Assessment of de novo copy-number variations in Italian patients with schizophrenia: Detection of putative mutations involving regulatory enhancer elements

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Abstract

Objectives: Variants appearing de novo in genes regulating key neurodevelopmental processes and/or in non-coding cis-regulatory elements (CREs), as enhancers, may increase the risk for schizophrenia. However, CREs involvement in schizophrenia needs to be explored more deeply.

Methods: We investigated de novo copy-number variations (CNVs) in the whole-genomic DNA obtained from 46 family trios of schizophrenia probands by using the Enhancer Chip, a customised array CGH able to investigate the whole genome with a 300-kb resolution, specific disease loci at a ten-fold higher resolution, and which was highly enriched in probes in more than 1,250 enhancer elements selected from Vista Enhancer Browser.

Results: In seven patients, we found de novo CNVs, two of which overlapped VISTA enhancer elements. De novo CNVs encompass genes (CNTNAP2, MAGI1, TSPAN7 and MET) involved in brain development, while that involving the enhancer element hs1043, also includes ZIC1, which plays a role in neural development and is responsible of behavioural abnormalities in Zic mutant mice.

Conclusions: These findings provide further evidence for the involvement of de novo CNVs in the pathogenesis of schizophrenia and suggest that CNVs affecting regulatory enhancer elements could contribute to the genetic vulnerability to the disorder.

In the last few years, different strategies have been combined to investigate the genetic contribution to the pathogenesis of schizophrenia. Large genome-wide association studies (GWASs) and high-throughput DNA sequencing technologies have identified common and rare single-nucleotide variants as well as rare copy-number variations (CNVs) as potential risk factors (for review see Chen et al. 2015). everal case-control studies have also reported an increased genomic burden in the case population for duplications and deletions sized 100 kb and larger at recurrent loci (International Schizophrenia Consortium 2008; Walsh et al. 2008). To date, at least 15 distinct genomic regions with changes in their copies have been associated with an increased risk for schizophrenia (Rees et al.2014).

In this scenario, it has been postulated that genetic variants may appear de novo to increase the population risk for the disorder (Xu et al. 2011, 2012; Fromer et al. 2014). The analysis of de novo variants in schizophrenia has included not only CNVs but also single-nucleotide and small InsDel variants (Girard et al.; Xu et al. 2011, 2012; Mulle et al. 2010). Specifically, rare de novo CNVs that disrupt genes involved in

signalling and neurodevelopmental pathways as well as in synaptic plasticity have been associated to schizophrenia (Walsh et al. 2008; Kirov et al. 2012). Moreover, some lines of evidence have suggested that sequence variants or CNVs in non-coding regions of the human genome, as those occurring in cis-regulatory elements (CREs) such as promoters or enhancers, could contribute to a wide spectrum of psychiatric conditions, including schizophrenia (Visel et al. 2004; Walsh et al. 2008; International Schizophrenia Consortium 2008; Cooper et al. 2011; Malhotra et al. 2011; Xiao et al. 2017; Li and Weiberger 2017), very probably by altering transcriptional regulation in the brain (Roussos et al. 2014). A CRE is a non-coding DNA sequence regulating the spatiotemporal expression of target genes localised near or distant to the CRE itself. GWASs have identified many disease- and trait-associated non-coding genetic variants enriched in regulatory DNA marked by deoxyribonuclease I (DNase I) hypersensitive sites (DHSs), which lie within non-coding CREs (Maurano et al. 2012). These disease- and trait- associated variants can perturb transcription factor recognition sequences, the allelic chromatin state and regulatory networks (Won et al.). Furthermore, Roussos et al. (2014) recently demonstrated that, in post-mortem human brain tissue, the risk variant of the L-type calcium channel (CACNA1C) gene, a well-established schizophrenia risk locus (Ripke et al. 2013), interacts physically with a distal enhancer and this is responsible for a reduced gene expression and transcriptional activity. Thus, it seems plausible that CRE mutations may contribute to the genetic risk for schizophrenia.

CREs elements have been conserved across species, and range from some hundred to few thousand base pairs (Bejerano et al. 2004). The detection of CNVs affecting these sequence motifs could be hampered by a size-dependent reduction in sensitivity and specificity of GWAS or whole-genome SNP-CGH array approaches (Zhang et al. 2011). We recently developed the Enhancer Chip (Savarese et al. 2012), a customised array CGH design that is specifically oriented to investigate CNVs in evolutionarily conserved genomic regions corresponding to putative enhancer sequence motifs. The Enhancer Chip not only allows the analysis of all the human genome with a 300-kb resolution, but also permits to investigate specific disease loci, including those related to psychiatric conditions, at a ten-fold higher resolution. In addition, it is also able to test, at high resolution, over 1,250 enhancer elements selected from the Vista Enhancer Browser (Visel et al. 2007). These evolutionarily conserved sequence motifs were experimentally validated for their capacity to drive, in vivo, the expression of a reporter gene in transgenic mouse embryos at day 11.5 (E11.5) in a well-established enhancer assay that links the human conserved fragment to a minimal mouse heat shock promoter fused to a lacZ reporter gene (Kothary et al. 1989; Nobrega et al. 2003; Pennacchio et al. 2006).

In the present study, we used this tool to analyse the whole-genomic DNA of an Italian cohort of schizophrenia patients and their parents. The analysis of these family trios provides further evidence for the involvement of de novo CNVs in the pathogenesis of schizophrenia, and suggests that copy-number changes affecting regulatory enhancer elements of the human genome should be considered as potential contributors to the genetic vulnerability to schizophrenia.

Materials and methods

Subjects

Study participants were part of a wider multicenter study, involving an Italian cohort of schizophrenia patients living in the community and consecutively seen at the outpatient units of 26 Italian university psychiatric clinics and/or mental health departments (Galderisi et al. 2014). According to that study, patients were enrolled on the basis of the following inclusion and exclusion criteria. Inclusion criteria were: (a) a diagnosis of schizophrenia according to DSM-IV, confirmed with the Structured Clinical Interview for DSM-IV – Patient version (SCID-I-P); (b) age range between 18 and 66 years; (c) no family history of schizophrenia; (d) living and available parents. Exclusion criteria were: (a) history of head trauma with loss of consciousness; (b) history of moderate to severe mental retardation or of neurological diseases; (c) history of alcohol and/or

substance abuse in the last 6 months; (d) current pregnancy or lactation; (e) inability to provide an informed consent.

Fifty-seven patients, 42 males and 15 females, aged 19–50 years (mean ± SD 31.2 ± 7.0 years), with their parents were included in the study. Patients were on stable antipsychotic treatment for at least 3 months: nine of them were treated with haloperidol (1–6 mg/day), ten with aripiprazole (15–30 mg/day), eight with clozapine (100–300 mg/day), twelve with olanzapine (5–25 mg/day), seven with paliperidone (6–12 mg/day), ten with risperidone (2–6 mg/day) and one with ziprasidone (160 mg/day). Patients underwent a comprehensive clinical assessment (Galderisi et al. 2014) that included: (a) the Positive and Negative Syndrome Scale (PANSS, Kay et al. 1987 that rates symptom severity; (b) the Brief Negative Symptom Scale (BNSS), which explores five negative symptoms domains (anhedonia, asociality, avolition, blunted affect and alogia) (Mucci et al. 2015); (c) the MATRICS (Measurement and Treatment Research to Improve Cognition in Schizophrenia) Consensus Cognitive Battery (MCCB), which assesses seven distinct cognitive domains (processing speed, attention/vigilance, working memory, verbal learning, visual learning, social cognition, and reasoning and problem solving) (Kern et al. 2008; Nuechterlein et al. 2008).

The study was approved by the Ethics Committee of the University of Campania 'Luigi Vanvitelli' and was carried out in accordance with the Declaration of Helsinki for experiments involving humans. All the participants signed a written informed consent to participate after receiving a comprehensive explanation of the study procedures and goals.

