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## **Title**

**High resolution array analysis: diagnosing pregnancies with abnormal ultrasound findings**

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## **ABSTRACT**

**Background:** Genome-wide high resolution array analysis is becoming established as a diagnostic test in the investigation of individuals with learning disability and congenital anomalies; many novel microdeletion and microduplication syndromes have already been identified. The diagnostic use of high resolution array genomic hybridisation analysis for prenatal testing remains to be systematically assessed.

**Methods:** We studied 106 prenatal samples with abnormal ultrasound and a normal karyotype using the Affymetrix GeneChip 6.0 array. 'Rare' DNA copy number changes (CNVs) were classified into three groups depending on their size, genomic location and the presence or absence of matched copy number changes in a large cohort of 3,000 control samples analysed for copy number changes using genotyping arrays.

**Results:** A total of 35 rare CNVs were identified. 10 (9%) of these are considered likely to represent pathogenic CNVs; 5 were syndromic and 5 were novel. 12 CNVs were detected in at least one control hybridisation and likely to be benign, and 13 CNVs were of unknown clinical significance. In addition, we identified one case of cryptic mosaicism for trisomy 10, one case of loss of heterozygosity (LOH) and showed that the Affymetrix GeneChip 6.0 array platform can detect triploidy.

**Conclusions:** We conclude that careful implementation of high resolution array testing would benefit at least 10% of obstetric patients with abnormal ultrasound findings and a normal karyotype result.

## **Key Words**

microarray analysis, prenatal diagnosis, ultrasonography

## INTRODUCTION

Routine prenatal ultrasound scanning in the first and second trimesters has become an integral component of antenatal care around the world. Detection of major fetal abnormalities can inform pregnancy management. In most instances, the decisions regarding further management of affected pregnancies are undertaken in conjunction with other diagnostic tests. One fifth of abnormal ultrasound cases will have a significant cytogenetics anomaly found, which will inform the parents and clinicians of subsequent morbidity and mortality[1, 2]. However, in a significant proportion of such pregnancies decisions have to be made without knowing the aetiology.

In postnatal cytogenetics of individuals with learning difficulties and congenital abnormalities, genome-wide microarray analysis has become an adjunct to karyotyping. This has increased the detection rate of submicroscopic pathogenic copy number changes associated with learning difficulties and congenital abnormalities by as much as 10-15%. A number of new submicroscopic copy number change syndromes have been identified[3-8]. We asked if a similar increase in diagnoses may be achieved in pregnancies with abnormal ultrasound findings were microarray testing to be implemented.

We have performed a retrospective analysis on 106 karyotypically normal referrals with abnormal ultrasound findings using the GeneChip 6.0 array from Affymetrix. This array platform provides uniquely high resolution coverage of the genome with over 1.8 million probes, using oligonucleotide targets that provide copy number information only and single nucleotide polymorphisms (SNPs) oligonucleotide targets which provide genotyping as well as copy number information. Genome-wide screening using this platform was expected to detect all known syndromes that result from copy number changes as well as new ones and to

provide additional genotyping information including the detection of loss of heterozygosity (LOH).

To deal with the interpretational challenge associated with higher density microarrays we used careful assessment of copy number changes observed in large publicly available datasets of normal individuals.

## **MATERIALS AND METHODS**

### **Samples**

All samples used in this study were referred for diagnostic cytogenetic analysis because of major ultrasound abnormalities or multiple soft markers detected by standard 2D ultrasonography. As part of the prenatal diagnostic work-up, all samples were cultured and karyotyped. The surplus cultured material from samples with normal G-banded karyotype analysis (450-550 banding resolution) and 22q11.2 FISH was frozen and stored in liquid nitrogen as part of the diagnostic management of the patient. DNA and additional cytogenetic slides were prepared from 106 retrospective, randomly selected, successfully reconstituted anonymised samples stored between 2001 and 2007. An abnormal triploid (69,XXY) sample was also analysed to assess the allelic difference algorithm of the analysis software for detection of triploidy.

Of the 106 samples included in the study, 87 were second and third trimester amniotic fluid samples, 15 were referred as first trimester chorionic villus (CV) biopsies, and 4 samples were post-termination placental biopsies. All samples were linked to detailed clinical information available at the time of referral for chromosome analysis. The referral categories included 34 samples for congenital heart abnormalities, 24 samples for multisystem abnormalities and multiple soft markers,

18 samples were referred for very large nuchal mass, cystic hygroma or hydrops fetalis, 16 samples for brain abnormalities including posterior fossa cysts, ventriculomegaly, agenesis of corpus callosum, 6 samples were referred for skeletal abnormalities or short long bones, 2 samples for isolated abdominal wall defects, and 6 samples were referred for other abnormalities.

A constraint of the ethical approval for this study was that there is no follow-up data on these cases as some may be identifiable, so only the clinical referral information can be linked to the results.

This anonymised study was approved by the Cambridgeshire 1 Research Ethics Committee and the Research and Development Review Board at the Cambridge University NHS Foundation Trust.

### **GeneChip 6.0 (Affymetrix) array hybridization**

DNA was extracted from cells using Qiagen's DNeasy Blood and Tissue kit following the manufactures instructions. RNase treatment was performed to remove the high levels of RNA in these cell types. DNA concentration and purity ( $A_{260-280} \sim 1.8-2.0$ ) was determined using a NanoDrop<sup>TM</sup> spectrophotometer (Thermo Fisher Scientific Inc., USA). Samples (20 $\mu$ l) diluted to  $\sim 100\text{ng}/\mu\text{l}$  were sent to Almac Diagnostics for further processing and hybridization to Affymetrix GeneChip 6.0 array (see Affymetrix Genomewide Human SNP Nsp/Sty 6.0 User Guide for details).

### **GeneChip 6.0 data analysis (see Affymetrix Genotyping Console 2.1 user manual for details)**

The .CEL intensity files were loaded into Genotyping Console v2.1 (Affymetrix UK Ltd.) for analysis. All samples passed the initial contrast QC metric ( $>0.4$ ) that

measures the ability of the intensity files to resolve SNPs into three genotyping clusters.

Copy number data were generated by comparing intensities for both SNP and copy number probes '*in silico*' to the HapMap control provided by Affymetrix. The resulting  $\log_2$  ratios were then analysed using a Hidden Markov Model (HMM) to generate copy number calls for each probe.

The quality of the  $\log_2$  data was assessed by the degree of variation, determined by the MAPD metric. MAPD is defined as the Median of the Absolute value of all Pairwise Differences between  $\log_2$  ratios for a given chip. High MAPD,  $>0.4$  (using the HapMap control) is considered to be the cut-off at which copy numbers can no longer be accurately called. None of the samples included in this study had a MAPD  $>0.4$ .

Using the copy number calls provided by Genotyping Console v2.1 as a guide, a more detailed analysis was performed by interrogation of the plots of  $\log_2$  ratios paying particular attention to CNV regions called by the HMM. A minimum  $\log_2$  ratio cut-off of  $\pm 0.3$  was used for autosomal CNVs. This excluded any false positive calls made by the HMM algorithm. Identified CNVs were checked against the Database of Genomic Variants (DGV) and gene tracks in Genotyping Console v2.1. CNVs were excluded from further analysis if they matched a known CNV exactly[9-12], matched no known CNV but fell outside gene boundaries, occurred in a known variable region but have not previously been reported as pathogenic CNVs (particularly within pericentromeric and subtelomeric polymorphic loci)[13], and finally if they significantly overlapped with polymorphic CNVs identified by McCarroll et al.[14] on the same array platform (see supplementary fig 1 for examples). CNVs

incorporating at least 25 probes and not identified as a known CNV were considered rare and recorded for further investigation.

