least three or more unrelated cases in the European American discovery cohort (frequency ≥ 0.278%). The threshold of three cases is selected because it is the minimal case frequency to provide reproducibility for the calls in a given region. This upfront exclusion is very similar to the inclusion threshold of 1% Minor Allele Frequency in GWA SNP genotype studies. This drastically cuts down on the number of test preformed to correct for genome wide testing.

Secondly, all CNVs were called simultaneously in both cases and controls and classified into CNVRs as defined in the manuscript. A total of 272 deletion and 174 duplication CNVRs were identified. Thirdly, to search for novel CNVs, all CNVRs observed in the Caucasian control cohort were filtered out to establish exclusivity in cases and carefully reviewed the raw data

(BAF and LRR) for accurate CNV calling and statistical significance as described in Methods. This left 12 deletion and 19 duplication CNVRs.

This dataset therefore defines the CNVRs from the discovery cohort that were used to test for novel Obesity CNVs. Replication of these CNVRs was then tested in the independent African American case-control dataset. 6 deletion and 7 duplication CNVRs survived the replication criteria (observed in an African American case and absent in the African American control set) and were subsequently experimentally validated with at least one independent method (QPCR and FISH). These results are shown in Table 3.

It is important to note that CNV calling is not unequivocally attained by any one platform for multiple reasons, including variations in DNA provided, array type, DNA processing, data processing, quality control, CNV calling algorithm, genomic features, genomic coverage and statistical presentation of regions. This can lead to high false positive rate upon initial inspection despite exhaustive efforts to standardize and control each confounding contribution.

CNV validation by quantitative PCR (QPCR)

Universal Probe Library (UPL; Roche, Indianapolis, IN) probes were selected using the ProbeFinder v2.41 software (Roche, Indianapolis, IN). Quantitative PCR was performed on an ABI 7500 Real Time PCR Instrument or on an ABI Prism™ 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). Each sample was analyzed in quadruplicate either in 25 µl reaction mixture (250 nM probe, 900 nM each primer, Fast Start TaqMan Probe Master from Roche, and 10 ng genomic DNA) or in 10 µl reaction mixture (100 nM probe, 200 nM each

primer, 1x Platinum Quantitative PCR SuperMix-Uracil-DNA-Glycosylase (UDG) with ROX from Invitrogen, and 25 ng genomic DNA). The values were evaluated using Sequence Detection Software v2.2.1 (Applied Biosystems, CA). Data analysis was further performed using the $\Delta\Delta C_T$ method. Reference genes, chosen from *COBL* (MIM 610317), *GUSB* (MIM 611499), and *SNCA* (MIM 163890), were included based on the minimal coefficient of variation and then data was normalized by setting a normal control to a value of 1. None of these rare CNVs could be effectively tagged by a single common SNP present on the array (Table 2).

CNV	SNP	r^2
chr5:83835179-83874339	rs13355569	0.012672
chr5:83835179-83874339	rs10041822	0.00577
chr19:10489548-10512171	rs7256672	0.000808
chr19:10489548-10512171	rs281423	0.000599
chr7:113843696-113859679	rs10255943	0.001823
chr7:113843696-113859679	rs7782412	0.001009
chr5:77039051-77076628	rs2359877	0.001682
chr5:77039051-77076628	rs2359875	0.001568
chr7:20708193-20711088	rs1108864	0.001978
chr7:20708193-20711088	rs6954985	0.001187
chr3:104059109-104092618	rs6787298	0.001848
chr3:104059109-104092618	rs938988	0.001083
chr12:16141980-16149714	rs6488792	0.003442
chr12:16141980-16149714	rs4763429	0.002848
chr10:118354808-118363319	rs4751986	0.001028
chr10:118354808-118363319	rs17095060	0.000927
chr13:56772821-56786987	rs9537876	0.026849
chr13:56772821-56786987	rs1475441	0.02579
chr20:5111828-5137472	rs17785402	0.003542
chr20:5111828-5137472	rs6038092	0.0034
chr12:1778115-1803814	rs11608641	0.001571
chr12:1778115-1803814	rs11611260	0.001276
chr4:35782722-35828015	rs6826501	0.000984
chr4:35782722-35828015	rs4833119	0.000724
chr17:49444406-49449022	rs807101	0.001083
chr17:49444406-49449022	rs9303339	0.00103
chr12:8322269-8401135	rs2058456	0.001399
chr12:8322269-8401135	rs10161484	0.001034
chr1:92164066-92168702	rs17131553	0.000749
chr1:92164066-92168702	rs643927	0.00062
chr13:42467085-42516530	rs9590722	0.001425
chr13:42467085-42516530	rs10492434	0.001386
chr7:16885697-17020565	rs7809828	0.001501

chr7:16885697-17020565	rs6949072	0.000957
chr9:115996089-116495996	rs1003859	0.004612
chr9:115996089-116495996	rs11999545	0.001678
chr5:53467427-53480255	rs271246	0.002212
chr5:53467427-53480255	rs12153556	0.001691
chr16:79761583-79838201	rs7199318	0.005738
chr16:79761583-79838201	rs8058311	0.005555
chr6:31567721-31580699	rs2596480	0.003791
chr6:31567721-31580699	rs2267637	0.003166
chr22:17405381-19770502	rs5748302	0.010889
chr22:17405381-19770502	rs16984071	0.001003
chr12:21446005-21459060	rs4762726	0.001848
chr12:21446005-21459060	rs10841939	0.00179
chr7:8710455-8984816	rs6962574	0.00223
chr7:8710455-8984816	rs10239677	0.001396
chr6:67391945-67395314	rs16897804	0.002653
chr6:67391945-67395314	rs7767899	0.001389
chr3:177280907-177303744	rs1461249	0.00236
chr3:177280907-177303744	rs12488992	0.002253
chr5:90155080-90157263	rs2914673	0.001697
chr5:90155080-90157263	rs1809814	0.00102
chr13:61409439-61413267	rs3121782	0.002359
chr13:61409439-61413267	rs9539012	0.001593
chr13:22255136-22381716	rs314862	0.001881
chr13:22255136-22381716	rs17077897	0.001741
chr2:21337060-21342588	rs11096672	0.001064
chr2:21337060-21342588	rs10198972	0.000911
chr12:46232064-46330773	rs2087343	0.00186
chr12:46232064-46330773	rs4760607	0.001848

Table 2: Best SNP genotype surrogates for the CNVs and their respective r^2 values, reflecting the level of linkage disequilibrium between the markers. Due to the common nature of SNPs >1% and the rare nature of CNVs <1%, it is challenging to identify SNP genotype surrogates for the CNVs. Most r^2 values of genotyped SNPs within 1Mb of the CNV were close to zero.

RESULTS

The instant study consisted of 1,080 Caucasian obese children (BMI ≥ 95th percentile), 2,500 Caucasian lean controls (BMI < 50th percentile), 1,479 AA obese children and 1,575 AA lean controls (2-18 years old) that met strictly established data quality thresholds for CNVs. However, all subjects had to be between -3 and +3 standard deviations of CDC corrected BMI in order to exclude outliers that could potentially be a result of measurement error or Mendelian causes of extreme

obesity. Indeed, this latter category specifically excludes individuals that fall in to the category previously reported for CNV discovery (Bochukova et al. (2010) Nature 463:666-70; Walters et al. (2010) Nature 463:671-5).

An average of 22.0 and 19.5 CNV calls per individual were made using the PennCNV software (Wang et al. (2007) Genome Res., 17:1665-74) in the European American (EA) cases and controls, respectively, with 93% of subjects having 8-45 CNV calls (Figure 3). Four different copy number states were called for EA case/control cohorts, including 919/2,109 homozygous deletions (copy number, or CN =0), 13,678/28,917 hemizygous deletions (CN =1), 6,767/16,689 one copy duplications (CN =3), and 344/1,139 two copy duplications (CN =4), respectively. Figure 2 shows an example of raw Illumina data as viewed in the BeadStudio software and the resulting CNV call. The CNV calls spanned from 3 to 7903 SNPs, with an average of 19 SNPs per CNV call, and their sizes ranged from 50bp to 33Mb, with an average size of 81kb.

93% of African American (AA) subjects also harbored 8-45 CNV calls (Figure 1). An average of 24.1 and 25.1 CNV calls per individual were made in AA cases and controls, respectively. Among them, 897/1,026 CN =0, 25,650/28,793 CN =1, 8,778/10,251 CN =3 and 418/535 CN =4 were identified. The CNV calls spanned from 3 to 7903 SNPs, with an average of 15 SNPs per CNV call, and their sizes ranged from 50bp to 33Mb, with an average size of 67kb.

To identify novel genomic loci potentially contributing to non-syndromic childhood obesity in the EA subjects, a segment-based scoring approach was applied that scans the genome for consecutive SNPs with

more frequent copy number changes in cases compared to controls. The genomic span for these consecutive SNPs delineates common copy number variation regions, or CNVRs.

Local ancestry was assessed using the 1MB region surrounding each CNV locus which included an average of 300 SNP genotypes, resulting in well clustered populations without significantly deviating individuals. Statistical local minimums are reported to narrow the association in reference to a region of nominal significance including SNPs residing within 1 Mb of each other. Resulting significant CNVRs were excluded if they met any of the following criteria: i) residing on telomere or centromere proximal cytobands; ii) arising in a "peninsula" of common CNV arising from variation in boundary truncation of CNV calling; iii) genomic regions with extremes in GC content which produces hybridization bias; or iv) samples contributing to multiple CNVRs.

34 putative CNVR loci (15 deletions and 19 duplications) were identified that were exclusively present in at least three EA cases ($P \leq 0.05$). However, three of the deletions proved to be false positives during the validation process with quantitative PCR (qPCR), a method commonly used for independent validation of CNVs (Table 3 and Figure 4). All of the experimental validations were conducted in a blinded manner by an independent investigator group to minimize any bias, which adds another level of confidence to the experimental validation results. Issues related to CNV recurrence, accuracy, locus dependence and difficulties related to GC content were alleviated by running qPCR in quadruplicate, observing low standard deviation across runs, assaying control samples for the same genomic

region and assaying the same samples in different genomic regions.