All the participants provided blood samples for genomic DNA extraction according to standard procedures. Concentration and purity of all DNA samples were assessed using spectrophotometer (Nanodrop ND 1000, Thermo Scientific Inc., Rockford, IL, USA) and the Amelogenin PCR test was carried out to exclude any erroneous sex assignation during data/sample collection. In addition, DNA samples were also genotyped with a panel of six autosomal highly heterozygous STR markers to validate kinship relationships in family trios.

According to the above criteria, whole-genomic DNA was obtained from 57 family trios, but only 46 of them passed quality control and were further investigated in this study.

Array CGH analysis

Genomic DNA was analysed using the Enhancer Chip, a customised array CGH developed using the Agilent SurePrint G3 8X60K format (Savarese et al. 2012), and able to investigate the whole genome with a 300-kb resolution, specific disease loci at a ten-fold higher resolution (for more details see Supplemental Table S2 in Savarese et al. 2012), and more than 1,250 highly enriched in probes in VISTA enhancer motifs for their potential pathogenic role.

Ten sex-matched anonymous DNA samples from healthy individuals were pooled at equimolar concentration and used as male or female reference DNA samples in all the array CGH experiments. Labelling and hybridisation were performed according to the manufacturer's specifications (Agilent Oligonucleotide Array-Based CGH for Genomic DNA Analysis protocol, version 6.1; Agilent Technologies, USA) and as previously reported (Piluso et al. 2011; Savarese et al. 2012). Scanned array images were analysed using Feature Extraction software (version 10.5.1.1; Agilent Technologies). Graphical overview and analysis of data were obtained using DNA Analytics as part of Agilent Genomic Workbench software (version 7.0.4.0; Agilent Technologies).

To identify copy-number changes we used the Aberration Detection Method 2 (ADM-2) algorithm (Agilent Technologies) for data that passed QC metrics testing. The ADM-2 algorithm identifies all aberrant intervals

in a given sample with consistently high or low log ratios based on the statistical score. The statistical score represents the deviation of the average of the log ratios from the expected value of zero, in units of standard deviation. The algorithm searches for intervals in which a statistical score based on the average quality weighted log ratio of the sample and reference channels exceeds a user specified threshold. The threshold is normally set to 6. In our experiments, the threshold was set between 5 and 7 and it was empirically chosen for each test, considering the noise reduction in control hybridisations (reference vs reference). The aberration filter was set to select aberrant regions with at least three targets showing the same direction in copy-number change, and to exclude aberrant regions if the average log2 ratio within the region was less than the value of Derivative Log Ratio Spread.

Identified CNVs were compared with the Database of Genomic Variants (http://dgv.tcag.ca/) and with the Decipher database (https://decipher.sanger.ac.uk/) to facilitate interpretation.

Validation experiments

Potentially pathogenic CNVs were further validated by reanalysing DNA samples on Enhancer Chip after dye-swapped labelling or alternatively using Agilent SurePrint G3 CGH ISCA v2 8x60K platform, when regions of interest were adequately covered by probes. In addition, CNV validation was also performed by quantitative amplification of specific genomic regions (primer pairs available in Supplementary Table S1 available online) on CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, USA) using iQ SYBR Green Supermix (Bio-Rad Laboratories), according to the manufacturer's instructions. Each assay was performed in triplicate and results were normalised and analysed using CFX Manager Software Version 1.5 (Bio-Rad Laboratories). For some of the identified CNVs, 5' and 3' breakpoint boundaries were further refined by Real-Time PCR (*Supplementary Table S1).*

Bioinformatic analysis

For genes in CNVs never described previously as potentially associated to psychiatric conditions, GeneMANIA (www.genemania.org) was used to generate protein-protein interaction (PPI) and co-expression networks (Mostafavi et al. 2008; Warde-Farley et al. 2010). This interface is able to create interaction networks, highlighting the most representative pathways, on the basis of data obtained from currently available genomics and proteomics databases. The algorithm contains two import components: a linear regression-based algorithm that is used to calculate a single, composite functional association network from multiple networks that were derived from different proteomic or genomic data sources and a component that predicts gene functions (Warde-Farley et al. 2010). Network nodes scores and gene list-specific weights are reported in *Supplementary Tables S2 and S3.*

Results

Evaluating array CGH results, all mechanisms of inheritance were considered. No rearrangements were found in accordance with an autosomal recessive or X-linked model of inheritance. We then considered de novo CNVs spanning more than 20 kb, in which no copy-number polymorphisms were present. To our knowledge, de novo CNVs here described were not previously reported.

We found two deletions and five duplications in seven unrelated schizophrenia patients (Table 1). In particular, a de novo heterozygous deletion of about 50 kb in size was found at 7q35 in the patient S-23 (Supplementary Figure S1). This deletion encompassed the intron 12 of CNTNAP2, for which haploinsufficiency was previously associated to epilepsy and schizophrenia (Friedman et al. 2008). The closer

CNTNAP2 exon fell in the 3' breakpoint boundary, but it did not show to be deleted by using qPCR (Supplementary Figure S1). Patient S-36 showed a de novo duplication about 350 kb long at 3p14.1, involving MAGI1 *(Supplementary Figure S1)*. This gene and the closely related MAGI2 have already been associated to schizophrenia (Karlsson et al. 2012; Koide et al. 2012). Male patient S-60 had a de novo duplication of about 50 kb in size at Xp11.4, involving the last six exons of TSPAN7, a gene recently associated to X-linked intellectual disability, as well as autism and schizophrenia (Noor et al. 2009; Piton et al. 2011; Utine et al. 2012) *(Supplementary Figure S1)*.

Table 1. CNVs in schizophrenia family trios.

Patient ID	Sex	Chr	Min (max) size (bp)	5' breakpoint boundary	3' breakpoint boundary	Probes	Log ratio	Type	Genes*	ID	Flanking genes	Expression pattern
				Chromosomal position of CNVs (hg19)						VISTA enhancer		
S-23	M	7	50311 (79413)	147521169-147539344	147589655-147600582	4	−0.99	Del	CNTNAP2			
S-36	M	3	352255 (521588)	65063442-65169975	65522230-65585030	5	0.59	Dup	MAGI1			
S-40	M	19	993139 (1010055)	5016130-5023081	6016220-6026185	18	0.60	Dup	KDM4B, PTPRS, ZNRF4, TINCR, SAFB2, SAFB, C19orf70, HSD11B1L, RPL36, LONP1, CATSPERD, PRR22, DUS3L, NRTN, FUT6, FUT3, LOC101928844, FUT5, NDUFA11, VMAC, CAPS, RANBP3			
S-45	M	3	234587 (252828)	147125699-147125777	147360364-147378527	6	0.78	Dup	ZIC1	hs1043	ZIC1, ZIC4	hindbrain, neural tube
S-60	M	X	50470 (1159732)	38484842-38497481	38547951-38644574	7	0.71	Dup	TSPAN7			
S-75	M	7	693624 (730152)	115897566-115913229	116606853-116627718	11	−0.56	Del	LOC102724434, CAV2, CAV1, LINC01510, **MET**, **CAPZA2**, ST7-AS1, **ST7**, ST7-OT4			
S-131	M	X	54892 (149000)	81464550-81464749	81519641-81613550	3	1.01	Dup		hs582	SH3BGRL-POU3F4	forebrain

Del: deletion; Dup: duplication. Log ratio: $\log_2$-based intensity ratio (loss or gain are indicated with values lower or higher than zero). *Genes highlighted in bold could be sensitive to haploinsufficiency, as they have a loss-of-function index (pLI) more than 0.9 (range 0–1) in ExAC Browser (exac.broadinstitute.org).