The allelic difference and loss of heterozygosity plots generated from the difference in allele intensity for each SNP were analysed to investigate parental consanguinity and as an aid to the interpretation of potential mosaicism. Genotyping was performed using the Birdseed v.2 algorithm. All samples had call rates greater than 97.5%.

### **FISH and Q-PCR**

Large deletions and duplications were confirmed with FISH using standard cytogenetic laboratory techniques and BAC clones from the 30K TPA clones set. 5µl of probe (approx 0.5µg DNA) was labelled with either Spectrum Orange or Green dUTP (Abbott Molecular UK), using Nick Translation Kit reagents (Abbott Molecular UK), and re-suspended in 4µl TE buffer. 1µl of probe was used per slide with 1µl Cot-1 and 8µl Hybridisation buffer (Hybrisol VII). Slides washed and counter stained with DAPI were analyzed using fluorescence microscopy (Olympus BX61, UK), and images recorded using SmartCapture 3 software (Digital Scientific, UK).

Small deletions (<100 kb) and duplications (<1.5 Mb) were confirmed by Q-PCR using GAPDH as a reference gene. Each Q-PCR reaction (20µl) contained 10µl POWER SYBR Green (2x) master mix (Applied Biosystems, USA), primers for the control gene (GAPDH) or gene of interest (0.3µM), ~100ng template DNA and DNase free water. Q-PCR was performed using Corbett Life Science's Rotor-Gene 6000 software (Australia) and standard Q-PCR cycling conditions: 10mins at 95°C, then 35 cycles of 15s at 95°C, 30s at 60°C and 30s at 72°C. Finally a product melt was performed by ramping the temperature back to 95°C.



### **RNA extraction and two step RT-qPCR**

RNA was extracted from cultured cells using Qiagen's RNeasy isolation kit. Purified RNA was either stored at  $-20^{\circ}\text{C}$  or used immediately as a template for total RNA RT-PCR using Qiagen's QuantiTect reverse transcription kit following the manufacturer's instructions. The level of expression was quantified using quantitative RT-PCR (as described above for Q-PCR) using cDNA specific primers.

### **Welcome Trust Case Control Consortium (WTCCC) sample analysis**

The WTCCC samples consist of 1500 cell lines from the 1958 Birth Cohort and 1500 DNA samples from the UK national repository of anonymised blood donor samples[15]. The 1958 Birth Cohort (or National Child Development Study) included all births in England, Scotland and Wales occurring within one week in March 1958. All samples used for the WTCCC were acquired during a follow-up consultation at the age of 44-45 years. The 1500 samples are representative of both genders and each geographical region. The National Blood Service samples included consenting participants from England, Scotland and Wales between 18-69 years. 1500 samples of similar gender and geographical distribution to the Birth Cohort samples were selected for the WTCCC.

The intensity files for the 3000 individuals were applied for and acquired from the Wellcome Trust Sanger Institute and used to examine the occurrence of 'rare' CNVs within a 'normal' population.

The GeneChip 250K\_Nsp array data generated by the WTCCC for the 3000 control samples was analyzed using Genotyping Console v2.0 (GTC). A sex-matched copy number analysis was performed using HapMap controls. NEXUS software

(BioDiscovery Inc., USA) was used to display multiple copy number files created by GTC using stringent parameters suggested by the manufacturer.

## RESULTS

In total, 35 DNA copy number changes (30 novel and 5 previously reported pathogenic CNVs) were identified for further analysis, 2 of these were found in two and 1 in three samples. Based on the size, gene content, previously reported cases in the literature and the presence or absence of matched CNVs in control samples (WTCCC and unaffected individuals identified in our laboratory in the course of a postnatal diagnostic follow-up), these rare CNVs were classified into three groups and are listed in tables 1-3.

### *Likely pathogenic rare CNVs*

Ten of the 35 rare CNVs were considered to be likely pathogenic (table 1, figs 1 and 2, supplemental fig 3). None of these CNVs were found in the 3000 WTCCC control samples or in the other unaffected individuals identified in our laboratory.

Four of the CNVs overlapped with previously published pathogenic microdeletions. A deletion (~0.5 Mb) at 17q21.31 was found in a sample referred due to a large ventricular septal defect (VSD). The 17q21.31 microdeletion syndrome is mainly characterised by hypotonia, dysmorphic facial appearance and developmental delay, with heart defects being less common features[3-5]. Two deletions were detected within 22q11.2. The first (~750 kb in size) was in a sample referred for a heart defect and was nested within the typical 3 Mb 22q11.2 microdeletion but did not include the DiGeorge critical region. Similar distal deletions have previously been reported with cardiac anomalies[16-18]. The second of the 22q11.2 deletions was an

**Table 1. Likely Pathogenic CNVs**

Copy No.	Loss/ Gain	Chr	Cytoband	Size (kb)	Start Position*	End Position*	Start Marker	End Marker
3	Gain	15	q21.1q21.1	729	42831204	43559740	SNP_A-8345787	SNP_A-1859646
1	Loss	15	q26.2q26.2	3571	92557725	96128332	CN_725893	SNP_A-8374247
1	Loss	16	q24.1q24.2	1989	84286000	86275006	CN_745007	CN_755946
1	Loss	17	q21.31q21.31	556	41014074	41569931	CN_734861	CN_737156
3	Gain	19	p13.2p13.2	4760	7628636	12388852	SNP_A-8539212	CN_789094
3	Gain	19	q13.41q13.42	529	58932027	59461178	CN_796537	SNP_A-8551967
1	Loss	19	q13.42q13.42	1597	59545138	61142344	CN_800868	SNP_A-8421559
1	Loss	22	q11.21q11.21	749	19046924	19795835	CN_900101	SNP_A-8376166
1	Loss	22	q11.21q11.22	1053	20247141	21300127	CN_900338	CN_873813
0	Loss	X	p22.2p22.2	60	14743675	14803466	CN_912494	CN_916888

**Table 2. Rare CNVs Identified in Control<sup>‡</sup> Array Hybridisations**

Copy No.	Loss/ Gain	Chr	Cytoband	Size (kb)	Start Position*	End Position*	Start Marker	End Marker
3	Gain	1	q21.1q21.1	2929	144643813	147572680	CN_436401	CN_005156
3	Gain	2	p12p12	151	79992846	80144175	CN_849251	CN_849310
3	Gain	2	q14.3q14.3	183	127635670	127818407	CN_814671	CN_814739
3	Gain	2	q24.1q24.2	305	159351110	159656347	CN_815439	SNP_A-1906806
1	Loss	4	q21.3q21.3	168	87712551	87880721	CN_1098901	CN_1098950
1	Loss	6	p22.1p22.1	71	26488457	26559532	SNP_A-8672948	SNP_A-2107841
1	Loss	7	q34q34	64	142538076	142601831	SNP_A-8504569	CN_1195107
1	Loss	17	p13.1p13.1	53	10175316	10228548	SNP_A-8316868	SNP_A-4259604
3	Gain	18	p11.31p11.23	255	7034693	7289938	SNP_A-8659367	CN_162287
3	Gain	X	p11.3p11.3	735	42658326	43393758	CN_975615	CN_944209
3	Gain	X	q22.3q22.3	535	105020902	105555463	CN_915981	SNP_A-4272644
3	Gain	X	q27.3q27.3	284	142191812	142476144	CN_923443	CN_925603