	European Americans	African Americans				
CNV	Cases impacted exclusively	SNP tested	Cases imp-acted	Controls imp-acted	BP from nearest gene	Nearest gene
DELETIONS						
chr5:83835179-83874339	**5**	**rs10051401**	**1**	**0**	**118812**	**EDIL3**
chr19:10489548-10512171	**3**	**rs11670254**	**5**	**0**	**0**	**S1PR5 (EDG8)**
chr7:113843696-113859679	**3**	**rs12705964**	**3**	**0**	**0**	**FOXP2**
chr5:77039051-77076628	**3**	**rs384109**	**2**	**0**	**0**	**TBCA**
chr7:20708193-20711088	**3**	**rs12700232**	**1**	**0**	**0**	**ABCB5**
chr3:104059109-104092618	**3**	**rs1144781**	**1**	**0**	**377734**	**ZPLD1**
chr12:16141980-16149714	3	rs1376332	0	0	60398	DERA
chr10:118354808-118363319	3	rs7905887	0	1	0	PNLIPRP1
chr13:56772821-56786987	3	rs7981921	74	94	130468	FLJ40296
chr20:5111828-5137472	3	rs17785402	0	0	0	BC015432, CDS2
chr12:1778115-1803814	3	rs11611260	2	1	0	CACNA2D4, LRTM2
chr4:35782722-35828015	3	rs4833119	1	1	0	CENTD1
DUPLICATIONS						
chr17:49444406-49449022	**3**	**rs17730346**	**2**	**0**	**186834**	**KIF2B**
chr12:8322269-8401135	**3**	**rs10770444**	**1**	**0**	**0**	**BC073935, CR611653**
chr1:92164066-92168702	**3**	**rs11165816**	**1**	**0**	**19018**	**BRDT**
chr13:42467085-42516530	**3**	**rs2057529**	**1**	**0**	**0**	**DNAJC15**
chr7:16885697-17020565	**3**	**rs847403**	**1**	**0**	**0**	**AGR3**
chr9:115996089-116495996	**3**	**rs942520**	**1**	**0**	**0**	**DFNB31, COL27A1, AKNA, ATP6V1G1, ORM1, ORM2, c9orf91**
chr5:53467427-53480255	**3**	**rs16882296**	**1**	**0**	**0**	**ARL15**
chr16:79761583-79838201	4	rs12933806	0	0	0	PKD1L2, BCMO1

chr6:31567721-31580699	3	rs3828903	0	0	0	MICB
chr22:17405381-19770502	3	rs2238790	3	4	0	COMT+
chr12:21446005-21459060	3	rs7953750	0	0	6367	SLCO1A2
chr7:8710455-8984816	3	rs1859416	0	0	0	NXPH1
chr6:67391945-67395314	3	rs183895	0	2	918106	EGFL11
chr3:177280907-177303744	3	rs9875122	0	0	274785	NAALADL2
chr5:90155080-90157263	3	rs7717221	0	0	0	GPR98
chr13:61409439-61413267	3	rs2183953	0	0	509359	PCDH20
chr13:22255136-22381716	3	rs9510354	0	0	0	BC048997, AK091626
chr2:21337060-21342588	3	rs870638	0	0	216610	APOB
chr12:46232064-46330773	3	rs739856	0	0	12568	RPAP3

Table 3: CNVs at the SNP level (qPCR validated but not filtered for presence in the public domain) exclusive to childhood obesity in the European American cohort and replication attempt in African Americans. Those CNVs that replicated exclusively in African American cases are highlighted in bold. Co-ordinates are derived from UCSC Build hg 18. The "BP from nearest gene" column lists zero if the CNV encompasses the gene listed or a value if the CNV is nearby the gene listed. The individual CNV calls are assigned as a SNP-based statistic. Therefore, contiguous SNPs showing case non-zero values and control zero values form a region of CNV which are the CNVRs listed. The SNP tested is the central SNP in the CNVR region to ensure exact overlap in replication.

In the replication attempt, 13 of the 31 validated CNVR loci (41.9%) were also found to be exclusively present in one or more AA cases (6 deletions and 7 duplications) (Table 3). Only 17 of the CNVR loci were unique to the instant cohort (i.e., not reported in controls by the Database of Genomic Variants), of which 8 (47.1%) also replicated exclusively in AA cases (6 deletions and 2 duplications) (Table 4). The use of a different racial group for replication purposes represents a higher bar than making similar attempts in

the same ethnicity. For example, when the common variant in *TCF7L2* (MIM 602228) associated with type 2 diabetes was also associated with the disease in Africans with a similar magnitude (Helgason et al. (2007) Nat. Genet., 39:218-225), it was considered much more established as having a role in the disease; in addition, the recent asthma GWAS finding was also observed in African Americans which gave us a much higher level of certainty of the observation (Sleiman et al. (2010) New Eng. J. Med., 362:36-44). Since many variants have been shown to have different frequencies in different ethnic groups, observation of the same CNV as exclusive in EA and AA cohorts separately for the same phenotype minimizes the potential for confounding effects of population stratification. Figure 3 visualizes representative loci most frequently impacted by deletions or duplications using the UCSC Genome Browser with Build hg18 (March 2006; genome.ucsc.edu/cgi-bin/hgGateway) of the human genome. It was also evaluated if any of the key CNVs co-segregated with obesity in families, where data was available, and were able to establish that at least four of the CNVs were inherited from an obese parent and at least four were *de novo* (Table 5).

	European Americans	African Americans				
CNV	Cases impacted exclusively	SNP tested	Cases imp-acted	Controls imp-acted	BP from nearest gene	Nearest gene
DELETIONS						
chr5:83835179-83874339	5	rs10051401	1	0	118812	EDIL3
chr19:10489548-10512171	3	rs11670254	5	0	0	S1PR5 (EDG8)
chr7:113843696-113859679	3	rs12705964	3	0	0	FOXP2
chr5:77039051-77076628	3	rs384109	2	0	0	TBCA

chr7:20708193-20711088	3	rs12700232	1	0	0	ABCB5
chr3:104059109-104092618	3	rs1144781	1	0	377734	ZPLD1
chr12:16141980-16149714	3	rs1376332	0	0	60398	DERA
chr10:118354808-118363319	3	rs7905887	0	1	0	PNLIPRP1
chr20:5111828-5137472	3	rs17785402	0	0	0	BC015432, CDS2
chr4:35782722-35828015	3	rs4833119	1	1	0	CENTD1
DUPLICATIONS						
chr17:49444406-49449022	3	rs17730346	2	0	186834	KIF2B
chr5:53467427-53480255	3	rs16882296	1	0	0	ARL15
chr12:21446005-21459060	3	rs7953750	0	0	6367	SLCO1A2
chr6:67391945-67395314	3	rs183895	0	2	918106	EGFL11
chr5:90155080-90157263	3	rs7717221	0	0	0	GPR98
chr13:61409439-61413267	3	rs2183953	0	0	509359	PCDH20
chr2:21337060-21342588	3	rs870638	0	0	216610	APOB

Table 4: CNVs at the SNP level (both not present in the public domain and qPCR validated) exclusive to childhood obesity in the European American cohort and replication attempt in African Americans. Those CNVs that replicated exclusively in African American cases are highlighted in bold. Co-ordinates are derived from UCSC Build hg18. The "BP from nearest gene" column lists zero if the CNV encompasses the gene listed or a value if the CNV is nearby the gene listed. These loci showed CN=1 for deletion loci and CN=3 for duplication loci across contributing samples. No other states such as CN=0 or CN=4 were observed at these loci. The individual CNV calls are assigned as a SNP-based statistic. Therefore, contiguous SNPs showing case non-zero values and control zero values form a region of CNV which are the CNVRs listed. The SNP tested is the central SNP in the CNVR region to ensure exact overlap in replication.

CNVR	Inheritance
chr1:162647580-162655215	*de novo*
chr5:83835179-83874339	From obese father
chr19:10489548-10512171	Not from mother
chr19:10489548-10512171	Not from mother

chr7:20708193-20711088	*de novo*
chr12:16141980-16149714	From obese father
chr10:118354808-118363319	From mother
chr13:56772821-56786987	Not from mother
chr4:35782722-35828015	Not from mother
chr17:49444406-49449022	From obese mother
chr17:49444406-49449022	Not from mother
chr17:49444406-49449022	*de novo*
chr13:42467085-42516530	From obese mother
chr7:16885697-17020565	From mother
chr5:53467427-53480255	Not from mother
chr16:79761583-79838201	Not from mother
chr16:79761583-79838201	From father
chr6:31567721-31580699	*de novo*
chr3:177280907-177303744	Not from mother
chr13:22255136-22381716	From mother
chr13:22255136-22381716	Not from mother
chr2:21337060-21342588	Not from mother
chr12:46232064-46330773	Not from mother

Table 5: Inheritance of CNVs observed, based on available family data.

In order to further establish the significance of these findings, the other tail of the distribution (i.e. exclusive CNVRs among the controls) was examined. Firstly, while exclusivity to three obese subjects constituted a nominally significant observation among EA cases, ten EA controls were required to reach the same significance threshold due to the abundance of controls in the study. Analyzed in this way, no exclusive deletions were observed and only four exclusive duplications in the EA controls were observed. However, none of these duplications replicated exclusively in the AA controls. As such, in contrast to these control observations, a highly significant over abundance of exclusive CNVRs was seen in the EA cases that go on to

replicate exclusively in the AA cases, revealing that there is less than a 12% false positive discovery rate.

Large rare deletions present in <1% of individuals and >500 kb in size as set previously (Bochukova et al. (2010) Nature 463:666-670) were evaluated and an excess of large rare deletions genome-wide was not observed (Table 6). This is not unexpected given the previous report only found significance when including developmental delay subjects but not when severe early-onset obesity was evaluated alone.

Sample Cohort	CNV Type	Case Frequency	Control Frequency	*P* value
European Americans	Deletions	0.0222	0.0224	1
	Duplications	0.0611	0.0588	0.817
	Deletions and Duplications	0.0824	0.0760	0.541
African Americans	Deletions	0.0128	0.0184	0.245
	Duplications	0.0345	0.0406	0.393
	Deletions and Duplications	0.0541	0.0584	0.638

Table 6: Comparison of large rare CNV load genome-wide.

The loci harboring exclusive deletions in cases of both ethnicities consisted of four genes directly impacted, namely *S1PR5* (endothelial differentiation, sphingolipid; MIM605146), *FOXP2* (forkhead box P2; MIM 605317), *TBCA* (tubulin-specific chaperone a; MIM 610058) and *ABCB5* (ATP-binding cassette, sub-family B, member 5; MIM 611785) while two deletions were close to *EDIL3* (EGF-like repeats- and discoidin I-like domains-containing protein 3; MIM 606018) and *ZPLD1* (zona pellucida-like domain containing 1), respectively.

The loci harboring exclusive duplications in cases from both ethnicities consisted of four genes directly impacted, namely *BC073935-CR611653*, *DNAJC15* (DnaJ

homolog, subfamily C, member 15), *AGR3* (breast cancer membrane protein 11 precursor) and *ARL15* (ADP-ribosylation factor-like 15) plus a gene cluster at 9q32. In addition, two duplications were close to *KIF2B* (kinesin family member 2B) and *BRDT* (testis-specific bromodomain protein).

Pairwise IBD, both global and local, yielded low values close to zero between the five cases with the EDIL3 deletion; although exact CNV breakpoint sharing between cases was not a necessary parameter of the analysis, it does indicate that a specific copy number polymorphism is observed. The two loci harboring exclusive duplications in cases from both ethnicities consisted of one gene directly impacted, namely ARL15 (ADP-ribosylation factor-like 15) and one closest to KIF2B (kinesin family member 2B).