For CNVs identified in patients S-40 and S-75, 5' and 3' breakpoint boundaries were further defined by qPCR (Table 1 and Supplementary Figure S2). We therefore investigated whether genes included in these de novo putatively damaging copy-number changes were clustered in functional or PPI networks, in some way related to schizophrenia. By performing GeneMANIA in silico analysis (Koide et al. 2012; Utine et al. 2012), we observed that, among 31 genes located in these CNVs, only eight appeared to be interconnected, defining two distinct functional and PPI networks (Figure 1). Interestingly, the heterozygous 700-kb long deletion at 7q31.2 (patient S-75), encompassed three genes (CAV1, CAV2 and MET), which are implicated in neuronal migration and development and whose variants have been associated with schizophrenia (Burdick et al. 2010; Allen et al. 2011). The other genes investigated seem to be in part co-expressed and integrated in interesting functions, such as Wnt signalling pathway, glycosylation, endocytosis and cell migration (Wurst and Prakash, 2014) *(Figure 1)*.
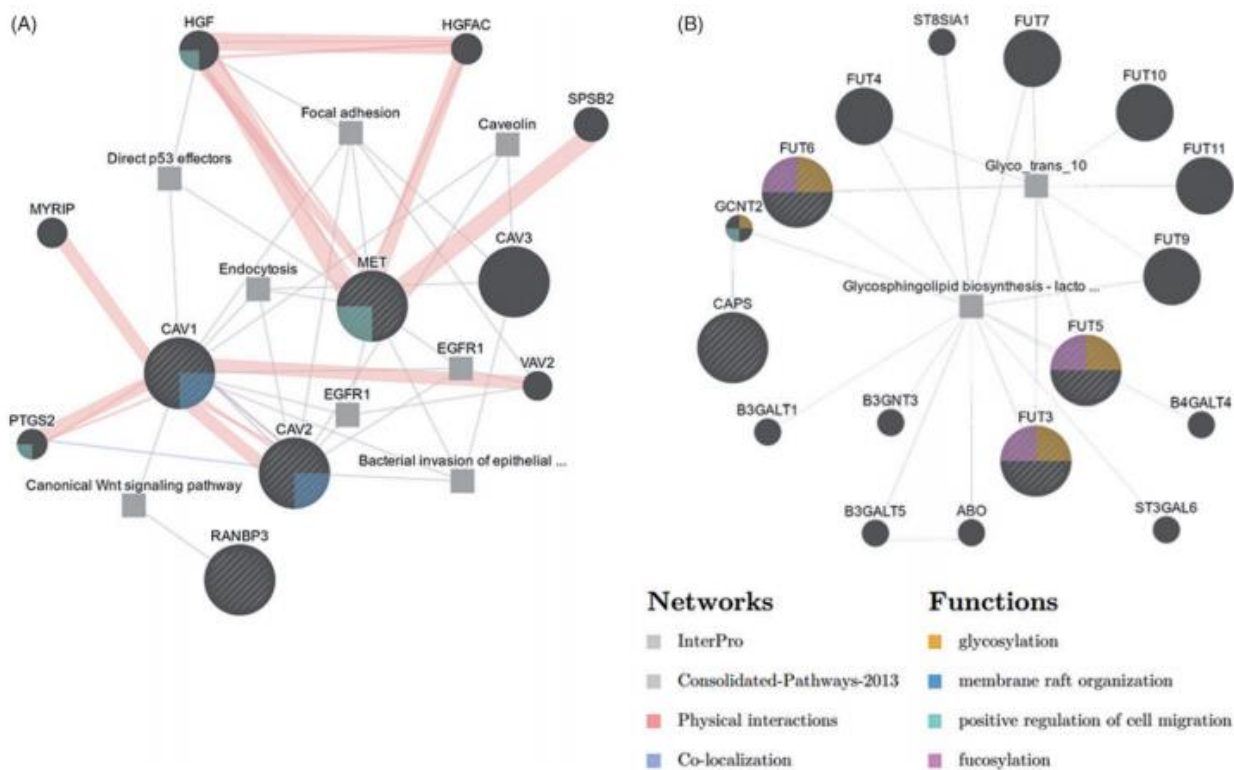
Figure 1. Gene network construction using GeneMania in silico analysis. Only eight out 31 genes from CNVs detected in patients S-40 and S-75, were clustered in two separate functional networks: (A) including *MET*, *CAV1* and *CAV2* (patient S-75); (B) including *FUT3*, *FUT5*, *FUT6* and *CAPS* (patient S-40). Genes of interest are highlighted by grey circles in halftone. Coloured lines indicate network association, according to the legend. Grey squares and lines identify specific pathways and functions among genes.

Enhancer Chip also permitted to identify two de novo CNVs overlapping VISTA enhancer elements. Patient S-45 showed a heterozygous 234-kb long duplication at 3q24, which includes ZIC1 and the regulatory element hs1043, while patient S-131 presented a heterozygous duplication of about 55 kb at Xq21.1, which involves the regulatory element hs582 *(Table 1 and Supplementary Figure S3).*

When patients carrying de novo CNVs were compared to those not carrying de novo CNVs, no significant differences emerged in PANSS, BNSS and MCBB scores.

Discussion

To our knowledge, this is the first genetic study of schizophrenia that includes an analysis of copy-number changes in CRE of the human genome. Our cohort of family trios from schizophrenia patients was investigated using the Enhancer Chip (Savarese et al.), a customised array CGH designed not only to analyse, at high resolution, the whole human genome, but also the potential pathogenic role of over 1,250 VISTA enhancer sequence motifs (Pennacchio et al. 2006). Array CGH analysis identified a total of seven de novo rearrangements in unrelated patients with schizophrenia. In two cases, a VISTA enhancer element was involved.

One patient (S-23) showed a de novo heterozygous deletion affecting only part of the intron 12 of CNTNAP2. This gene is highly conserved and expressed in different areas of the brain, where it encodes for Caspr2 protein, which has been implicated in neuronal migration (Allen et al. 2011). Mutations in CNTNAP2 have already been described in members of Old Order Amish families presenting a neuropathological phenotype

with cortical dysplasia and focal epilepsy, as well as in two other unrelated families with Pitt-Hopkins-like syndrome (Strauss et al. 2006; Wurst and Prakash, 2014). In addition, several studies indicate that CNTNAP2 is an autism susceptibility gene (Alarcón et al. 2008; Zweier et al. 2009). Recently, Friedman et al. (2008) first demonstrated an association between CNTNAP2 and schizophrenia, describing different gene deletions in three unrelated patients with schizophrenia and autism. Like our patient who showed a deletion involving only the intron 12 of CNTNAP2, one of Friedman's patients had a small deletion of the CNTNAP2 intron 3. The pathogenic significance of these intronic rearrangements remains to be clarified. For patient S-23, the deleted region of intron 12 seems to be enriched in putative transcription factor binding sites conserved in the human/mouse/rat alignment, as well as DNaseI hypersensitivity clusters that could perturb CNTNAP2 expression or act at distance, affecting other genes (http://genome.ucsc.edu).

However, functional analysis of this intronic CNTNAP2 deletion has been hampered by the CNTNAP2 expression pattern restricted to the nervous system (Poliak et al. 1999). At present, this rearrangement should only be considered as a variant of uncertain significance.

The de novo microduplication at 3p14.1, identified in patient S-36, encompasses MAGI1. MAGI1 encodes a postsynaptic scaffolding protein interacting with a multitude of molecules that have been implicated in the pathogenesis of bipolar disorders or schizophrenia, such as neuroligins, β-catenin, neuregulin receptor ErbB4 and glutamate receptors (Nishimura et al. 2002; Iida et al. 2004; Buxbaum et al. 2008; Emtage et al. 2009). Deletions and duplications of MAGI1 have recently been described in a study of 277 DNA samples from 48 families with a clinical diagnosis of bipolar disorder or schizophrenia (Karlsson et al. 2012). In this study, the authors followed up an apparently highly penetrant CNV from a family-based analysis, investigating 4,084 unrelated cases and carrying out a pooled analysis on a total of 10,925 patients and 16,747 controls. Statistical data analysis supported the hypothesis that 3p14 could be a region of interest for schizophrenia (Karlsson et al. 2012).