**Table 3. Rare CNVs Not Identified in Control<sup>‡</sup> Array Hybridisations**

Copy No.	Loss/ Gain	Chr	Cytoband	Size (kb)	Start Position*	End Position*	Start Marker	End Marker
3	Gain	2	q11.2q11.2	136	100885342	101021754	SNP_A-8363841	CN_781396
1	Loss	3	p26.2p26.2	191	4465777	4656908	CN_1019660	SNP_A-2215036
1	Loss	4	q13.1q13.1	73	62018599	62091738	CN_1074069	CN_1074096
1	Loss	8	q21.11q21.11	442	77982322	78424626	SNP_A-1852526	CN_1277827
1	Loss	8	q22.2q22.2	50	99614669	99664963	CN_1327739	CN_1327758
3	Gain	9	p24.3p24.3	198	336739	534519	SNP_A-4267299	SNP_A-8435185
3	Gain	12	q14.1q14.1	1238	59716019	60954182	SNP_A-8393568	CN_622744
3	Gain	13	q12.3q12.3	241	29263783	29505076	SNP_A-8427957	SNP_A-1875934
1	Loss	13	q21.2q21.2	85	59445984	59530649	CN_632878	CN_632907
4	Gain	15	q21.1q21.1	94	43705221	43799561	CN_691975	SNP_A-8689230
3	Gain	16	p13.2p13.2	175	8769713	8944958	SNP_A-8328707	CN_732050
3	Gain	X	p22.12p22.12	392	19470418	19862134	CN_914967	CN_917170
1	Loss	X	q24q24	90	118239441	118329782	SNP_A-8450878	CN_922882

\* Physical positions utilize the hg17(b36) build of the human genome sequence

‡ WTCCC and unaffected parents identified in our laboratory

approximately 1 Mb deletion extending from 22q11.21 to 22q11.22 found in a sample referred for multiple VSDs and hypospadias. This deletion is consistent with 22q11.2 distal deletion syndrome[6] and a patient with multiple VSDs and a clinical diagnosis of Goldenhar syndrome has been reported previously with an overlapping deletion of similar size[19]. The fourth case was a deletion of ~3.5 Mb at 15q26.2 detected in a sample referred for hypoplastic left heart. Deletions of this region have been described with congenital diaphragmatic hernia (CDH), congenital heart disease, and kidney defects[20, 21]. It is interesting that the deletion incorporates the NR2F2 gene (MIM\*107773) reported to be involved in angiogenesis and heart development[22]. Although CDH was not identified by ultrasound in this case, CDH is detected prenatally in only about 60% of patients and heart 'hypoplasia' is among anomalies frequently found in association with CDH[23].

The smallest pathogenic CNV identified was a 60 kb deletion at Xp22.2 detected in a placental biopsy of a male fetus with hydrocephalus, cleft lip and palate, absent radii, short forearms, and intestinal atresia. The deletion incorporated the entire FANCB gene (MIM\*300515) gene. The ultrasound abnormalities and the absence of FANCB gene in the fetus are consistent with the clinical diagnosis of Fanconi anaemia and X-linked VACTERL association with hydrocephalus (MIM#314390)[24].

There were 5 other novel CNVs that were considered to be likely pathogenic. A deletion of ~2 Mb within the long arm of chromosome 16 with breakpoints at 16q24.1 and 16q24.2 was detected in a sample referred for hypoplastic left heart and dilated renal pelvices. This deletion encompasses several protein coding genes including cytochrome C oxidase genes COX4NB and COX4I1 (MIM\*123864), IRF8 (MIM\*601565), FOXF1 (MIM\*601089), FOXC2 (MIM\*602402), FOXL1

(MIM\*603252), MAPL3b, ZCCHC14, JPH3 (MIM\*605268). Haploinsufficiency of the forkhead genes FOXF1 and FOXC2 may be responsible for the heart and renal abnormalities found on prenatal ultrasound in this pregnancy. A duplication of ~730 kb within 15q21.1 was identified in an amniotic fluid sample referred for posterior ventriculomegaly, echogenic intracardiac focus, and echogenic bowel. This involved several genes, namely, SORD (MIM\*182500), SHF, DUOX1 (MIM\*606758), DUOX2 (MIM\*606759), SLC28A2 (MIM\*606208), GATM (MIM+602360), SPATA5L1, PLDN (MIM\*604310), SLC30A4 (MIM\*602095). This case was considered pathogenic because of the size, absence in the WTCCC control samples and no major overlap with CNVs recorded in the DGV. Three abnormalities were identified that involved chromosome 19, a deletion and two duplications, all involving several genes (fig 2). A deletion at 19q13.42 (~1.6 Mb) was identified in a sample referred for first trimester cystic hygroma. This deletion overlaps with a previously reported significantly larger interstitial duplication with breakpoints at q13.2 and q13.4 in a fetus with cystic hygroma[25]. For the duplications identified, the breakpoints of the duplication at 19p13.2 (~4.76 Mb) in a fetus with cleft lip and palate, renal pyelectasis, and bilateral talipes are similar to the breakpoints reported in DECIPHER for a patient (00001857) with a *de novo* deletion and multiple congenital abnormalities, suggesting that dosage sensitive genes exist in this region and it may be a site of recurrent pathogenic rearrangement. The ~530 kb duplication within 19q13.4 identified in a case referred with multiple ultrasound abnormalities including polyhydramnios, micrognathia, abnormal right pinna, absent stomach bubble and given a provisional diagnosis of Treacher Collins syndrome has not been previously reported.

### *Rare CNVs identified in control array hybridisations*

Twelve of the rare CNVs were considered likely to be benign in the context of this study. They were identified in one or more WTCCC control samples or were previously identified in our laboratory as being inherited from a normal parent (table 2, supplemental fig 2). It is likely that these CNVs would be inherited and not related to the observed ultrasound abnormalities.

Two CNVs, a deletion mapped to chromosome 7q34 and a duplication mapped to Xq27.3, could not be observed in the WTCCC controls due to the limited resolution of the 250K\_Nsp array (*i.e.*, no SNPs) in these genomic regions. Both of these have been previously detected using Affymetrix GeneChip 6.0 array in our laboratory and found to be inherited from a phenotypically normal parent.

All of the other autosomal CNVs and the Xp11.3 apparent duplication were found in at least one control WTCCC hybridisation with similar breakpoints. The ~3 Mb duplication within 1q21.1 identified in a sample referred for two soft markers detected on ultrasound (choroid plexus cysts and unilateral renal pyelectasis) has been published previously and has been associated with a mild phenotype in some instances[26, 27]. Similar 1q21.1 duplications were found in three WTCCC control individuals and in a diagnostic setting appropriate counselling could be offered without further family follow-up.

A further apparent duplication of ~535 kb at Xq22.3 was found in a female sample with multisystem abnormalities detected on ultrasound. A similar duplication was also found in a single female control hybridisation (WTCCC). As a result it was assumed that this CNV does not contribute significantly to the fetal abnormalities in this pregnancy. However, as this duplication has not been observed in a normal male, its pathogenicity can not be fully excluded.