The majority of genes harboring at the loci uncovered in this study have not been implicated in obesity previously. However, the most notable finding is with *ARL15*, which was recently uncovered in a GWA study of adiponectin levels, with the same risk allele also being associated with a higher risk of coronary heart disease and type 2 diabetes (Richards et al. (2009) PLoS Genet., 5:e1000768).

In addition, although members of the human Forkhead-box (FOX) gene family have been strongly implicated in metabolic traits (Katoh et al. (2004) Int. J. Oncol., 25:1495-500), mutations in FOXP2 are best known for causing developmental speech and language disorders in humans (Vernes et al. (2008) N. Engl. J. Med., 359:2337-45). Although autism was recorded for one EA case and ADHD for one AA case (the only one of the replicated loci with reported co-morbidities), none of the children impacted by FOXP2 CNVs had evidence of

classical syndromes. The connection between the development of human language and speech and childhood obesity is not clear but one could hypothesize that problems with speech could lead to greater likelihood of social isolation and thus less activity. Indeed, this theory is supported by the fact that there are clear speech and language characteristics in children with Prader-Willi syndrome, who generally present with morbid obesity (Akefeldt et al. (1997) J. Intellect Disabil. Res., 41:302-11; Kleppe et al. (1990) J. Speech Hear. Disord., 55:300-9).

The EA cohort was used for discovery due to the much larger control group available in this ethnicity to observe and establish exclusivity most comprehensively in the discovery setting. However, in order to explore the possibility of additional exclusive loci, the AA cohort was also analyzed as the discovery cohort and attempted to replicate in the EA cohort (Table 7). Although exclusivity to five AA cases was required to achieve nominal significance in the discovery stage in this setting due to fewer lean controls in this cohort (Table 8), evidence for a deletion at the EPHA6-UNQ6114 locus (MIM 600066; e.g., www.ncbi.nlm.nih.gov/omim/600066) was observed, that was exclusive to AA cases.

	African Americans		European Americans			
CNV	Cases impacted exclusively	SNP tested	Cases imp-acted	Controls imp-acted	BP from nearest gene	Nearest gene
DELETIONS						
chr3:97523082-97528441	**5**	**rs9843398**	**1**	**0**	**487674**	***EPHA6,UNQ6114***
chr19:10477303-10512171	**5**	**rs12460981**	**3**	**0**	**0**	***S1PR5***
chr3:105183599-105186764	6	rs2166826	0	3	0	*BC070396*
chr5:84245129-84399973	6	rs1154964	2	3	528762	*EDIL3*

chr8:115226631-115278440	5	rs1515685	0	0	708213	*CSMD3*
chr6:120618851-120652337	5	rs9320745	0	2	764685	*AK097101*
DUPLICATIONS						
chr3:120225538-120284544	5	rs7638463	5	12	0	*IGSF11*

Table 7: African American cohort as discovery cohort and European Americans as replication. Those CNVs that replicated exclusively in European American cases are highlighted in bold. Note that a minimum of 5 CNVs exclusive in African American Cohort were required to reach nominal significance ($P \leq 0.05$) in discovery, mainly due to the lower number of lean African Americans in the cohort relative to lean European Americans.

EA Cases Harboring	EA Cases Not Harboring	EA Controls Harboring	EA Controls Not Harboring	*P* value (Fisher's Exact Test Two Tailed)
0	1080	0	2500	1
1	1079	0	2500	0.301676
2	1078	0	2500	0.09095
3	**1077**	**0**	**2500**	**0.027402**
4	**1076**	**0**	**2500**	**0.00825**
5	**1075**	**0**	**2500**	**0.002483**
6	**1074**	**0**	**2500**	**0.000746**
7	**1073**	**0**	**2500**	**0.000224**
8	**1072**	**0**	**2500**	**$6.74x10^{-5}$**
9	**1071**	**0**	**2500**	**$2.02x10^{-5}$**
10	**1070**	**0**	**2500**	**$6.06x10^{-6}$**

AA Cases Harboring	AA Cases Not Harboring	AA Controls Harboring	AA Controls Not Harboring	*P* value (Fisher's Exact Test Two Tailed)
0	1479	0	1575	1
1	1478	0	1575	0.484283
2	1477	0	1575	0.234448
3	1476	0	1575	0.11346
4	1475	0	1575	0.054889
5	**1474**	**0**	**1575**	**0.026545**
6	**1473**	**0**	**1575**	**0.012833**
7	**1472**	**0**	**1575**	**0.006202**
8	**1471**	**0**	**1575**	**0.002996**
9	**1470**	**0**	**1575**	**0.001447**
10	**1469**	**0**	**1575**	**0.000698**

Table 8: Discovery significance justification for frequency thresholds.

To date, there has been a notable paucity of GWA studies in childhood obesity, with studies exclusively uncovering loci in the adult setting (Frayling et al. (2007) Science 316:889-94; Grant et al. (2008) PLoS ONE 3:e1746; Hinney et al. (2007) PLoS ONE 2:e1361; Dina et al. (2007) Nat. Genet., 39:724-6; Scuteri et al. (2007) PLoS Genet., 3:e115; Loos et al. (2008) Nat. Genet., 40:768-75; Thorleifsson et al. (2009) Nat. Genet., 41:18-24; Willer et al. (2009) Nat. Genet., 41:25-34), and no study to date has reported CNVs that are significantly associated with the non-syndromic trait as opposed to the syndromic form (Bochukova et al. (2010) Nature 463:666-70; Walters et al. (2010) Nature 463:671-5). As such, the instant study represents the first large-scale, unbiased genome-wide scan of CNVs in pediatric obesity. The loci uncovered in this present study are for the first time exclusively observed in childhood obesity and replicated in an independent case control data set from a different ethnicity. The results are given extra credibility as the well-established FTO (Frayling et al. (2007) Science 316:889-94; Grant et al. (2008) PLoS ONE 3:e1746; Hinney et al. (2007) PLoS ONE 2:e1361; Dina et al. (2007) Nat. Genet., 39:724-6; Scuteri et al. (2007) PLoS Genet., 3:e115) locus is very strongly associated with both BMI and obesity in the EA cohort (Grant et al. (2008) PLoS One, 3:e1746; Zhao et al. (2009) Examination of all type 2 diabetes GWAS loci reveals HHEX-IDE as a locus influencing pediatric BMI. Diabetes). Interestingly, no association was observed with any CNVs previously reported in extreme syndromic pediatric obesity with developmental delay (Bochukova et al. (2010) Nature 463:666-70). In addition, the previously reported common CNV flanking NEGR1 (MIM 613173) or encompassing

SH2B1 (MIM 608937) (Willer et al. (2009) Nat. Genet., 41:25-34) were not further studied.

Taken together, the instant unbiased approach to assess the entire genome has revealed novel genes impacted by CNVs that are exclusive to cases in two different ethnicities and have not previously been directly implicated in the context of obesity and await further characterization. Further functional studies may be performed to more fully characterize the function of the genes at these loci in relation to childhood obesity.

While certain of the preferred embodiments of the present invention have been described and specifically exemplified above, it is not intended that the invention be limited to such embodiments. Various modifications may be made thereto without departing from the scope and spirit of the present invention, as set forth in the following claims.

What is claimed is:

1. A method for diagnosing an increased risk for non-syndromic obesity in a Caucasian child subject, said method comprising determining the copy number of at least one gene in a biological sample from said subject,

wherein said gene is selected from the group consisting of EDIL3, S1PR5, FOXP2, TBCA, ABCB5, ZPLD1, DERA, PNLIPRP1, PRR20A, BC015432, CDS2, CACNA2D4, LRTM2, CENTD1, KIF2B, BC073935, CR611653, BRDT, DNAJC15, AGR3, DFNB31, COL27A1, AKNA, ATP6V1G1, ORM1, ORM2, C9orf91, ARL15, PKD1L2, BCMO1, MICB, COMT, SLCO1A2, NXPH1, EGFL11, NAALADL2, GPR98, PCDH20, BC048997, AK091626, APOB, RPAP3, and EPHA6,

wherein the presence of a variation in the copy number of at least one of said genes is indicative of an increased risk for obesity in a Caucasian child subject.

2. The method of claim 1, wherein said gene is selected from the group consisting of EDIL3, S1PR5, FOXP2, TBCA, ABCB5, ZPLD1, KIF2B, BC073935, CR611653, BRDT, DNAJC15, AGR3, DFNB31, COL27A1, AKNA, ATP6V1G1, ORM1, ORM2, C9orf91, and ARL15.

3. The method of claim 1, wherein said gene is selected from the group consisting of EDIL3, S1PR5, FOXP2, KIF2B, ARL15, and DNAJC15.

4. The method of any one of claims 1 to 3, wherein said variation in copy number is a deletion.

5. The method of any one of claims 1 to 3, wherein said variation in copy number is a duplication.

6. A method for diagnosing an increased risk for non-syndromic obesity in a child subject of African ancestry, said

method comprising determining the copy number of at least one gene in a biological sample from said subject,

wherein said gene is selected from the group consisting of EDIL3, S1PR5, FOXP2, TBCA, ABCB5, ZPLD1, KIF2B, BC073935, CR611653, BRDT, DNAJC15, AGR3, DFNB31, COL27A1, AKNA, ATP6V1G1, ORM1, ORM2, C9orf91, ARL15, and EPHA6,

wherein the presence of a variation in the copy number of at least one of said genes is indicative of an increased risk for obesity in a child subject of African ancestry.

7. The method of claim 6, wherein said gene is selected from the group consisting of EDIL3, S1PR5, FOXP2, TBCA, ABCB5, ZPLD1, KIF2B, BC073935, CR611653, BRDT, DNAJC15, AGR3, DFNB31, COL27A1, AKNA, ATP6V1G1, ORM1, ORM2, C9orf91, and ARL15.

8. The method of claim 6, wherein said gene is selected from the group consisting of EDIL3, S1PR5, FOXP2, KIF2B, ARL15, and DNAJC15.

9. The method of any one of claims 6 to 8, wherein said variation in copy number is a deletion.

10. The method of any one of claims 6 to 8, wherein said variation in copy number is a duplication.

11. A method for identifying agents which are therapeutic for the treatment of non-syndromic obesity in a Caucasian child subject, said method comprising:

a) providing cells expressing at least one copy number variant, wherein said copy number variant is of at least one gene selected from the group consisting of EDIL3, S1PR5, FOXP2, TBCA, ABCB5, ZPLD1, DERA, PNLIPRP1, PRR20A, BC015432, CDS2, CACNA2D4, LRTM2, CENTD1, KIF2B, BC073935, CR611653, BRDT, DNAJC15, AGR3, DFNB31, COL27A1, AKNA, ATP6V1G1, ORM1,

ORM2, C9orf91, ARL15, PKD1L2, BCMO1, MICB, COMT, SLCO1A2, NXPH1, EGFL11, NAALADL2, GPR98, PCDH20, BC048997, AK091626, APOB, RPAP3, and EPHA6;

b) contacting the cells of step a) with a test agent; and

c) determining whether said agent modulates an activity of said gene, wherein the modulation of the activity of the copy number variant towards wild-type activity is indicative of a therapeutic agent.