For the de novo duplication at Xp11.4, which involves TSPAN7 and was observed in patient S-60, it is worth noting that this gene is related to synaptic functions and encodes a cell-surface protein of the tetraspanin family, involved in cell growth and motility (Bassani et al. 2012). Rare variants of TSPAN7 have been found to be associated with X-linked intellectual disability in patients with autism spectrum disorders, and have been suggested to have a role also in schizophrenia (Noor et al. 2009; Piton et al. 2011; Utine et al.).

In patient S-75, the de novo heterozygous deletion at 7q31.2 included CAV1, CAV2 and MET, whose variants have been already associated to schizophrenia in different ways. In particular, the CAV1 and CAV2 genes encode caveolins, which are multi-functional scaffolding proteins of the inner surface of caveolae, small invaginations of the plasma membrane, and are involved in essential cellular functions including vesicular trafficking, lipid homeostasis and signal transduction (Ostrom 2002; Cohen et al. 2004). Recently, an insertional mutation disrupting CAV1 has been identified as a rare structural variant associated with schizophrenia (Allen et al. 2011). Moreover, it has been observed that CAV1 knockout mice exhibited increased sensitivity to the psychotomimetic effects of phencyclidine (Allen et al.), a phenomenon also observed in patients with schizophrenia (Lahti et al. 1995), and that they were unresponsive to the normalising effects of the atypical antipsychotics clozapine and olanzapine on phencyclidine-induced disruption of pre-pulse inhibition, a deficit that has been linked to abnormalities of sensorimotor gating observed in patients with schizophrenia (Javitt and Freedman 2015). These findings support a role for this

gene in the pathophysiology of schizophrenia. The MET proto-oncogene, instead, is a cancer-related gene involved in the metastasis of several forms of cancer, the repair and development of peripheral organs (Birchmeier et al. 2003; Tahara et al. 2003), as well as in the cortical and cerebellar development (Ieraci et al. 2002). Interestingly, MET seems to be sensitive to the haploinsufficiency, with a loss-of-function index (pLI) equal to 1 in the ExAC Browser (exac.broadinstitute.org). Recently, Burdick et al. (2010) examined 21 SNPs of this gene in a cohort of schizophrenic patients and found that individuals carrying at least one copy of the most common haplotype were significantly less likely to develop schizophrenia as compared with those carrying no copies. Furthermore, MET haplotype carriers had significantly better cognitive performance than non-carriers, although the results did not reach statistical significance.

For patient S-40, the de novo heterozygous duplication at 19p13.13 encompassed 22 genes (Table 1), some of which clustered in glycobiology pathway, including sphingolipid metabolism and glycosyltransferases (Figure 1(B)). Disruption of sphingolipid metabolism could result in widespread effects, related to diverse pathological deficits already described in schizophrenia, including myelination and oligodendrocyte function (Narayan et al. 2009). Three fucosyltransferase genes (FUT3, FUT5 and FUT6) map in the duplicated region (Costache et al. 1997), and plasma α1,3-fucosyltransferase deficiency was detected in Japanese patients with schizophrenia (Yazawa et al. 1999; Tanaka et al. 2001). Post-translational protein modifications can have a role in the pathophysiology of schizophrenia. α1,6-fucosyltransferase (Fut8–/–)-deficient mice exhibited multiple behavioural abnormalities consistent with a schizophrenia-like phenotype (Fukuda et al. 2011), while dysregulated fucosyltransferase expression was observed in the superior temporal gyrus of elderly patients with schizophrenia compared with healthy controls (Mueller et al. 2017).

In schizophrenia, most of the reported rare de novo mutations seem to be enriched in genes related to chromatin remodelling, synaptic organisation and plasticity, as well as to be associated to autism or intellectual disability (Fromer et al. 2014; McCarthy et al. 2014). Some of genes encompassed in de novo CNVs reported here (CNTNAP2, MAGI1, TSPAN7, CAV1 and CAV2) seem to have similar characteristics (Horresh et al. 2008; Head et al. 2010; Zheng et al. 2011; Bassani and Passafaro 2012).

The most innovative finding of our study is that the Enhancer Chip allowed us to detect rearrangements covering enhancer elements. The 234-kb long duplication at 3q24, which we detected in patient S-45, includes the enhancer element hs1043 and its flanking gene ZIC1. Interestingly, ZIC1 is a member of a zinc-finger protein family that plays a crucial role in neural development (Aruga 2004). In addition, behavioural abnormalities have been observed in Zic mutant mice, resembling human neurological and psychiatric conditions (Ogura et al. 2001). In addition, ZIC1 and other 11 genes were found differentially expressed in the mouse brain after treatment with valproate at human therapeutic concentrations (Chetcuti et al. 2006). Therefore, ZIC1 dysregulation could be involved in the therapeutic action of this drug, which acts as mood stabiliser in people with bipolar or schizoaffective disorder. Interestingly, the regulatory enhancer element hs1043 was able to drive reporter gene expression in hindbrain (rhombencephalon) and neural tube of transgenic mouse embryos at stage E11.5 (experimental data for hs1043 are freely available at https://enhancer.lbl.gov/).

The 55-kb long duplication found at Xq21.1 (patient S-131) seems deserted of genes, only including the regulatory element hs582, representing the unique evolutionarily conserved sequence motif in this interval. To the present, we can only speculate on pathogenic significance of this arrangement. Nevertheless, hs582

was able to drive reporter gene expression in forebrain of transgenic mouse embryos at stage E11.5 (experimental data for hs582 are freely available at https://enhancer.lbl.gov/).

Interestingly, the increased density of probes in enhancer elements also permitted to better characterise the 5' breakpoint boundary that, for both rearrangements, falls into the enhancer element, possibly perturbing its function *(Supplementary Figure S3).*

At present, it is not simple to explain the possible role of enhancer elements in the pathophysiology of human brain disorders, because little is known about their gene targets. However, previous studies have established the important regulatory role of the enhancers on transcriptome organisation during neurodevelopment and adulthood (Wenger et al. 2013; Andersson et al. 2014). Moreover, Roussos et al. (2014) suggested that risk-allele-specific changes in enhancer elements could affect the spatiotemporal organisation of the transcriptome that, when combined with other derangements, could lead to schizophrenia.

Very recently, a high-resolution three-dimensional analysis of chromatin contacts during human corticogenesis permitted large-scale annotation of previously uncharacterised regulatory elements, identifying hundreds of genes that physically interact with enhancers and that are associated with human cognitive function (Won et al. 2016). Some non-coding variants identified in GWAS schizophrenia studies (Schizophrenia Working Group of the Psychiatric Genomics Consortium 2014) were enriched in some of these selected chromatin regions, supporting their role in the dysregulation of specific genes related to human brain development and cognition. Enhancer elements that we found in CNVs, seem to be not enriched in the above reported experimental model.

The lack of precise data on copy-number changes affecting enhancer elements is due to the absence of appropriate methods for their identification. Our study represents a first attempt in using a dedicated tool, the Enhancer Chip, to investigate the role of regulatory elements in the pathogenesis of schizophrenia. However, the relevance of mutations in enhancer elements for the pathophysiology of schizophrenia needs to be established in larger studies including also control groups.

As for data presented here, rare CNVs can only explain a very limited number of schizophrenia cases and do not seem to confer peculiar phenotypic aspects, since patients carrying these de novo CNVs did not differ from those not carrying them on symptom severity, psychopathological negative dimensions and cognitive functions. Whole-exome sequencing (WES) studies are now providing the most extensive datasets of de novo mutations in schizophrenia (Xu et al. 2011; Fromer et al. 2014; Iossifov et al. 2014; McCarthy et al. 2014; Kranz et al. 2015; Rees et al. 2015), extending the number of genes and biological pathways likely involved in this and related disorders. WES is the future perspective to complete the genetic investigation of this cohort of schizophrenia patients.