### *Rare CNVs of unknown significance*

Thirteen of the 35 rare CNVs identified in this study were novel and not detected in any of the control samples (table 3, fig 3). The sizes of these CNVs ranged from ~50-1238 kb but their gene content was low (1-3 genes only). In order to determine if these CNVs are of clinical significance further studies including family follow-up would be necessary.

Two rare CNVs on the X chromosome were detected in a female sample referred for a single umbilical artery and choroid plexus cysts detected on second trimester ultrasound scan. This sample had a duplication (~392 kb) at Xp22.12 including the SH3KBP1 (MIM\*300374) gene and a deletion (~90 kb) at Xq24 including the PGRMC1 (MIM\*300435) gene. The clinical relevance of both these CNVs in the context of a female pregnancy is unknown and further family follow-up would be required in a diagnostic setting.

Two CNVs listed in table 3 included genes known to cause recessive diseases. A deletion CNV of ~442 kb on chromosome 8q21.11 contains the PXMP3 gene (MIM+170993). Defects in the function of this gene are known to cause Zellweger syndrome (MIM#214100). This sample was referred for cystic hygroma at 33 weeks gestation, which could be one of the prenatal features of a peroxisomal disorder[28]. Zellweger syndrome is usually diagnosed postnatally by biochemical testing, which demonstrates abnormally high levels of very long chain fatty acids in the tissues and body fluids. Biochemical testing performed on cultured amniocytes from this sample after long-term storage produced normal levels of very long chain fatty acids indicating that the second allele was functioning normally.

A deletion of ~191 kb on chromosome 3p26.2 has a distal breakpoint in the SUMF1 gene (MIM\*607939) and a proximal breakpoint in the ITPR1 gene (MIM\*147265). Biallelic mutations in SUMF1 cause multiple sulfatase deficiency (MIM#272200), MSD. Partial allelic deletions of SUMF1 and ITPR1 have been described previously[29], as well as large intragenic ITPR1 deletions associated with SCA16[30]. Cytogenetically visible distal 3p deletions have been reviewed recently [31, 32] and found to be associated with variable phenotypes. This sample was referred for severe early onset intrauterine growth retardation, posterior fossa cyst, and oligohydramnios. The biochemical test for MSD on this sample failed due to culture fatigue.

An apparent ~198 kb duplication on chromosome 9p24.3 was detected in an amniotic fluid sample referred for multiple cardiac abnormalities. The CNV appears to disrupt the DOCK8 (MIM+611432) and ANKRD15 genes with the telomeric breakpoint between exons 8-10 in DOCK8 and centromeric breakpoint in the first intron of the ANKRD15 gene. A deletion and translocation involving the DOCK8 gene have been recently reported in two patients with mental retardation and developmental delay[33]. A deletion (~225 kb) of the ANKRD15 has been reported in a single family with congenital cerebral palsy[34]. There is no known association of either of these genes with cardiac defects. However, the duplication in this sample was confirmed to be in *cis* (supplemental fig 3) and it would be prudent to consider further familial studies to assess the clinical significance of this CNV in a diagnostic setting.

*Identification of mosaic aneuploidy / LOH / triploidy*



In addition to the 10 cases with likely pathogenic CNVs, one case, a CV with cystic hygroma, was positive for trisomy 10 mosaicism (fig 4) increasing the overall abnormality rate to approximately 10%. It was not possible to investigate the prevalence of trisomy 10 cells in the original diagnostic specimen to exclude cultural artefacts or confined placental mosaicism, however mosaic trisomy10 has been reported previously in a fetus referred for a cystic hygroma[35] and this was therefore considered the likely cause of the ultrasound abnormality.

Using the allelic difference and LOH plots in Genotyping Console v2.1, a single sample was identified with extended LOH tracks on all chromosomes (fig 4). The overall extent of the LOH was determined to be ~10% of the autosomal complement. This is similar to that observed in populations where consanguineous marriages occur[36]. This degree of LOH increases the likelihood that the cerebral ventriculomegaly seen on ultrasound in the fetus was caused by an autosomal recessive disease, which are more prevalent in consanguineous populations.

The triploid control sample produced a very distinct allelic difference plot (fig 4). Instead of producing three genotyping clusters, one each for heterozygous AB, homozygous AA and BB calls, this triploid sample produced four genotype clusters: AAA, AAB, BBA, and BBB. This information is particularly relevant for the future design of diagnostic array platforms aiming to replace rather than complement full karyotype analysis at prenatal diagnosis.

### *Expression studies*

Several of the CNVs in tables 2 and 3 include a single protein coding gene with no apparent dosage sensitive effects and it is likely that most if not all are inherited from an unaffected parent. To determine if nonsense mutations of the remaining allele lead

to biallelic inactivation of any of these genes we used real-time quantitative RT-PCR to validate the overall changes in the gene expression and to determine if the non-deleted alleles are targeted for nonsense-mediated decay. We selected four genes, LPHN3 (latrophilin3), PTPN13 (MIM\*600267), PXMP3 (MIM+170993), and STK3 (MIM\*605030), mapped to deletion CNVs at chromosomes 4q13.1, 4q21.3, 8q21.1, and 8q22.2, respectively. These genes were expected to be ubiquitously expressed in most tissues without transcription activation treatment. Indeed, all four genes showed similar levels of expression in both controls and the sample carrying the deletion (fig 5) indicating that the transcript from the second allele was stable.

## **DISCUSSION**

In this consecutive series of pregnancies with abnormal ultrasound findings, 'likely' pathogenic abnormalities were detected in 10% (11 of 106) of the samples with normal cytogenetics results. Two previous studies on samples with abnormal ultrasound or fetal development have shown detection rates of 1.3% (2/151) and ~10% (5/49) [37, 38], but the numbers are small and more studies are needed to determine the true prevalence of cytogenetically undetected, pathogenic DNA copy number changes in prenatal abnormal ultrasound referrals.

We found that single gene deletions/duplications of less than 100 kb in size could be detected with confidence using the GeneChip 6.0 array (fig 1) and we identified no false positive copy number calls. Furthermore we demonstrated that SNP based targets allow the detection of triploidy and the easy visualisation of mosaic trisomy (fig 4).

In recent reviews of the potential application of microarrays for clinical use in prenatal diagnosis, implementation of targeted arrays has been favoured [39-41]. Our

study suggests that the design of a prenatal targeted array needs to allow for the fact that prenatal samples referred due to abnormal ultrasound findings have a variety of structural defects under-represented in postnatally ascertained patients and there may be important genomic regions currently overlooked by targeted arrays designed for postnatal testing. Only four of the ten pathogenic CNVs identified in this study involved previously described microdeletion syndromes, and all of these cases had cardiac anomalies and were at the more severe end of the clinical spectrum for these conditions. Three of the novel pathogenic CNVs identified mapped to chromosome 19; two of these were referred for multiple structural fetal abnormalities and one for cystic hygroma, all associated with a significant risk of poor pregnancy outcome. In contrast, interstitial deletions and duplications involving chromosome 19 are rarely detected postnatally[42]. Moreover, since new microdeletion/duplication syndromes continue to be identified on postnatal higher resolution arrays, the coverage of a targeted prenatal array would need to be continually reviewed.