12. The method of claim 11, wherein said cells of step a) are of a non-human animal.

13. A method for identifying agents which are therapeutic for the treatment of non-syndromic obesity in a child subject of African ancestry, said method comprising:

a) providing cells expressing at least one copy number variant, wherein said copy number variant is of at least one gene selected from the group consisting of EDIL3, S1PR5, FOXP2, TBCA, ABCB5, ZPLD1, KIF2B, BC073935, CR611653, BRDT, DNAJC15, AGR3, DFNB31, COL27A1, AKNA, ATP6V1G1, ORM1, ORM2, C9orf91, ARL15, and EPHA6;

b) contacting the cells of step a) with a test agent; and

c) determining whether said agent modulates an activity of said gene, wherein the modulation of the activity of the copy number variant towards wild-type activity is indicative of a therapeutic agent.

14. The method of claim 13, wherein said cells of step a) are of a non-human animal.

15. An array for use in detecting copy number variants associated with an increased risk for non-syndromic obesity in a Caucasian child subject, said array comprising probes which specifically hybridize to at least six genes selected from the

group consisting of EDIL3, S1PR5, FOXP2, TBCA, ABCB5, ZPLD1, DERA, PNLIPRP1, PRR20A, BC015432, CDS2, CACNA2D4, LRTM2, CENTD1, KIF2B, BC073935, CR611653, BRDT, DNAJC15, AGR3, DFNB31, COL27A1, AKNA, ATP6V1G1, ORM1, ORM2, C9orf91, ARL15, PKD1L2, BCMO1, MICB, COMT, SLCO1A2, NXPH1, EGFL11, NAALADL2, GPR98, PCDH20, BC048997, AK091626, APOB, RPAP3, and EPHA6.

16. The array of claim 15, wherein said genes are selected from the group consisting of EDIL3, S1PR5, FOXP2, TBCA, ABCB5, ZPLD1, KIF2B, BC073935, CR611653, BRDT, DNAJC15, AGR3, DFNB31, COL27A1, AKNA, ATP6V1G1, ORM1, ORM2, C9orf91, and ARL15.

17. The array of claim 15, wherein said genes are selected from the group consisting of EDIL3, S1PR5, FOXP2, KIF2B, ARL15, and DNAJC15.

18. The array of any one of claims 15 to 17 comprising at least one probe to each of the genes listed.

19. The array of claim 18 consisting of at least one probe to each of the genes listed.

20. An array for use in detecting copy number variants associated with an increased risk for non-syndromic obesity in a child subject of African ancestry, said array comprising probes which specifically hybridize to at least six genes selected from the group consisting of EDIL3, S1PR5, FOXP2, TBCA, ABCB5, ZPLD1, KIF2B, BC073935, CR611653, BRDT, DNAJC15, AGR3, DFNB31, COL27A1, AKNA, ATP6V1G1, ORM1, ORM2, C9orf91, ARL15, and EPHA6.

21. The array of claim 20, wherein said genes are selected from the group consisting of EDIL3, S1PR5, FOXP2, TBCA, ABCB5,

ZPLD1, KIF2B, BC073935, CR611653, BRDT, DNAJC15, AGR3, DFNB31, COL27A1, AKNA, ATP6V1G1, ORM1, ORM2, C9orf91, and ARL15.

22. The array of claim 20, wherein said genes are selected from the group consisting of EDIL3, S1PR5, FOXP2, KIF2B, ARL15, and DNAJC15.

23. The array of any one of claims 20 to 22 comprising at least one probe to each of the genes listed.

24. The array of claim 23 consisting of at least one probe to each of the genes listed.

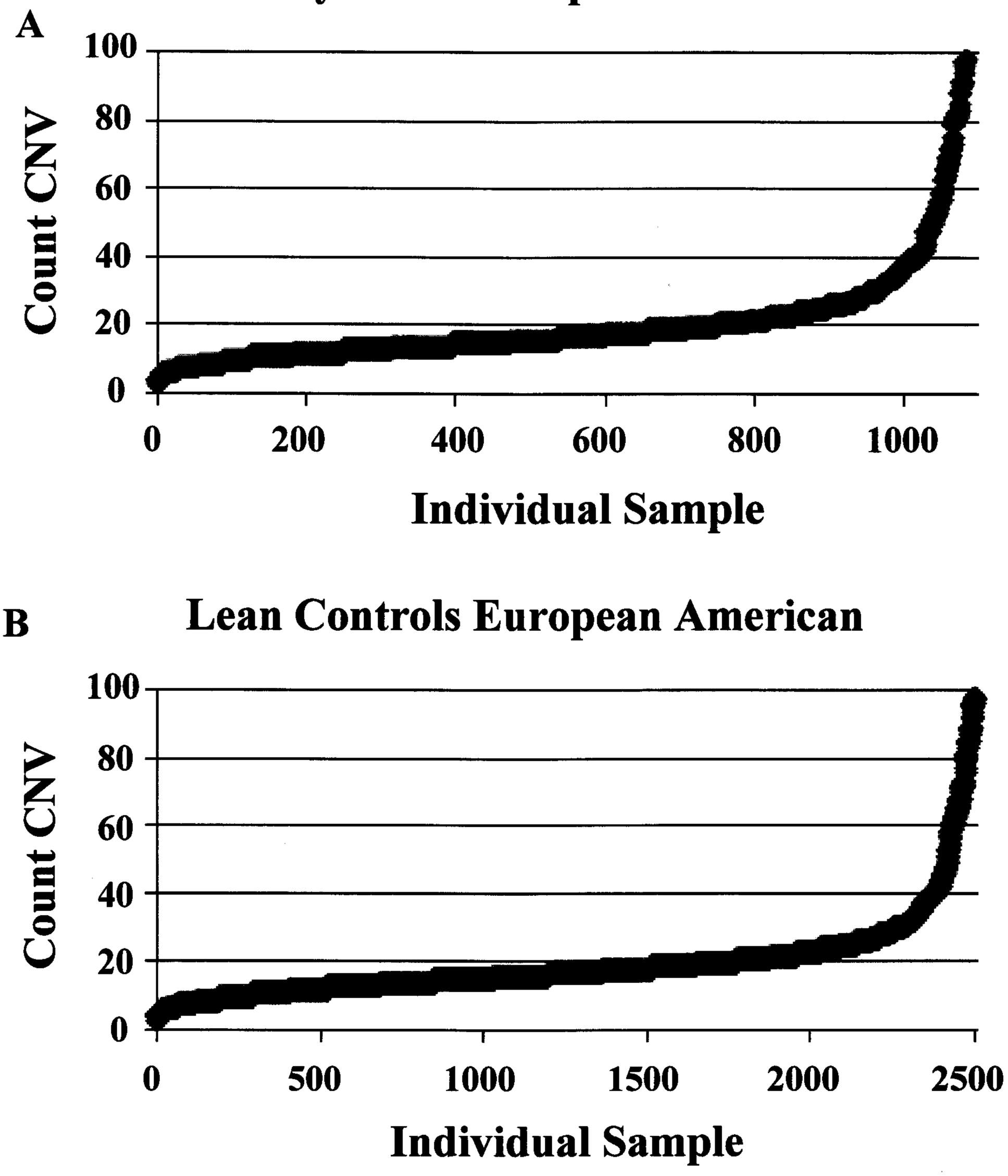
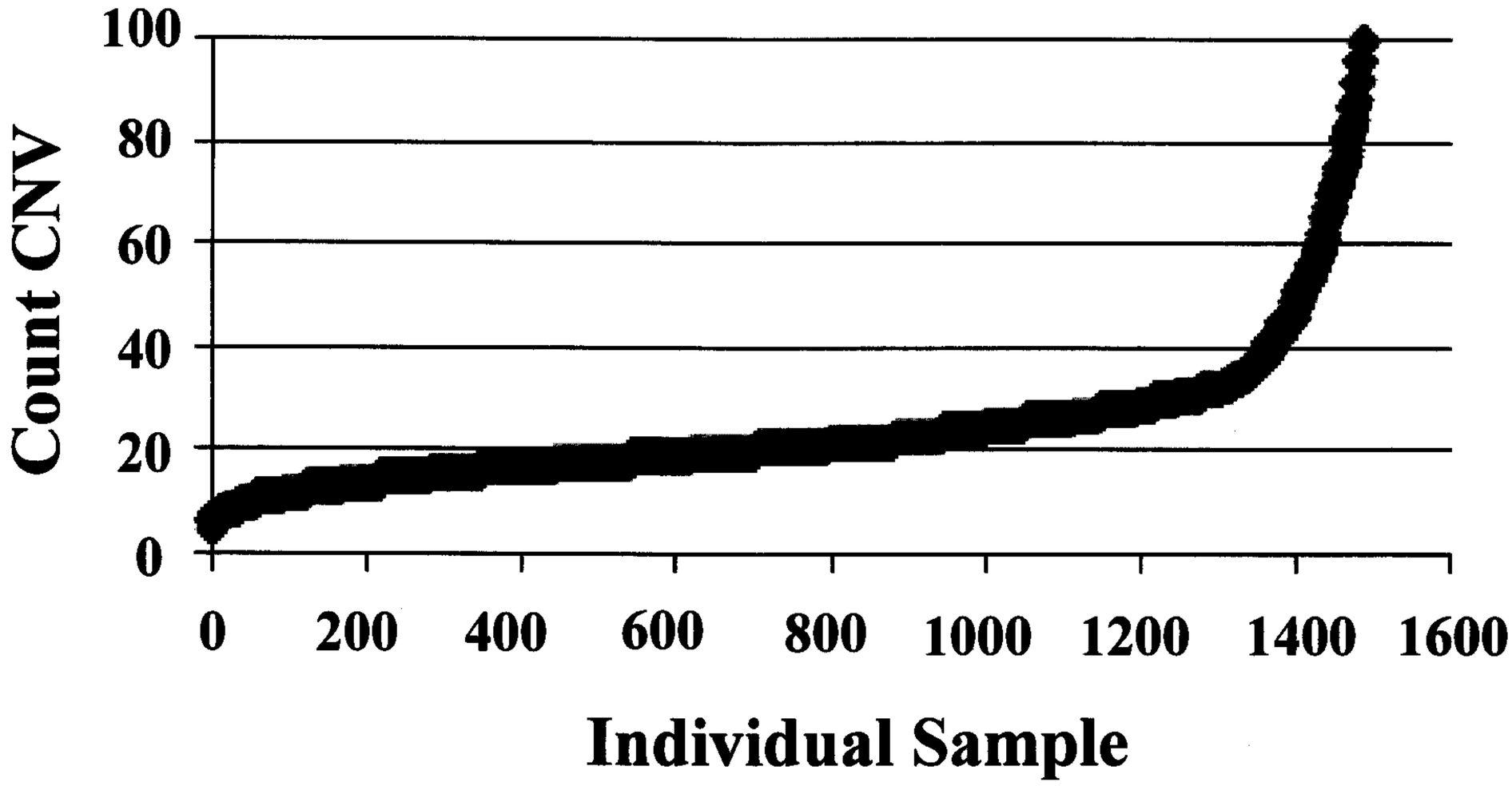