GWA, CNVs and NGS studies have permitted to identify recurrent and rare genetic variants that contribute to schizophrenia and, in some cases, overlap other psychiatric disorders and neurodevelopmental syndromes (Modai and Shomron, 2016). Genetic analysis strongly suggests that schizophrenia is biologically heterogeneous, but it also provides the way to better classify patients in unique subgroups, useful to test

new drugs or the efficacy of those now in use (Giegling et al. 2017; Matsumoto et al. 2017). Relatively homogeneous groups, defined using WES and array CGH, could constitute an initial step for building more accurate risk-prediction models in the attempt to develop personalised medicine approaches (Fraguas et al. 2017).

In conclusion, in a cohort of Italian schizophrenia patients, we found a total of seven de novo CNVs consisting of two deletions and five duplications. Two of these rearrangements that involved MAGI1 and TSPAN7 genes have been already described in association to schizophrenia and/or bipolar disorder as well as to X-linked intellectual disability with autistic spectrum. For the others, some of the involved genes are known to play critical roles in brain development, neuronal migration and synaptogenesis, which are processes supposed to be involved in the risk of schizophrenia. Only in two cases did we observe rearrangements of enhancer modulatory elements whose function in the human brain still await to be identified. These findings provide a significant contribution to the identification of rare CNVs associated to schizophrenia and pave the way to further investigations of the role of enhancer elements in the pathophysiology of this disorder.

Appendix

Members of the Italian Network for Research on Psychoses who participated in this study include: Valentina Montefusco, Giuseppe Plescia, Giuseppe Piegari, Eleonora Merlotti, Monica Cimmino, (University of Campania 'Luigi Vanvitelli', Naples); Marco Savarese (Telethon Institute of Genetics and Medicine, Naples); Enrico D'Ambrosio (University of Bari); Anna Rita Atti (University of Bologna); Paolo Valsecchi (University of Brescia); Maria Salvina Signorelli (University of Catania); Tiziano Acciavatti (University of Chieti); Mario Altamura (University of Foggia); Costanza Arzani (University of Genoa); Gaetano Callista, Francesca Pacitti (University of L'Aquila); Lucio Oldani (University of Milan); Carla Gramaglia (University of Eastern Piedmont, Novara); Elena Tenconi (University of Padua); Annalisa Camerlengo (University of Parma); Liliana Dell'Osso (University of Pisa); Roberto Brugnoli, Fabio Di Fabio (Sapienza University of Rome); Cinzia Niolu (Tor Vergata University of Rome); Giulio Corrivetti (Department of Mental Health, Salerno); Simone Bolognesi (University of Siena); Cristina Montemagni (University of Turin).

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Supplementary Information

Supplementary Table S1 – *Primer pairs for CNV validation by qPCR and genomic coordinate of each amplicon.*

Sample ID	Forward primer	Reverse primer	Genomic position (Hg19)
S-23	AATGACCGACCCAGTTGAATGTG	GCGCGTGTCTTCATACCTCAAAG	chr7:147520393-147520615
	AACCTCTTCCTCCTGCCTGTTC	GTCCATTTCGCTTGCACATCCA	chr7:147546718-147546948
	CACTACAGCCATTACAACCGGC	AAGCAGATGTGAGAGGAGCTGG	chr7:147568653-147568939
	TCGTGAAACCAAGCATTTACCACT	GGAGAGGCATGGTCAAGGTGAT	chr7:147583965-147584183
	AAGTGGGTGTAAGTGTGGCAGT	**CTCACCATTGCTTGTAGTCCGC**	**chr7:147600582-147600818**
	TCCCTGTCCTGACTCACATTCTT	GGCTCTGTTCCCACTTCTCCTAG	chr7:147602219-147602390
S-36	TTTCAACAAGAGGGCCCGAAAAC	TGCCCAACCTAAGATCTTGAAGG	chr3:65062072-65062303
	AGGCTGTGATTTGCATTTGGGG	TTTCTTTACTGGTTGCCTGGGG	chr3:65349525-65349727
	ATAGCCACACATGCATTGCCTG	CTGGAAAGGATGGCAGCAATGA	chr3:65361602-65361853
	GGGTAAGACAGAGGCCAGGATG	TTCTTGCCCCTCTCACTTGCTT	chr3:65376787-65377063
	ACTTCTCCTTCCCTTCCCCTGA	TGTCTGAGTTGTGTGGGTGGAG	chr3:66038511-66038786
S-40	AGTTCTGTTCCGCATCCATCCT	GGCCTTAAGACGCAGACACCTA	chr19:5015744-5015982
	GGAGATGACCCTGGATGATCTGG	**CGGGATTCACTTGTCTCTGGTCT**	**chr19:5023081-5023343**
	TCTGGGGAGAATTAGCCTGCAC	CACTCACCAGGACTTAGGCTCC	chr19:5047402-5047687
	CCTCCTCCGGCTTCTAGGTATC	GCAACCCTTCCTCAAATCCAGC	chr19:5339046-5339332
	CCCCAGGCCCTATCTCAATTCA	GCCTGGCATCAACTAAGCCATC	chr19:5625458-5625706
	GCTCTCACCTCACTCCTGACAG	CCCTCCTGTCAAGCGAGAAAGA	chr19:5941478-5941690
	TGTAAATGGGAGTGGGAGAGCAG	**TCCAGACATCCTTTCCTCCCAAC**	**chr19:5958256-5958507**
	CTTCCTTAAGCCCTCCCACAAG	**CTCTCAGTCGGCGGGAAAAGGAG**	**chr19:5978098-5978348**
	GTCCTCACCTCTCCCATCACAG	**GCACGTCTTAGGGATCACTCAGCC**	**chr19:5997063-5997362**
	TAACTGCAAGCACTCCAGAGGTG	**TTACAACCACTACCTTCGGCACT**	**chr19:6015967-6016220**
	TGGAAGAAAGAGTTGGGCAGCT	GTTCATCTCCTCCCGCTTCAGT	chr19:6026881-6027138
S-45	GGCCATTCATCAACCCCAACTTT	GGTTCGGGACTTGGCTTTCTTTA	chr3:147123249-147123462
	GGAACAATAGCGCGTTGGGAAAT	GAAAGCGCTTAAGTTCCCAGGTT	chr3:147126109-147126340
	TTCCAAACCGTGTTCAGGGATTC	ACATGTGCTGAGAGACTGTGACA	chr3:147292376-147292581
	TGCAAGCAGAAAGTTGGAGGAGA	TCCGCTTACTATGGTCCCCTACT	chr3:147345419-147345618
	GCTGGGAATCTTGGGGTTTAAGC	TGCCTCTGAAACAATGCTTCCTG	chr3:147379610-147379830
S-60	GTCACACTTGGGATGCATGGATT	AACATTTTGATGGCTGGTGGGG	chrX:38483284-38483527
	TGGGTTTTGAAATGGAGCAAGGA	TGAGTTTTGAAAGCGGGTGAGG	chrX:38499323-38499582
	GCTTCACACTCTCCTCTCCCTC	CACCAGTTTCAGCATCCATGGG	chrX:38525347-38525566
	CCAGTCTCTGCTCCACCTTTCA	ACCACAGTGAGGAGCTAACAGG	chrX:38547344-38547600
	GCCAGGAGTGAAATACAGAACGG	ACAGCTGAACACCATCTTGACTG	chrX:38645071-38645285
S-75	ATCGCATTGTCAGGCTGCAAATT	GGAGCTGATGTAAAGGCCACTCT	chr7:115885269-115885470
	TATGTCGTAACTGCAAGTGTGGC	**ACCAATGCTTGATTCTGGACAGG**	**chr7:115889079-115889328**
	ACAGCTTGATCTCTCACCCTCTT	**TATGGCTCGATACTTCTGGGTGC**	**chr7:115897303-115897566**
	AGTGTTGAGCTGTGGTTTAGGGT	TGTGTCATGTGGTGGTTTGTGG	chr7:116003210-116003453
	GAGTGCAGGGTGGAGGTGTTAT	GAAGTGATGGTGGCAGTTTGGG	chr7:116165516-116165733
	GTTCAAACACCCACAAGCCCTG	ACCTTCTTCCCAGTGATAACCAGT	chr7:116379889-116380140
	AAGAGAAGGTAAGAGTCGCGGG	TTTTCGGAGTACACACCCTACAAG	chr7:116502697-116502947
	TCTCTTGTTCACGTCTGGCATCA	**GAGGTCAGGAGTTCGAGATCAGC**	**chr7:116627718-116628014**
	GCCCTGCTATTGTCTTACTGCTAC	**GGTCATGAAGCCAGACAATGCAG**	**chr7:116660261-116660543**
	TCCGTCTCCTGAGTTCAAGCAAT	**TGGGTCCAGCAAAGAGAAGTTGT**	**chr7:116695340-116695618**
	CATTGTTTCCCTGCCATCCACTC	CCCCTGCAATTTACGAAGCAACT	chr7:116715559-116715779
S-131	GGGCTGATAGTGAAGATGACCACT	TTTGACACTGTCCAGGCTCTCT	chrX:81464200-81464470
	GTGACATGGAGGGGTACACTGAA	TGTCCCAACCCTGAATGCCAATC	chrX:81466425-81466624
	GGGCAGTTAGTGTGATTTTGGGG	CCAGAAGCAAAGCCACATGAGAC	chrX:81485224-81485427
	AAGGCAGCAGGAAGGAGAAGTT	TGTGATATGGTTTGGCTGGTACC	chrX:81505126-81505355
	TCACCCCACATAGTCTCTGTTTTGT	TCACAGTTTCTTCCACCTGTCGA	chrX:81614551-81614777