Although, testing for copy number changes using the GeneChip 6.0 array has the advantage that this platform can detect smaller previously undescribed pathogenic CNVs than lower resolution arrays, it was expected that more novel CNVs of unknown significance would be detected. The use of a large control population dataset in this study halved (from 20 to 10) the number of novel CNVs of unknown significance that were initially identified for further investigation. This may reflect the recent suggestion that higher resolution array studies have shown that many of the common CNVs in the DGV are smaller and are less likely to contain significant genes than was originally thought to be the case [14]. With this CNV screening strategy, the proportion of CNVs with unknown clinical significance (table 3) compared with those likely to be pathogenic (table 1) was similar to that in most published series, using

lower resolution or targeted arrays, in which about half of the rare (non-polymorphic) CNVs detected require parental follow up to help determine their significance[43-46].

Two interpretational issues that would need to be considered before diagnostic prenatal arrays could be applied prospectively were identified in this study. Several previously undescribed copy number changes were identified that included only one or very few genes (table 3). For most of these the relationship, if any, between gene functionality and the ultrasound anomalies is not known. Two of these deletions included known autosomal recessive genes. Biallelic mutations in these genes result in severe phenotypes. Although we tested but failed to confirm defects of the second allele in our two cases, the possibility of detecting recessive disorders that cause abnormal fetal development needs to be borne in mind before implementing prenatal high resolution AGH. In particular, how to rapidly move from sample collection to producing a definitive result within the time-frame required for management of the affected pregnancy. We also found in this study five X chromosome CNVs of no or unknown clinical significance (tables 2 and 3). Although the knowledge about benign DNA copy number variation has increased significantly [14], there is very little known about the variants detected on the X chromosome and their pathogenic significance in the context of different sexes.

Our study indicates that rare DNA copy number changes are detected in about 30% of prenatal referrals with either major fetal abnormalities or multiple soft markers when high resolution hybrid arrays are applied. About a third of these rare CNVs are likely to be pathogenic abnormalities (table 1). This finding is similar to the detection rates in postnatal referrals, although the genome-wide distribution of pathogenic CNVs might be different. These additional data would help families to make informed decisions for their pregnancies and generate the recurrence risks for

future pregnancies. About one third of the detected rare CNVs could be identified in large control population datasets (table 2). These CNVs are therefore likely to be inherited from an unaffected parent and their laboratory follow up would not improve the medical management of the obstetric patients. The detection rate of rare CNVs with unknown clinical significance (table 3) in our study was relatively high and it would be prudent to determine their inheritance status in a diagnostic setting. It is likely that for a significant proportion of these it will not be easily possible to interpret their significance. More resources would be required for pre- and post-test counselling for these imbalances that cannot yet be interpreted. The inclusion of such CNVs in public domain CNV databases is vital to determine phenotypes which may otherwise be too complex to model in experimental systems.

At the present time, approximately 70% of all abnormal ultrasound findings remain without an aetiological diagnosis. Our study suggests that careful implementation of high resolution AGH analysis to standard practice could significantly improve the diagnostic yield in pregnancies with abnormal ultrasound findings, as well as advance our knowledge of the genetic basis of abnormal fetal development.

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Affymetrix: <http://www.affymetrix.com/support/technical/index.affx>

Almac Diagnostics: [www.almacgroup.com/diagnostics/](http://www.almacgroup.com/diagnostics/)

Corbett Life Science: <http://www.corbettlifescience.com/>

Database of Genomic Variants: <http://projects.tcag.ca/variation/>

Decipher: <https://decipher.sanger.ac.uk/>

Ensembl: <http://www.ensembl.org/>

GENECARDS: <http://www.genecards.org/>

NCBI: <http://www.ncbi.nlm.nih.gov/>

BioDiscovery: <http://www.biodiscovery.com/>

OMIM: <http://www.ncbi.nlm.nih.gov/Omim/>

Qiagen: [www.Qiagen.co.uk/geneglobe](http://www.Qiagen.co.uk/geneglobe)

Welcome Trust Case Control Consortium (WTCCC): <http://www.wtccc.org.uk/>

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## Figure Legends

**Figure 1** Nexus plots of SNP 6.0 data showing deletions and duplications in the 10 likely pathogenic copy number variations (CNVs) from table 1. Calling threshold changes are shown along the y-axis, where green lines indicate duplications and red lines indicate deletions. Location in Mb is provided along the x-axis. Panel (A) shows whole chromosome plots for the CNVs greater than 1Mb and panel (B) shows zoomed in views for the CNVs of less than 1Mb in size.

**Figure 2** Large likely pathogenic CNVs (>500 kb) confirmed by FISH. A and B: 19p13.2 duplication CNV in interphase nucleus (A) and partial metaphase (B). The BAC clone RP11-109L17 clone labelled in spectrum red shows 3 signals in the nucleus and an enlarged signal on one chromosome 19 in metaphase. The control clone RP11-384 (spectrum green) maps to 19q13.42. 16q24.1 deletion CNV in partial metaphase confirmed with RP11-56511 probe in spectrum green (no signal on one chromosome 16), and control probe RP11-368N21 (spectrum red) that maps to (16p12) (C). 19q13.42 deletion confirmed by RP11-384G4 clone (spectrum green), control probe RP11-109L17 (spectrum red) maps to chromosome 19p13.2) (D).

**Figure 3** Rare CNVs of unknown clinical significance from table 3. Nexus plots of SNP 6.0 data showing deletions and duplications in 12 patients. Calling threshold changes along y-axis, where green lines indicate duplications and red lines indicate deletions. Size in Mb is provided along the

x-axis. Panel (A) shows zoomed in views of deletions and panel (B) zoomed in views of duplications.

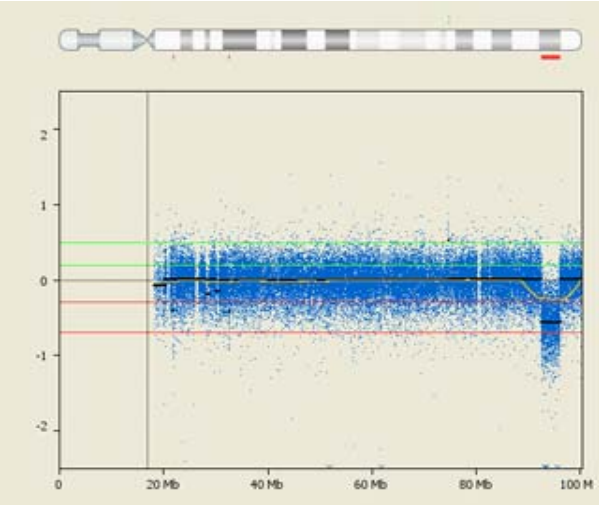
**Figure 4** 'Allele Difference' plots in Genotyping Console v2.1 showing the identification of trisomy 10 mosaicism and triploidy (A, B) and LOH (C). Part of chromosome 10 is shown in A and part of chromosome 11 in B for the mosaic trisomy 10 (violet), triploid (blue) and a normal (orange) sample. For the mosaic trisomy 10 sample the abnormal allele difference pattern is observed for chromosome 10 (A) and is not present for chromosome 11 (B). For the triploid sample both chromosomes 10 and 11 show an abnormal allele difference pattern (in panels A and B). In panel (C), the 'Allele Difference' plot is shown for chromosome 18. This sample shows a lack of heterozygous calls (row 1), which is further illustrated in the LOH plot (row 2). The same reference sample as in A and B (orange) illustrates the normal heterozygosity pattern for this chromosome (row 3) and absence of LOH calls (row 4).