A
Obesity Cases European American
Count CNV
100
80
60
40
20
0
0
200
400
600
800
1000
Individual Sample
B
Lean Controls European American
Count CNV
100
80
60
40
20
0
0
500
1000
1500
2000
2500
Individual Sample

Figure 1

C



Lean Controls African Americans

D

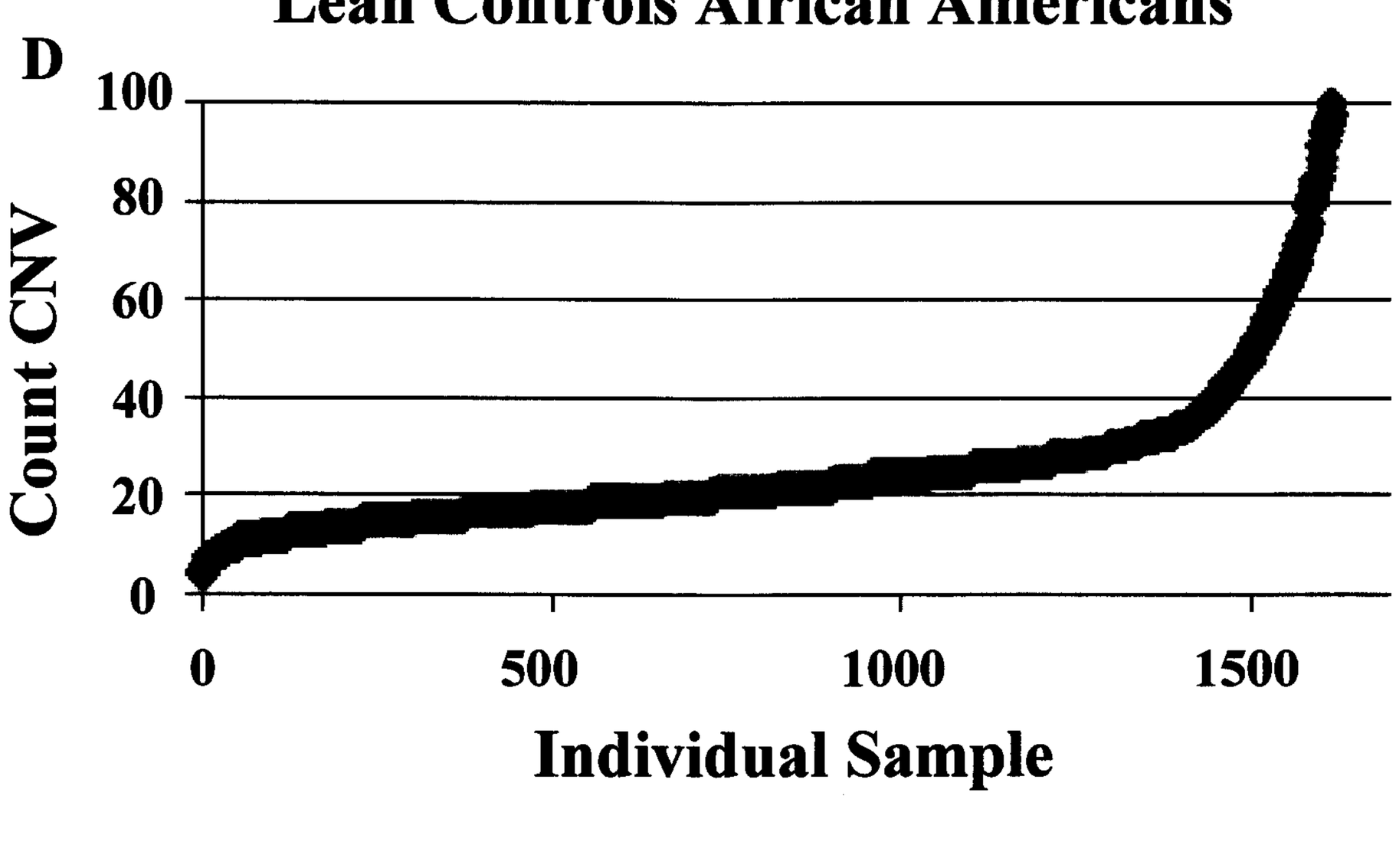


Figure 1

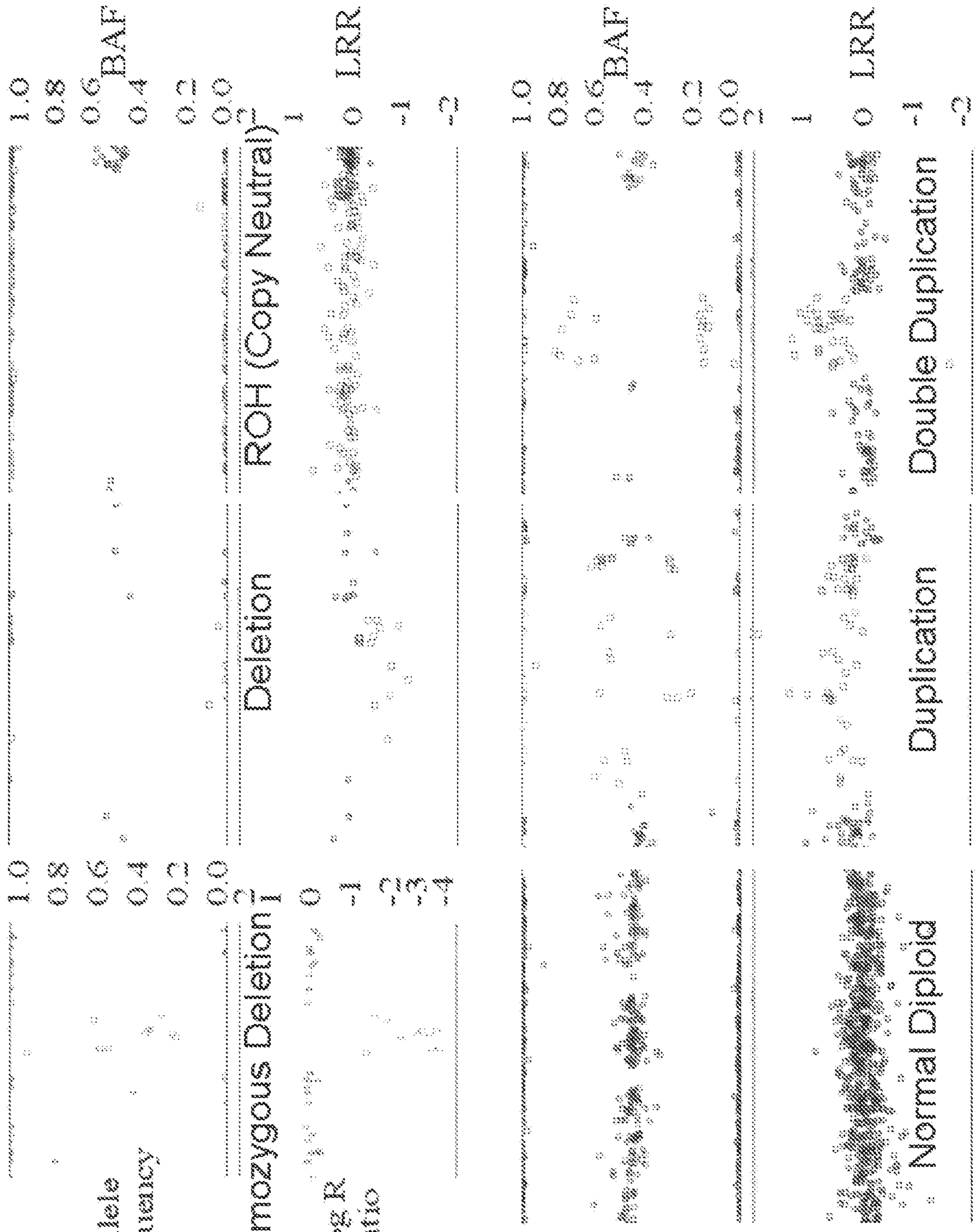
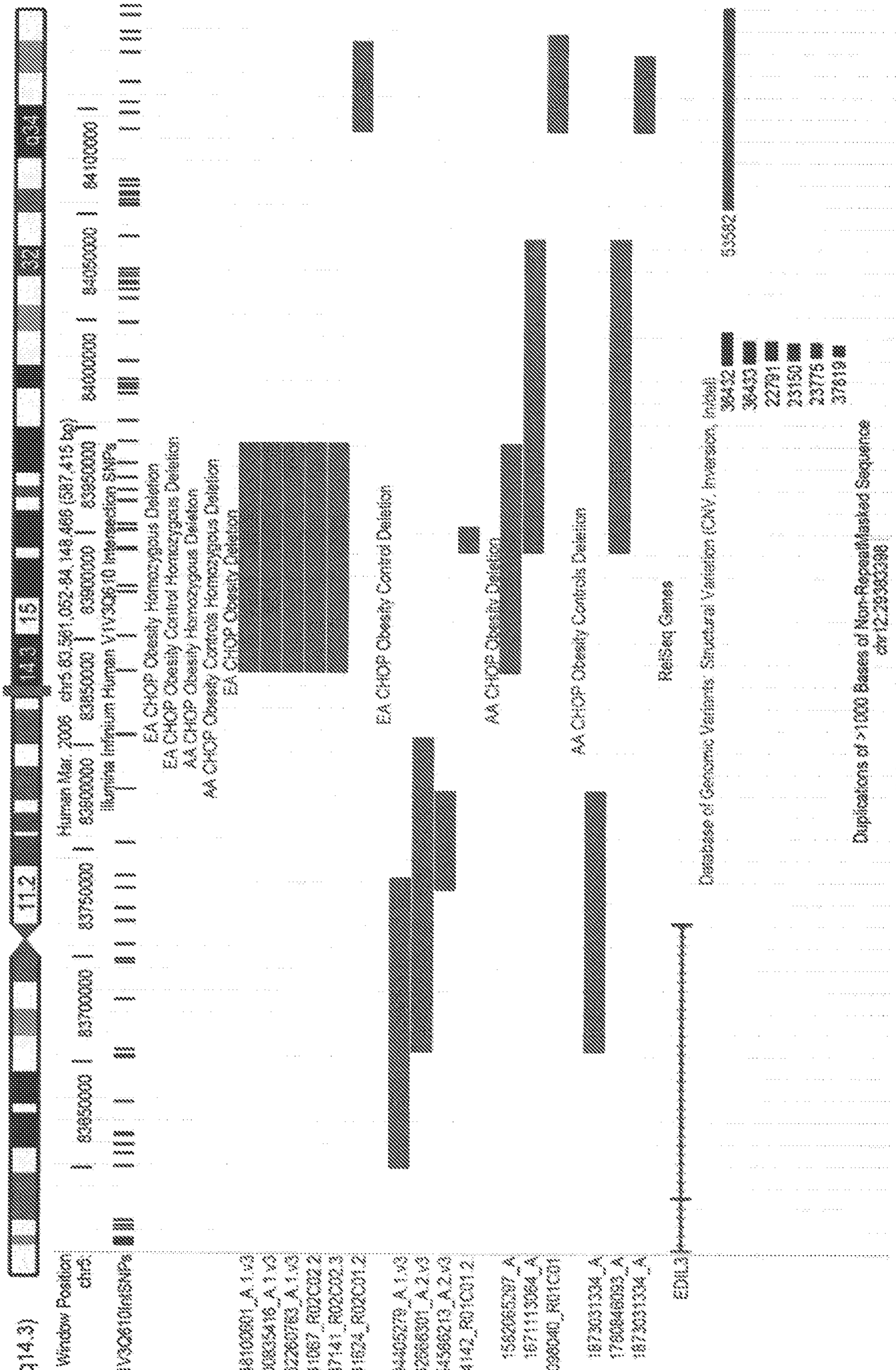