Note: Primers in bold were used to further define 5' and 3' breakpoint boundaries.

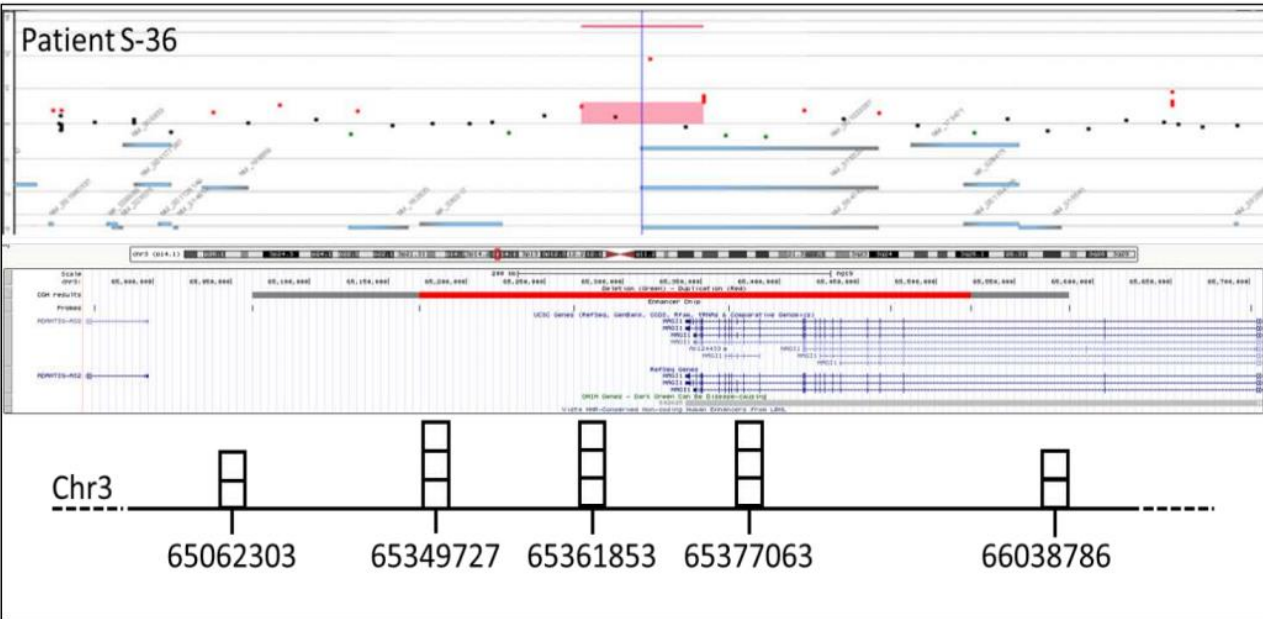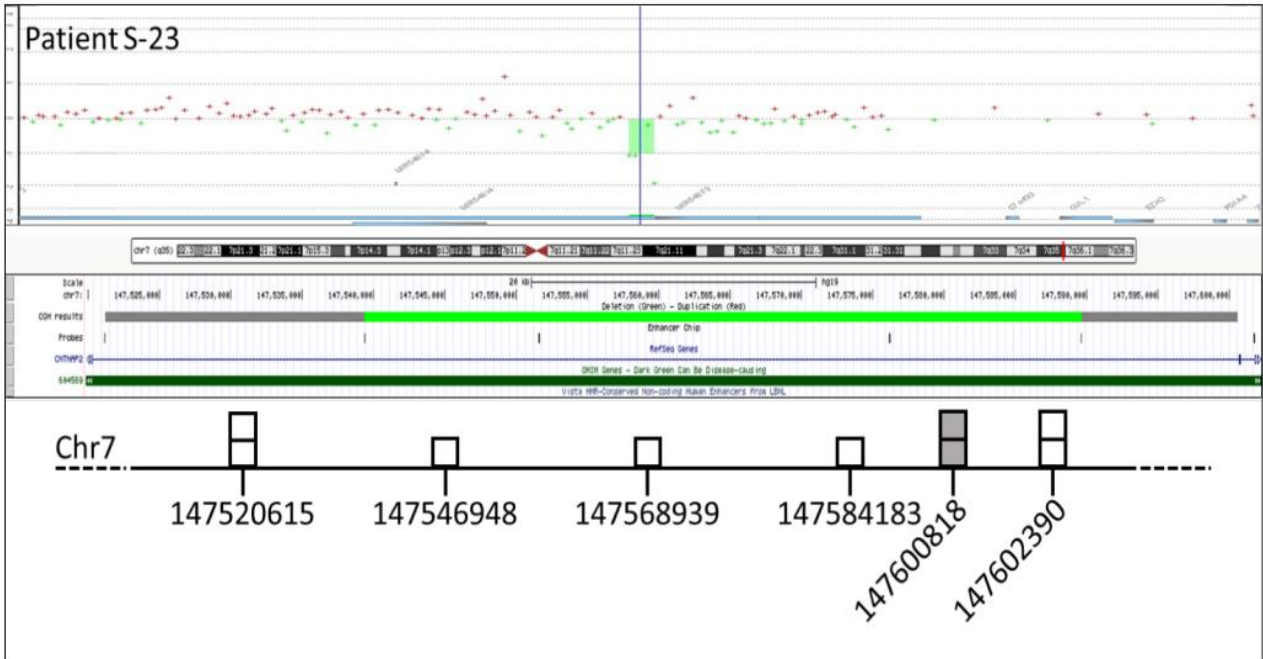**Supplementary Table S2** – *Network scores generated by GeneMANIA.*