**Figure 5** Genes of interest are expressed. Rotor-Gene software plots show PXMP3 indicated in bright green (A), STK3 indicated in violet (B), LPHN3 indicated in light blue (C) and PTPN13 indicated in violet (D). Four controls were used, indicated in orange, salmon, yellow and purple (A and B). Three controls were used, indicated in orange, salmon and yellow (C and D).

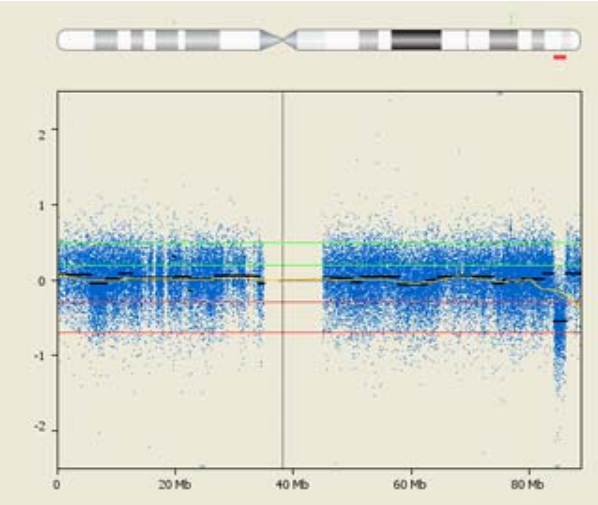
# Figure 1/panel A

A

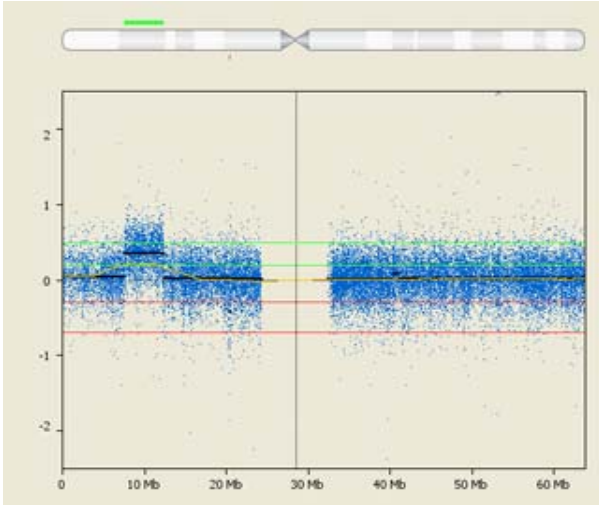
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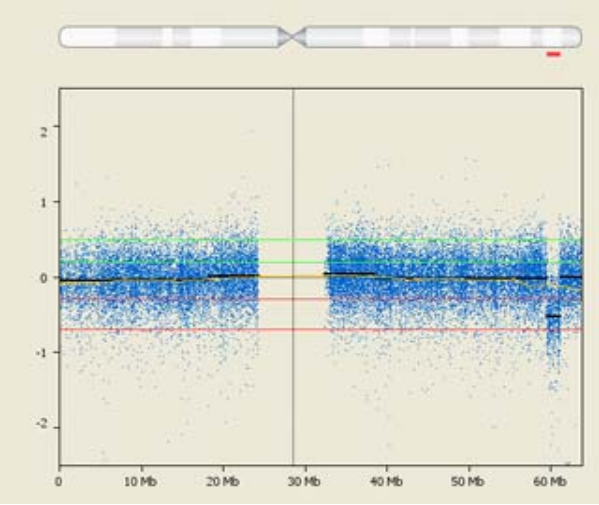
del(16)(q24.1q24.2)



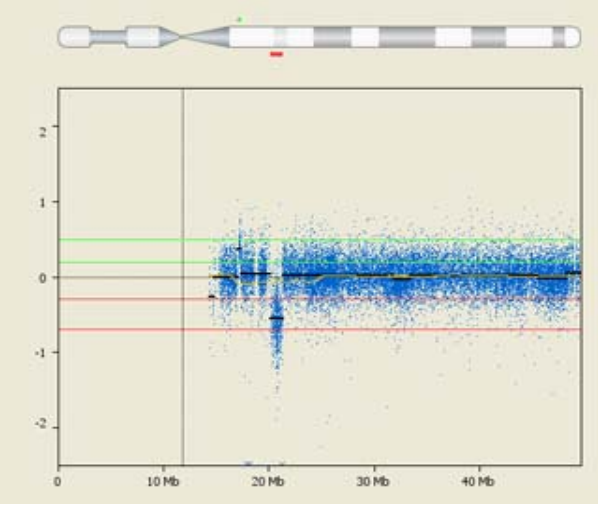
dup(19)(p13.2p13.2)



del(19)(q13.42q13.42)



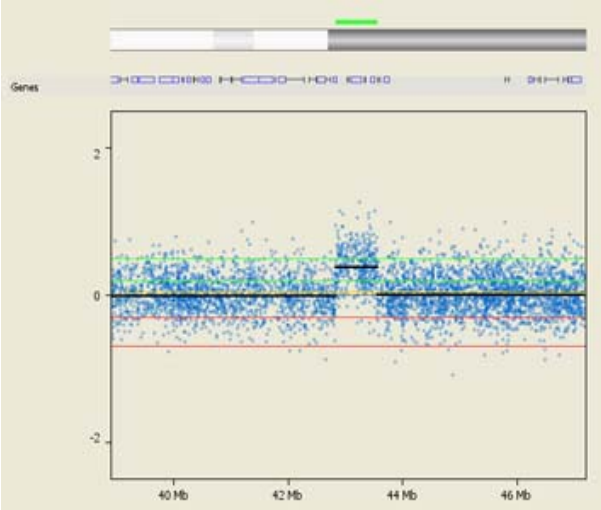
del(22)(q11.21q11.22)



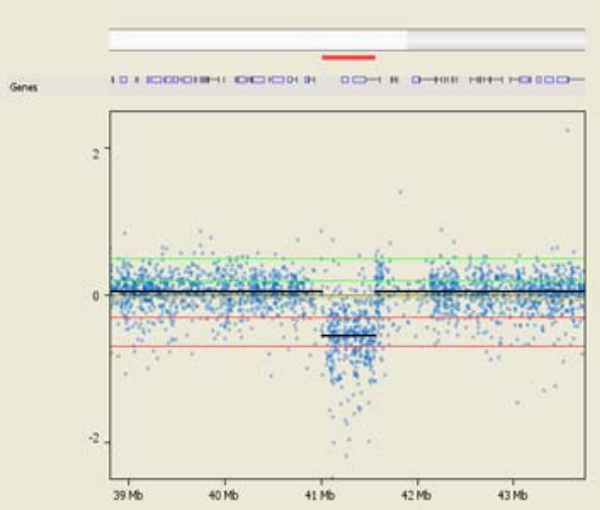
# Figure 1/panel B

**B**

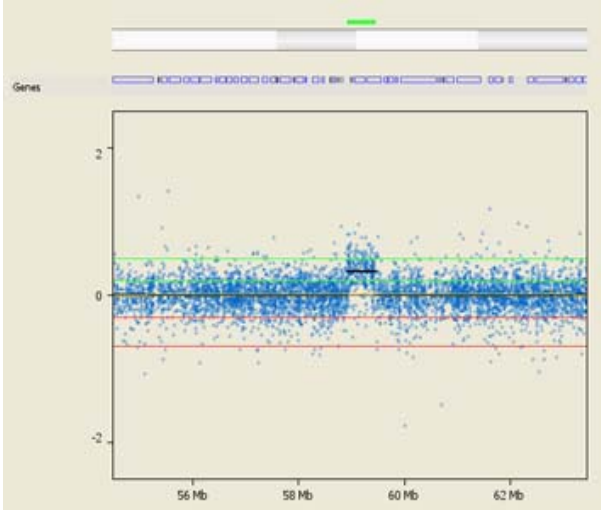
dup(15)(q21.1q21.1)