Double Duplication
Duplication
Normal Diploid
Deletion
Homozygous Deletion
BAF
LRR
ROH (Copy Neutral)
B Allele Frequency
Log R Ratio

Figure 2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18723031334_A
EBL3
Database of Genomic Variants: Structural Variation (CNV, Inversion, Indel)
Duplications of >1000 Bases of Non-Repeatmasked Sequence
chr12:29360288
Human Mar. 2006 chr5 83,521,052-84,148,466 (627,415 bp)
Human Human V1V3Q610 Intersection SNPs
Illumina Infinium HumanHap650Y
EA ChOP Obesity Homozygous Deletion
EA ChOP Obesity Homozygous Deletion
EA ChOP Obesity Control Homozygous Deletion
AA ChOP Obesity Homozygous Deletion
AA ChOP Obesity Controls Homozygous Deletion
EA ChOP Obesity Deletion
EA ChOP Obesity Deletion
EA ChOP Obesity Control Deletion
AA ChOP Obesity Deletion
AA ChOP Obesity Deletion
AA ChOP Obesity Controls Deletion
RefSeq Genes
band
q13
q14.1
q14.2
q14.3
q15
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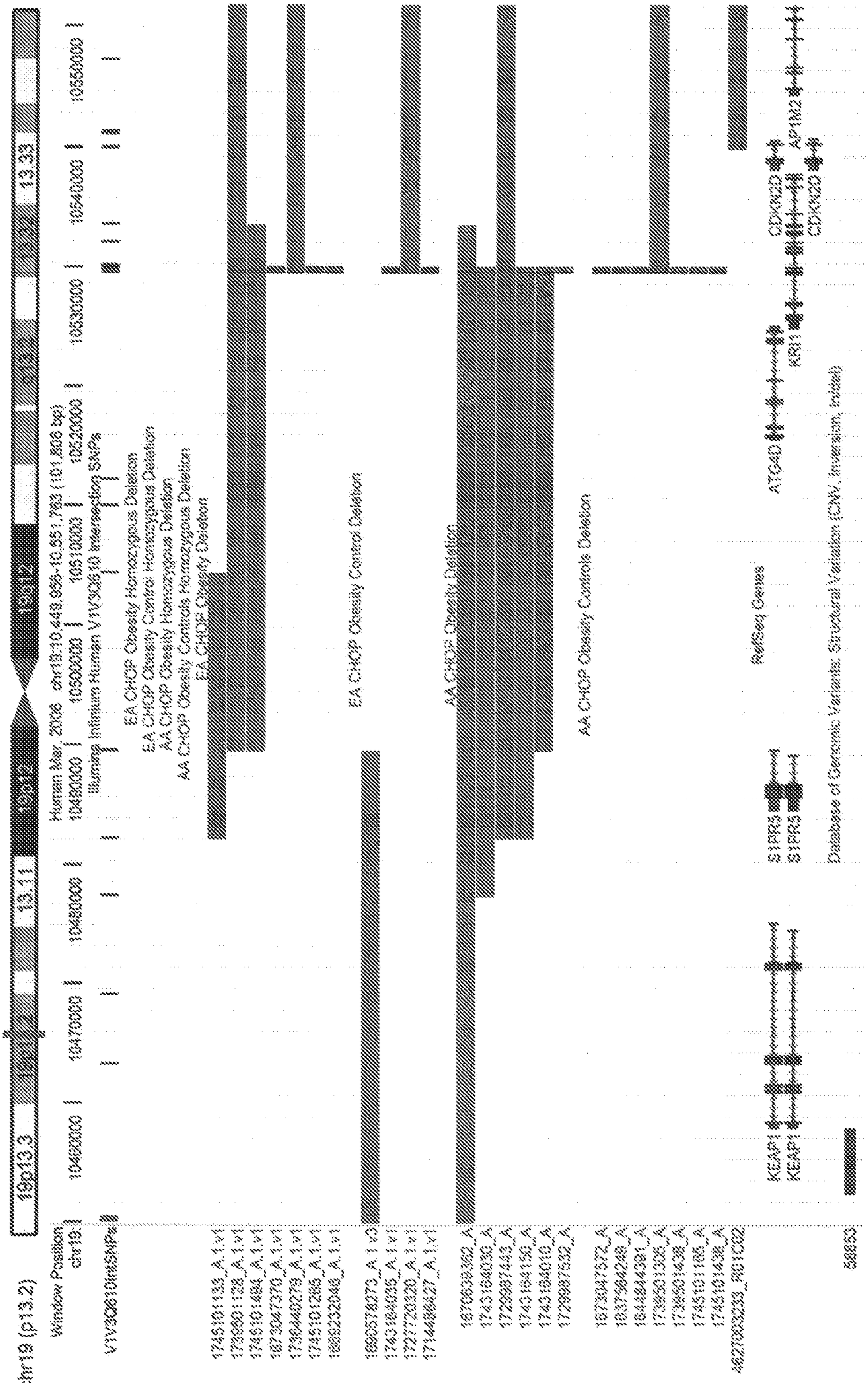
Figure 3A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Human Mar. 2006 (NCBI36/hg18) Assembly
Illumina Infinium HumanVariation 550v3 SNPs
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AA CHOP Obesity Deletion
EA CHOP Obesity Control Deletion
AA CHOP Obesity Control Deletion
EA CHOP Obesity Homozygous Deletion
AA CHOP Obesity Homozygous Deletion
EA CHOP Obesity Controls Homozygous Deletion
AA CHOP Obesity Controls Deletion
RefSeq Genes
ATG4D
KR1
CDKN2D
AP1M2
Database of Genomic Variants: Structural Variation (CNV, Inversion, Indel)
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S1PR5
KEAP1
KEAP1
SSBP3