Entrez Gene ID	Gene name	Node type	Score	log score	Annotations	Annotation name
828	CAPS	query	0.991752474	-0.008281725		
8498	RANBP3	query	0.914639024	-0.089225801		
858	CAV2	query	0.811386054	-0.209011316	GO:0001937|GO:0045121|GO:0031579	negative regulation of endothelial cell proliferation|membrane raft|membrane raft organization
4233	MET	query	0.791608452	-0.233688388	GO:1900408|GO:1900407|GO:0051272|GO:0040017|GO:0016323|GO:2000147|GO:0030335	negative regulation of cellular response to oxidative stress|regulation of cellular response to oxidative stress|positive regulation of cellular component movement|positive regulation of locomotion|basolateral plasma membrane|positive regulation of cell motility|positive regulation of cell migration
2527	FUT5	query	0.765274717	-0.267520403		
2525	FUT3	query	0.765274717	-0.267520403		
2528	FUT6	query	0.762588435	-0.271036797		
857	CAV1	query	0.691095945	-0.369476615	GO:0001937|GO:0045121|GO:0016323|GO:0031579	negative regulation of endothelial cell proliferation|membrane raft|basolateral plasma membrane|membrane raft organization
859	CAV3	result	0.151157368	-1.889433814	GO:0045121|GO:0031579	membrane raft|membrane raft organization
170384	FUT11	result	0.11050156	-2.20272564		
84750	FUT10	result	0.110357446	-2.204030674		
2526	FUT4	result	0.110002163	-2.207255251		
2529	FUT7	result	0.109873619	-2.208424487		
10690	FUT9	result	0.109304793	-2.213615031		
84727	SPSB2	result	0.034973952	-3.353151731		
3082	HGF	result	0.021486793	-3.840316829	GO:1900408|GO:1900407|GO:0051272|GO:0040017|GO:2000147|GO:0030335	negative regulation of cellular response to oxidative stress|regulation of cellular response to oxidative stress|positive regulation of cellular component movement|positive regulation of locomotion|positive regulation of cell motility|positive regulation of cell migration
5743	PTGS2	result	0.015363916	-4.175733659	GO:0051272|GO:0040017|GO:2000147|GO:0030335	positive regulation of cellular component movement|positive regulation of locomotion|positive regulation of cell motility|positive regulation of cell migration
25924	MYRIP	result	0.015350237	-4.176624393		
7410	VAV2	result	0.014096647	-4.26181831		
3083	HGFAC	result	0.012660977	-4.369230654		
10317	B3GALT5	result	0.007647767	-4.873341589		
2651	GCNT2	result	0.007643653	-4.873879694	GO:0051272|GO:0040017|GO:2000147|GO:0030335	positive regulation of cellular component movement|positive regulation of locomotion|positive regulation of cell motility|positive regulation of cell migration
10402	ST3GAL6	result	0.007638172	-4.874596967		
28	ABO	result	0.007630649	-4.875582377		
	Glyco_tran_10_N	attribute	0.198391167			
	Glyco_trans_10	attribute	0.198391167			
	Caveolin	attribute	0.138701642			
	Caveolin_CS	attribute	0.138701642			
	Glycosphingolipid biosynthesis - lacto ...	attribute	0.071646979			
	Canonical Wnt signaling pathway	attribute	0.05052492			
	Bacterial invasion of epithelial ...	attribute	0.034294837			
	Endocytosis	attribute	0.011596725			
	Focal adhesion	attribute	0.011005448			
	EGFR1	attribute	0.005319758			

Supplementary Table S3 – *Weighted scores generated by GeneMANIA.*

Gene 1	Gene 2	Weight	Type	Source
SPSB2	MET	4.83116331	Physical Interactions	IREF-DIP
HGFAC	HGF	3.386444515	Physical Interactions	IREF-DIP
HGF	MET	2.76758343	Physical Interactions	IREF-DIP
CAV1	CAV2	2.299959452	Physical Interactions	IREF-DIP
MYRIP	CAV1	2.299959452	Physical Interactions	IREF-DIP
PTGS2	CAV1	2.299959452	Physical Interactions	IREF-DIP
VAV2	CAV1	2.299959452	Physical Interactions	IREF-DIP
ERBB3	MET	2.103094322	Physical Interactions	IREF-DIP
HGFAC	MET	1.747208473	Physical Interactions	IREF-DIP
PRKCDBP	CAV3	1.228596699	Physical Interactions	IREF-INTACT
SH2D3C	CAV1	1.00436608	Physical Interactions	IREF-DIP
HRNR	RANBP3	0.876615571	Physical Interactions	IREF-INTACT
PRKCDBP	CAV1	0.689932813	Physical Interactions	IREF-DIP
CAV1	CAV2	0.373495482	Physical Interactions	IREF-INTACT
SH2D3C	MET	0.231061027	Physical Interactions	IREF-INTACT
PRKCDBP	CAV1	0.215641058	Physical Interactions	IREF-INTACT
HGF	MET	0.163430297	Physical Interactions	IREF-INTACT
VAV2	ERBB3	0.034900197	Physical Interactions	IREF-INTACT
CAV1	CAV2	0.024445356	Co-localization	Schadt-Shoemaker-2004
VAV2	MET	0.022967509	Physical Interactions	IREF-INTACT
ABO	B3GALT5	0.019472395	Co-localization	Johnson-Shoemaker-2003
PTGS2	CAV2	0.017576341	Co-localization	Schadt-Shoemaker-2004
HGF	MET	0.012446812	Physical Interactions	IREF-BIOGRID
SPSB2	MET	0.009075939	Physical Interactions	IREF-BIOGRID
GCNT2	CAPS	0.008978404	Co-localization	Johnson-Shoemaker-2003
CAV1	CAV2	0.007579912	Co-localization	Johnson-Shoemaker-2003
SH2D3C	CAV1	0.007178768	Co-localization	Johnson-Shoemaker-2003
CAV1	CAV2	0.00305537	Physical Interactions	IREF-BIOGRID
PTGS2	CAV1	0.002239148	Physical Interactions	IREF-BIOGRID
VAV2	ERBB3	0.001391299	Physical Interactions	IREF-BIOGRID