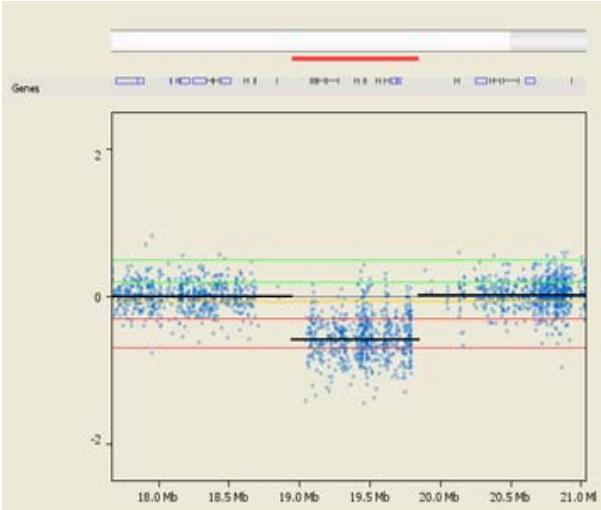
del(17)(q21.31q21.31)



dup(19)(q13.41q13.42)



del(22)(q11.21q11.21)



del(X)(p22.2p22.2)

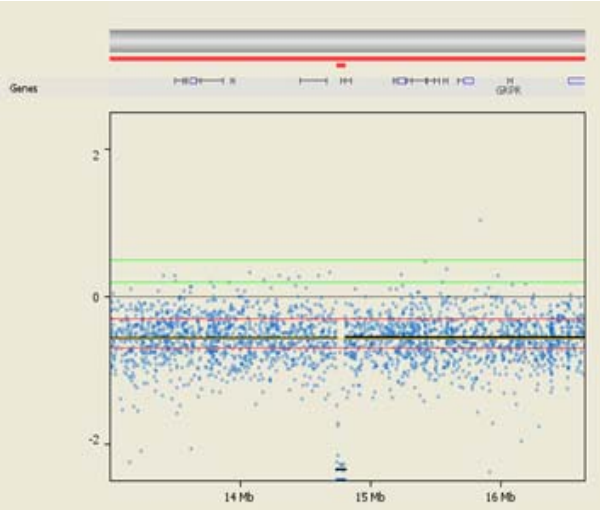
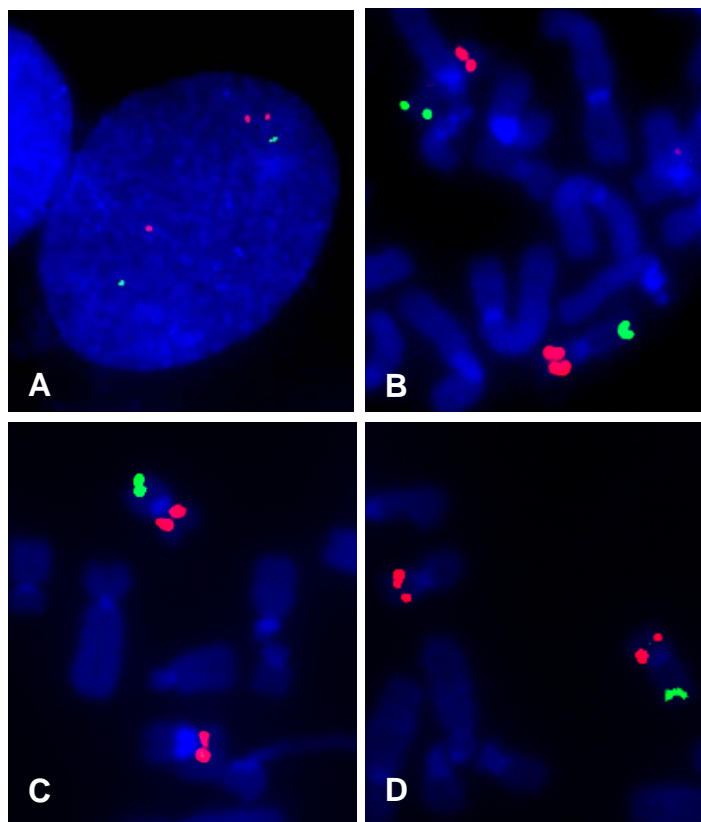


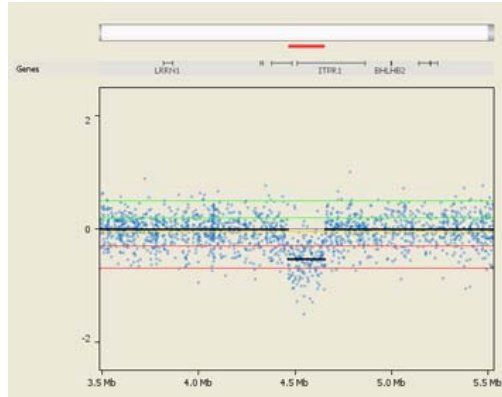
Figure 2



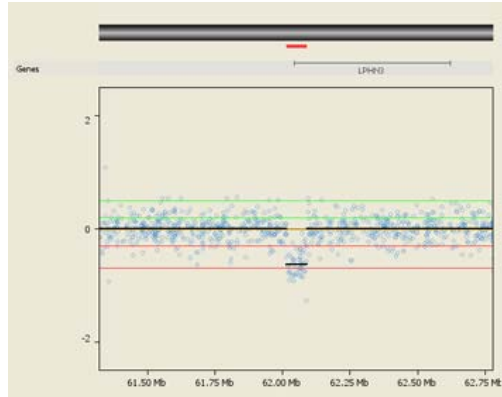
# Figure 3/panel A

A

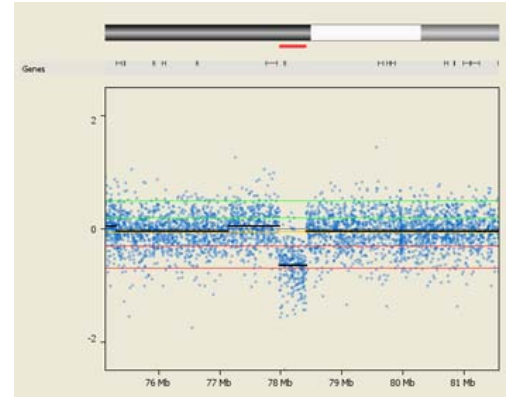
del(3)(p26.2p26.2)