Figure 3B

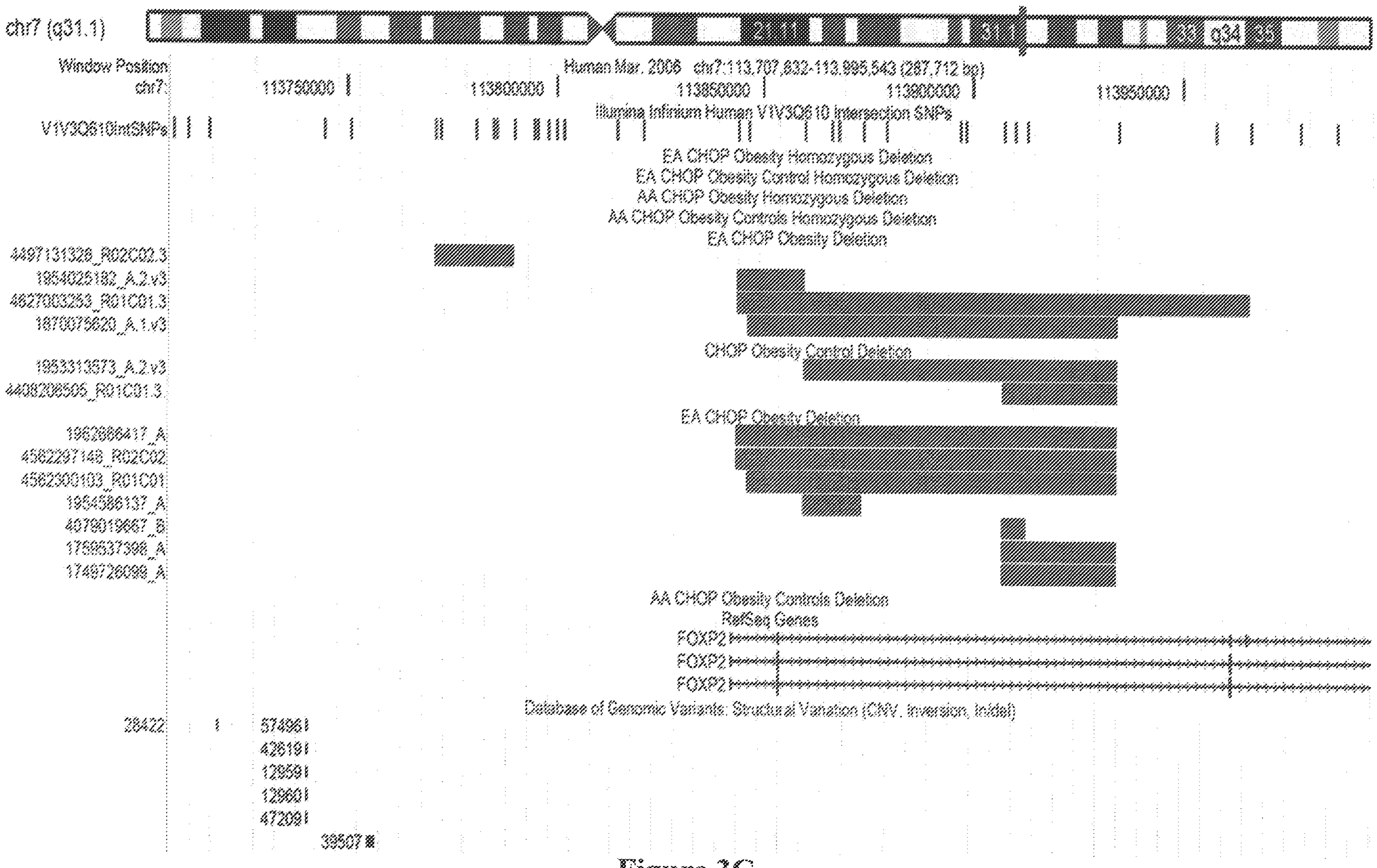

Figure 3C

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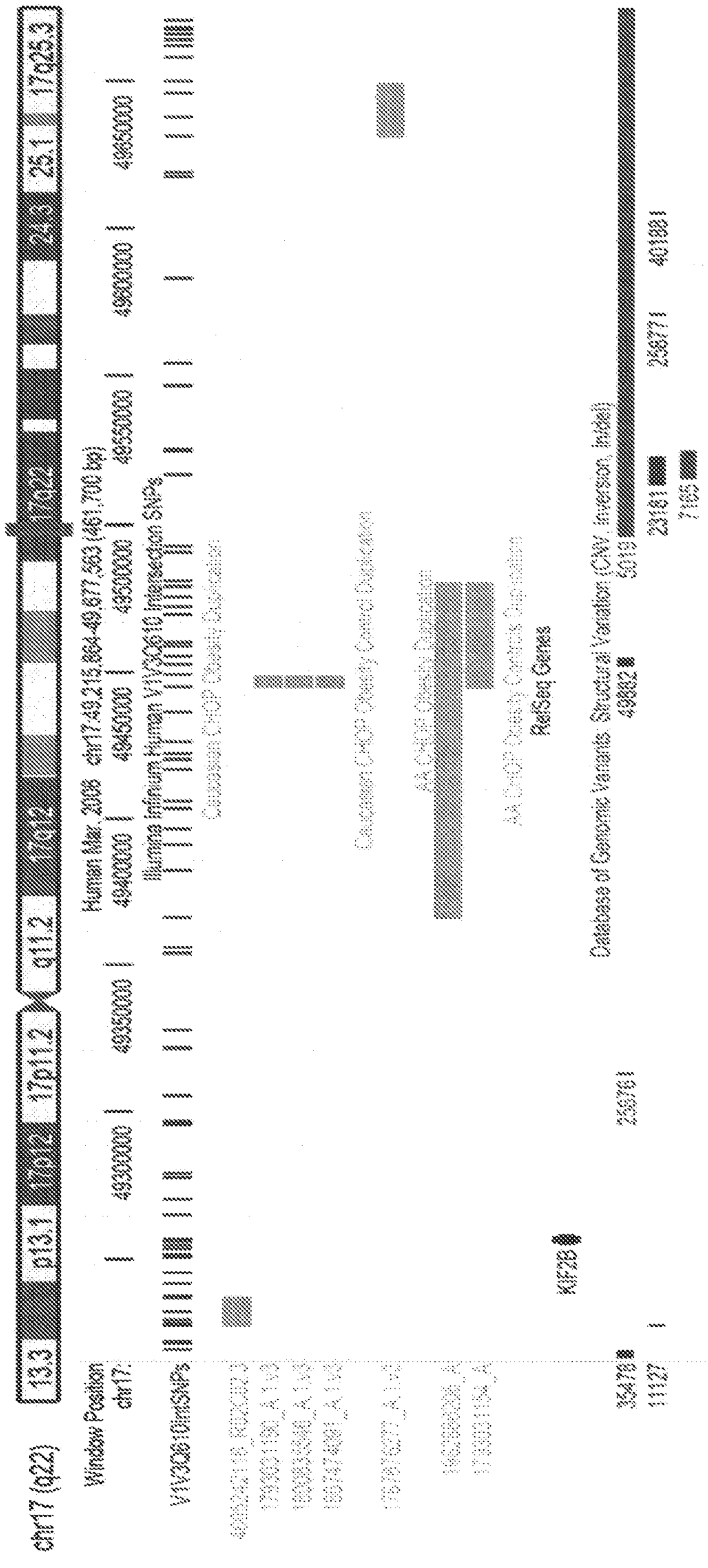
CA 02790502 2012-08-20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Human Mar. 2006 chr17:49,215,864-49,877,583 (661,720 bp)
Cancer Copy Number Variation
Copy Number Gains
Copy Number Losses
AA Copy Number Gains
Refseq Genes

Figure 3D

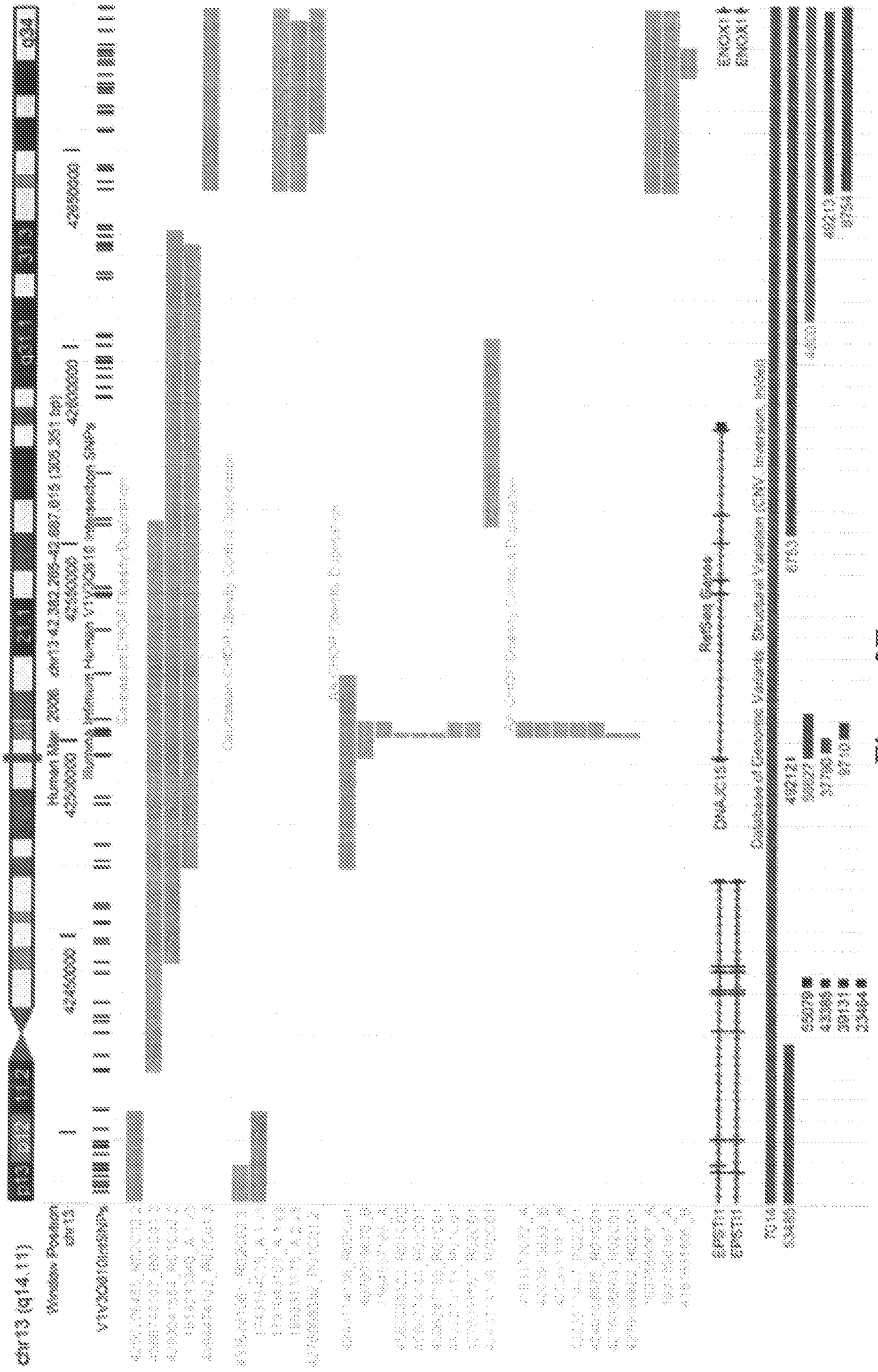

Figure 3E

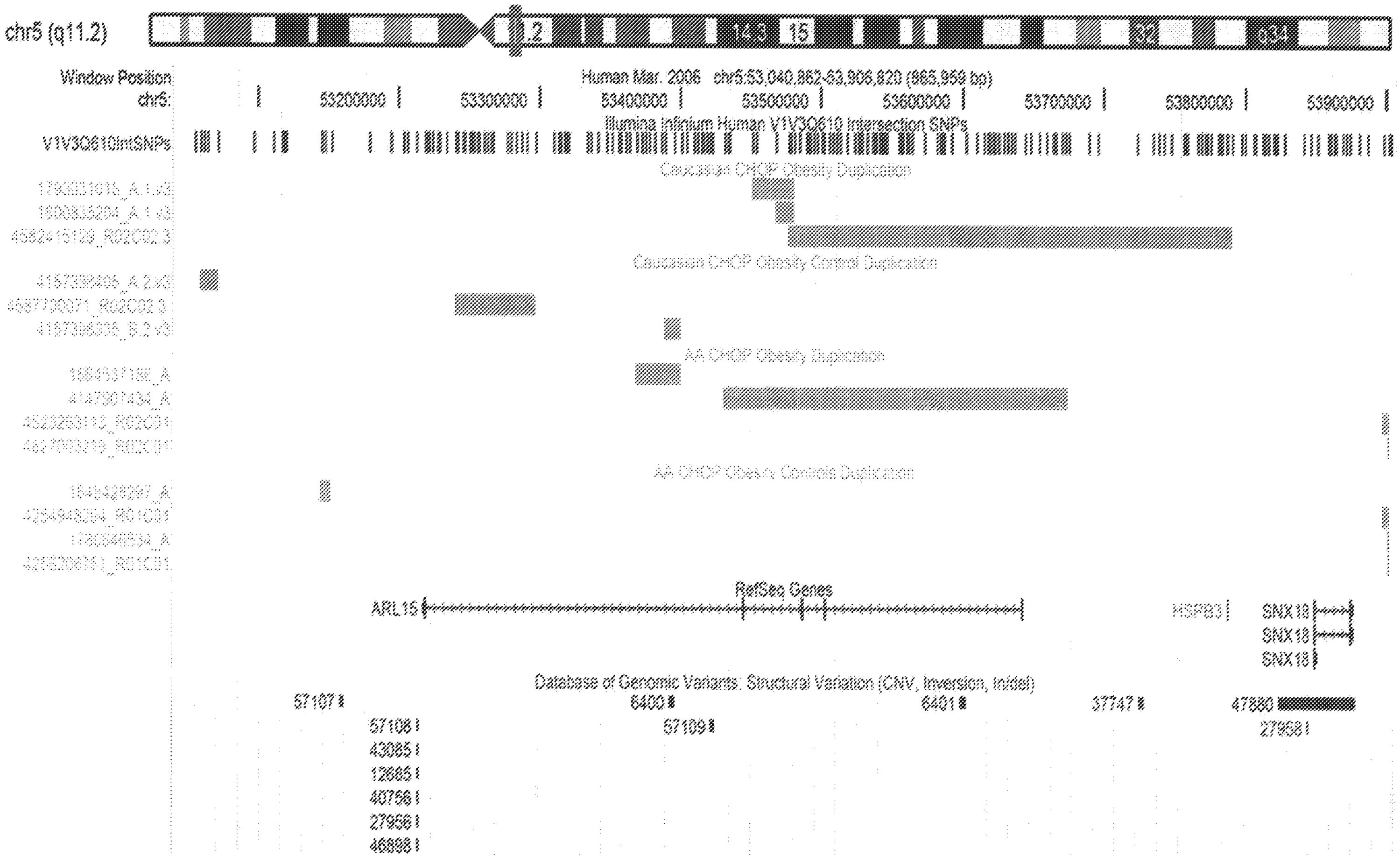

Figure 3F

SUBSTITUTE SHEET (RULE 26)