Supplementary Figure S1 – *Enhancer Chip profile and validation by real-time PCR of CNVs detected in patients S-23, S-36 and S-60*
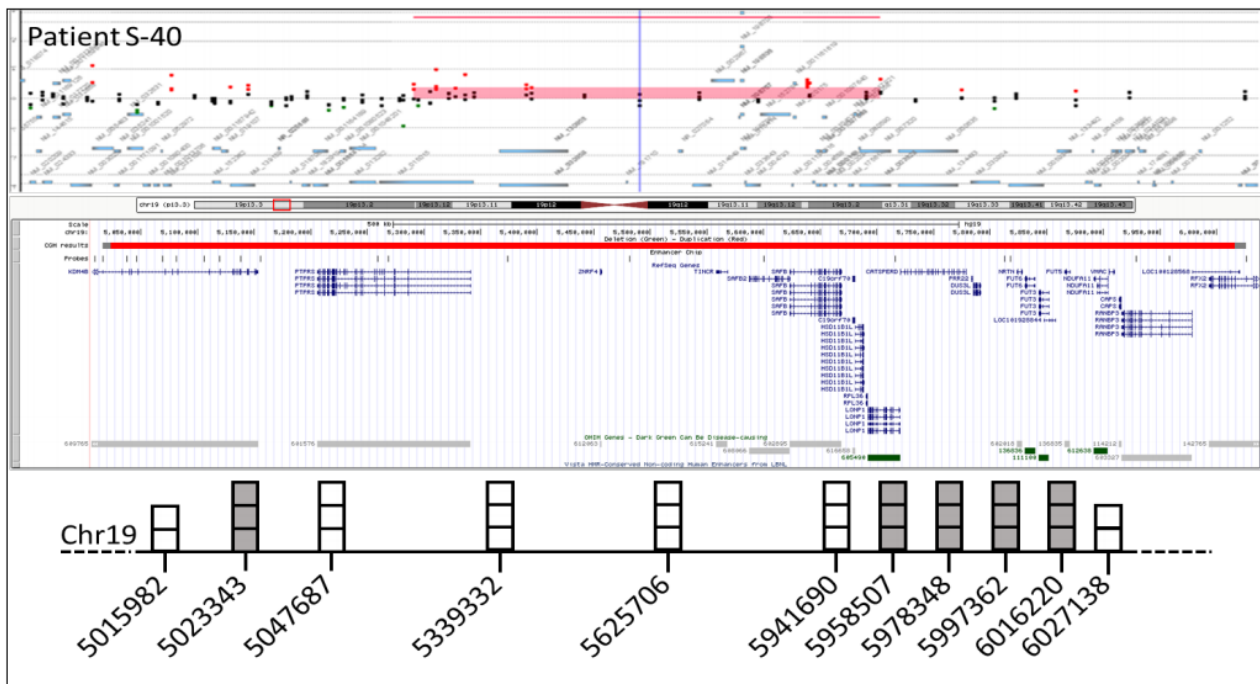
For each patient, in the gene view (top) the probe distribution and signal intensity are shown with a green or red bar indicating deletion or duplication, respectively. In the UCSC graphic view (middle), a similarly colored bar corresponds to the minimal aberration length, while the flanking grey bars indicate the 5' and 3' breakpoint boundaries as determined using Enhancer Chip and qPCR. The results of genomic quantification by real-time PCR are schematically represented (bottom). The middle base-pair position of each amplicon along chromosome is surmounted by a square corresponding to the number of detected copies. Gray squares highlight qPCR results for better defining 5' and 3' breakpoint boundaries.
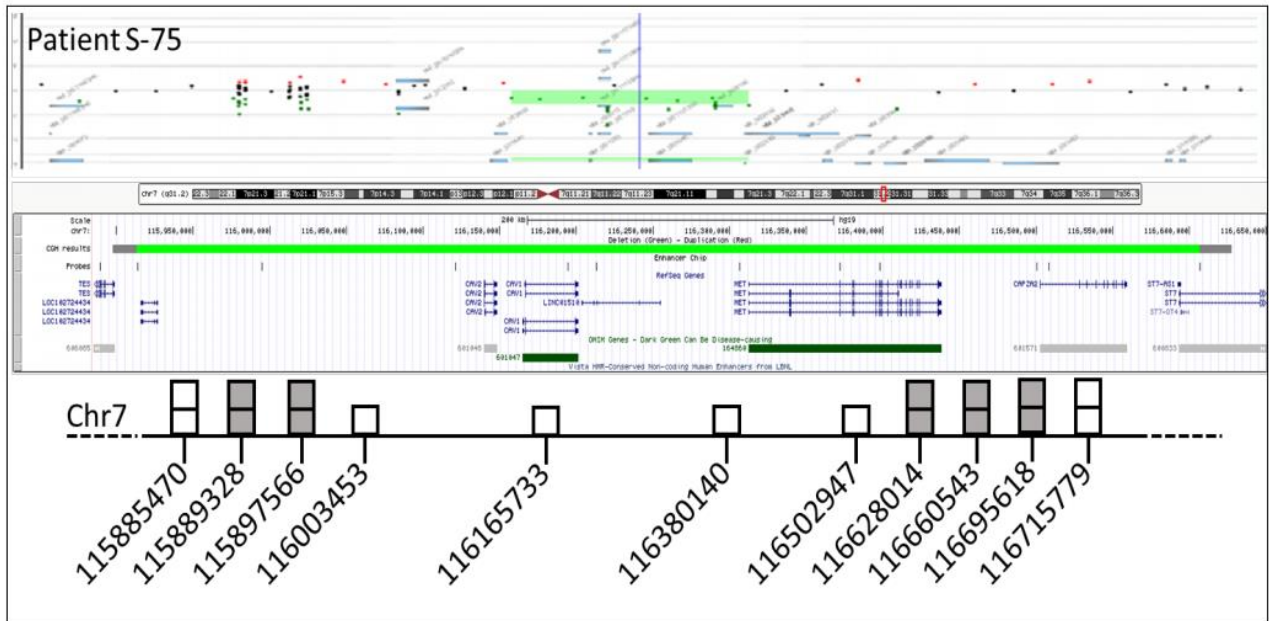
Supplementary Figure S2 – *Enhancer Chip profile and validation by real-time PCR of CNVs detected in patients S-40 and S-75*

For each patient, in the gene view (top) the probe distribution and signal intensity are shown with a green or red bar indicating deletion or duplication, respectively. In the UCSC graphic view (middle), a similarly colored bar corresponds to the minimal aberration length, while the flanking grey bars indicate the 5' and 3' breakpoint boundaries as determined using Enhancer Chip and qPCR. The results of genomic quantification by real-time PCR are schematically represented (bottom). The middle base-pair position of each amplicon along chromosome is surmounted by a square corresponding to the number of detected copies. Gray squares highlight qPCR results for better defining 5' and 3' breakpoint boundaries.
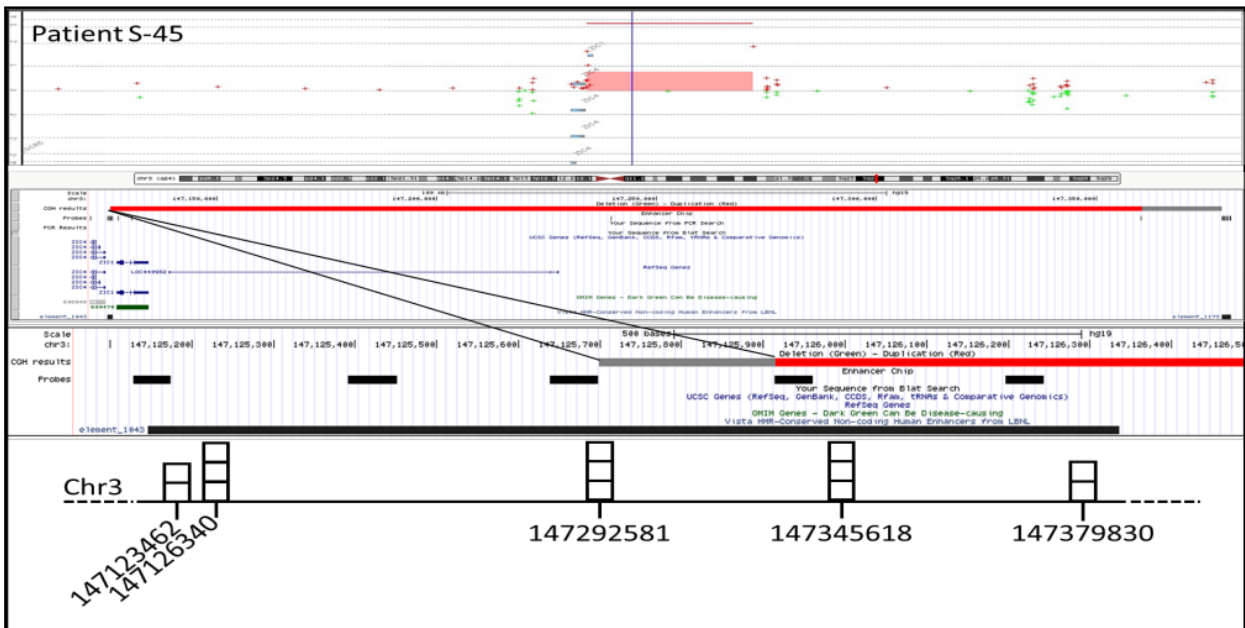
Supplementary Figure S3 – *Enhancer Chip profile and validation by real-time PCR of CNVs detected in patients S-45 and S-131 and involving VISTA enhancer elements*

For each patient, in the gene view (top) the probe distribution and signal intensity are shown with a green or red bar indicating deletion or duplication, respectively. In the UCSC graphic view (middle), a similarly colored bar corresponds to the minimal aberration length, while the flanking grey bars indicate the 5' and 3' breakpoint boundaries as determined using Enhancer Chip. The 5' breakpoint boundary is zoomed to highlight that it falls into enhancer sequence motif. The results of genomic quantification by real-time PCR are schematically represented (bottom). The middle base-pair position of each amplicon along chromosome is surmounted by a square corresponding to the number of detected copies.

Patient S-131

ChrX

81464470
81466624

81485427

81505355

81614777