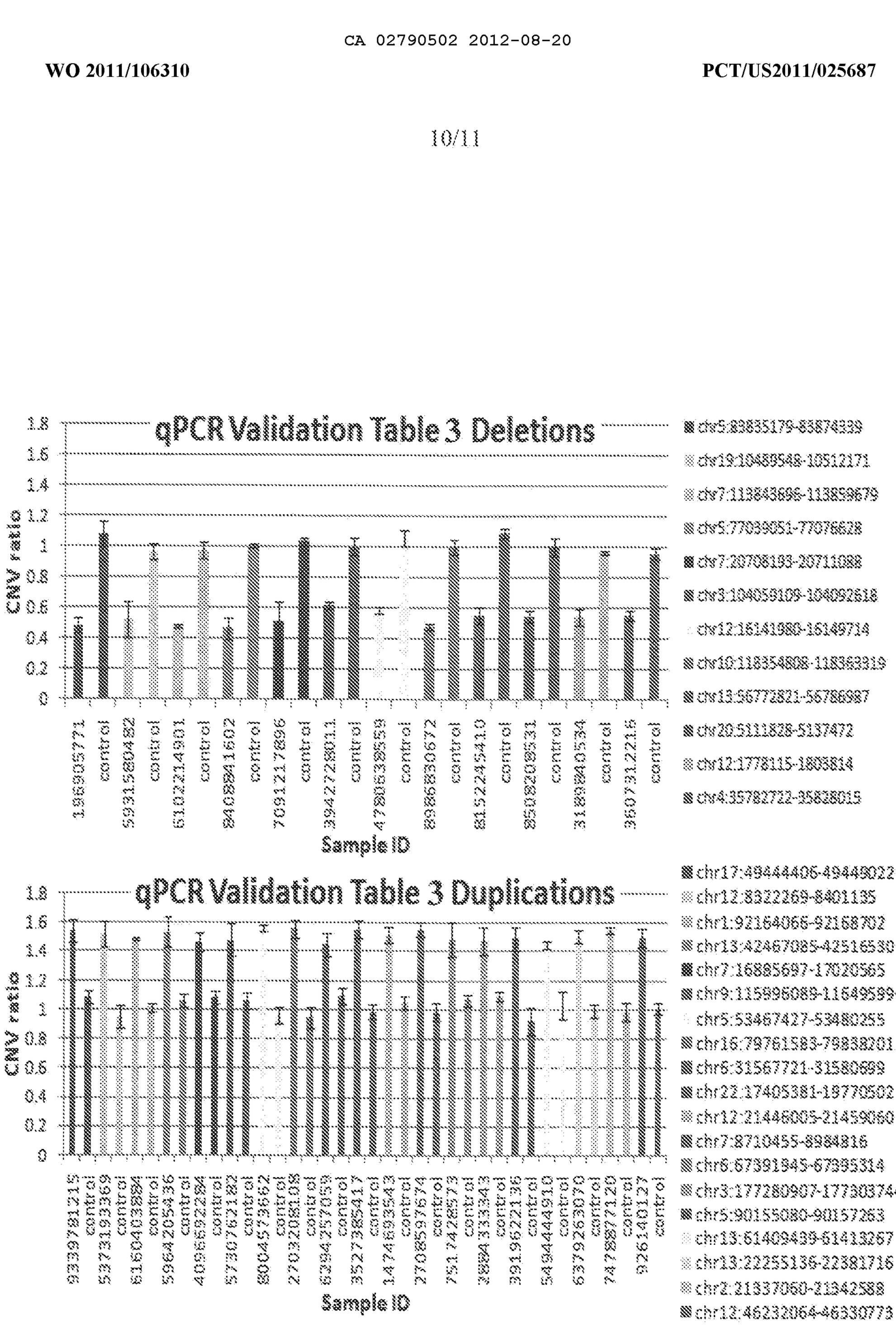
qPCR Validation Table 3 Deletions
CNV ratio
Sample ID
chr5:83835179-83874339
chr19:10489548-10512171
chr7:113843696-113859679
chr5:77039051-77076628
chr7:20706193-20711088
chr3:104059109-104092618
chr12:16141980-16149714
chr10:118354808-118363919
chr13:56772821-56786987
chr20:51111828-51137472
chr12:1778115-1803814
chr4:35782722-35828015
qPCR Validation Table 3 Duplications
CNV ratio
Sample ID
chr17:49444406-49449022
chr12:8322269-8401135
chr1:92164066-92168702
chr13:42467085-42516530
chr7:16885697-17020565
chr9:115995089-116495998
chr5:53467427-53480255
chr16:79761583-79838201
chr6:31567721-31580699
chr22:17405381-19770502
chr12:21446005-21459060
chr7:8710455-8984816
chr6:67391945-67395314
chr3:177280907-177303744
chr5:90155080-90157253
chr13:61409439-61413267
chr13:22255136-22381716
chr2:21337060-21342588
chr12:46232064-46330773

Figure 4

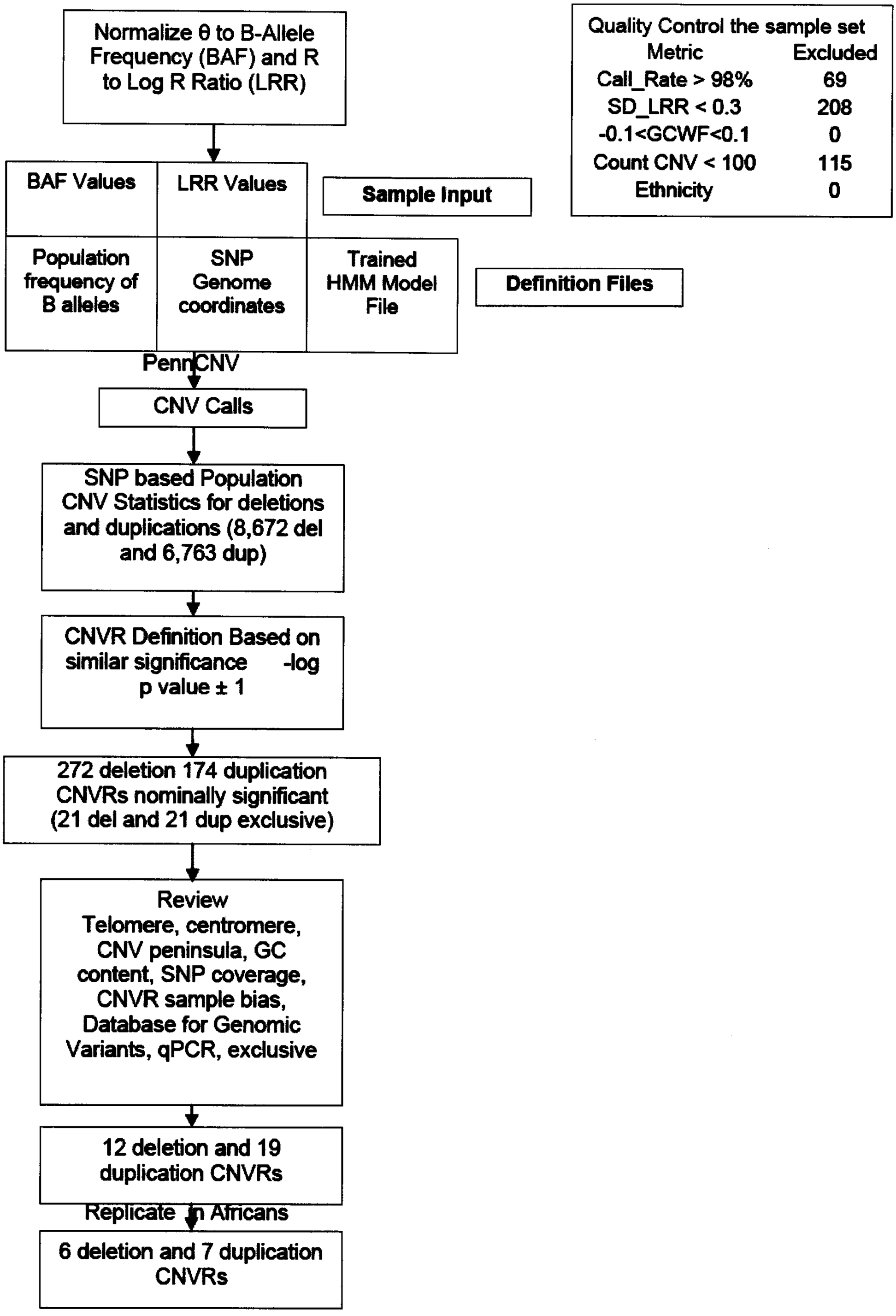
Normalize θ to B-Allele Frequency (BAF) and R to Log R Ratio (LRR)
BAF Values
LRR Values
Sample Input
Population frequency of B alleles
SNP Genome coordinates
Trained HMM Model File
Definition Files
PennCNV
CNV Calls
SNP based Population CNV Statistics for deletions and duplications (8,672 del and 6,763 dup)
CNVR Definition Based on similar significance -log p value ± 1
272 deletion 174 duplication CNVRs nominally significant (21 del and 21 dup exclusive)
Review Telomere, centromere, CNV peninsula, GC content, SNP coverage, CNVR sample bias, Database for Genomic Variants, qPCR, exclusive
12 deletion and 19 duplication CNVRs
Replicate in Africans
6 deletion and 7 duplication CNVRs
Quality Control the sample set
Metric Excluded
Call_Rate > 98% 69
SD_LRR < 0.3 208
-0.1<GCWF<0.1 0
Count CNV < 100 115
Ethnicity 0

Figure 5