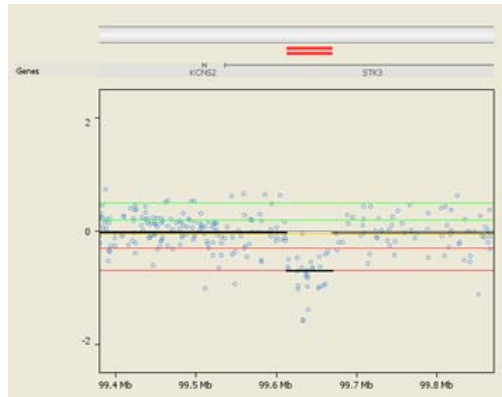
del(4)(q13.1q13.1)



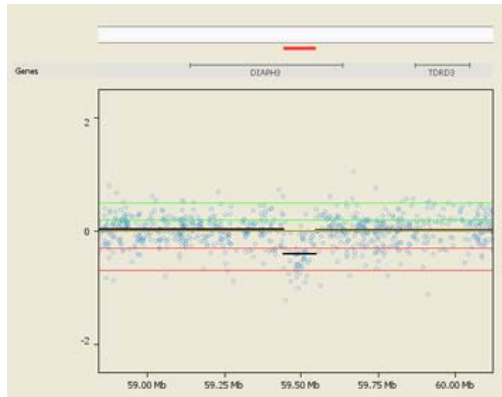
del(8)(q21.11q21.11)



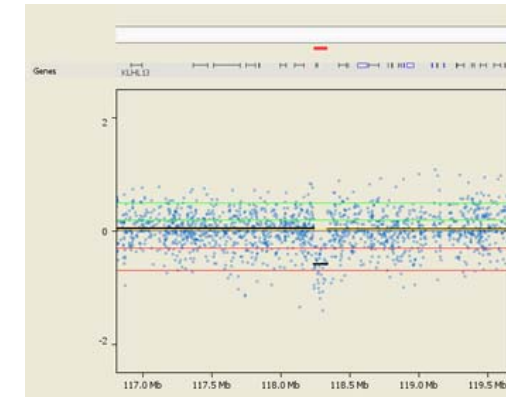
del(8)(q22.2q22.2)



del(13)(q21.2q21.2)



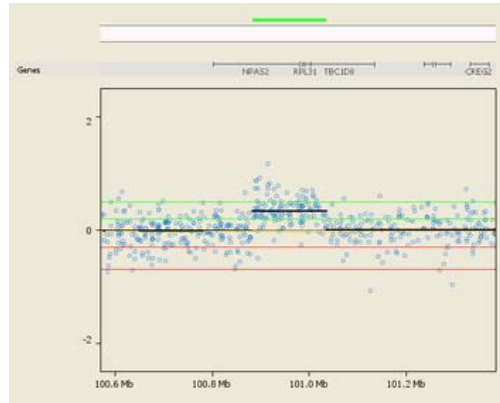
del(X)(q24q24)



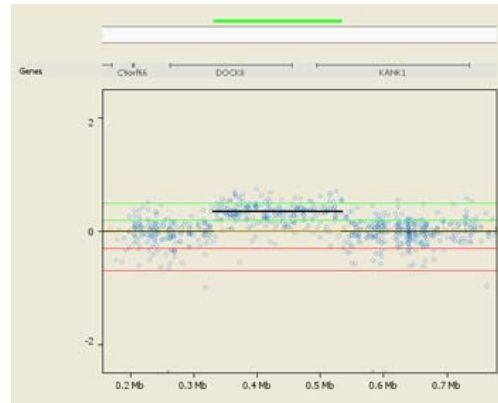
# Figure 3/ panel B

**B**

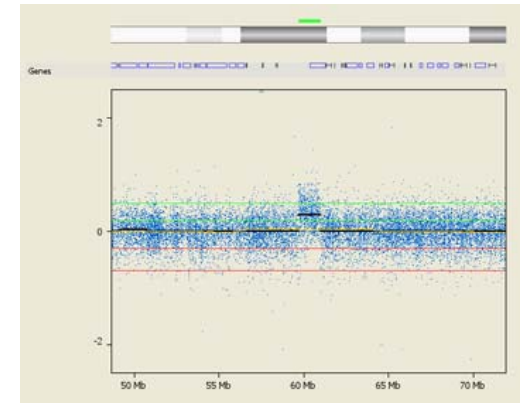
dup(2)(q11.2q11.2)



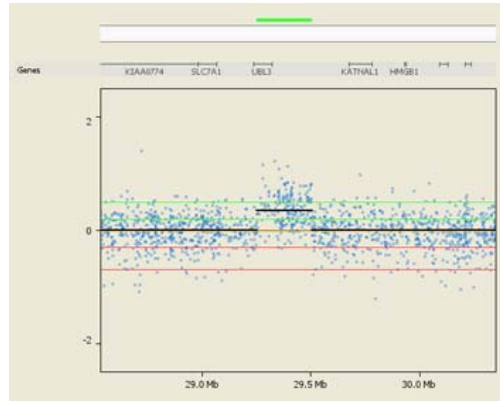
dup(9)(p24.3p24.3)



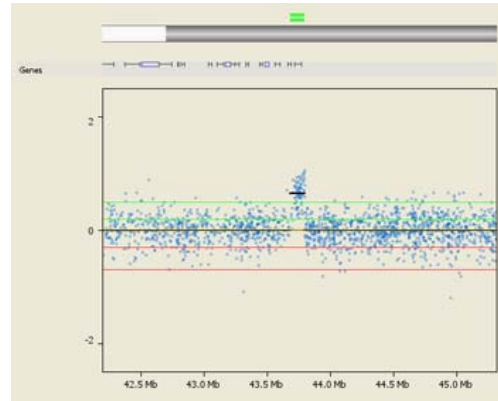
dup(12)(q14.1q14.1)



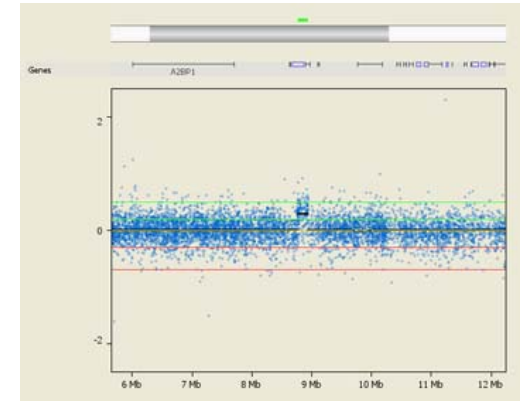
dup(13)(q12.3q12.3)



dup(15)(q21.1q21.1)



dup(16)(p13.2p13.2)



dup(X)(p22.12p22.12)

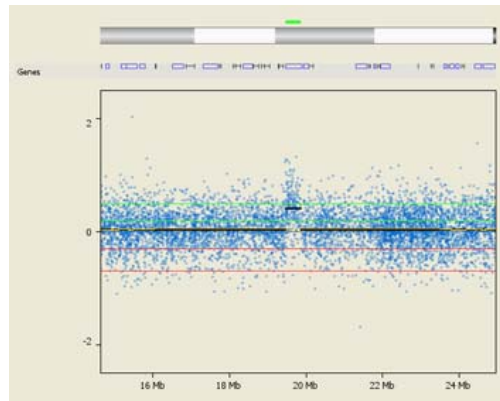




Figure 4

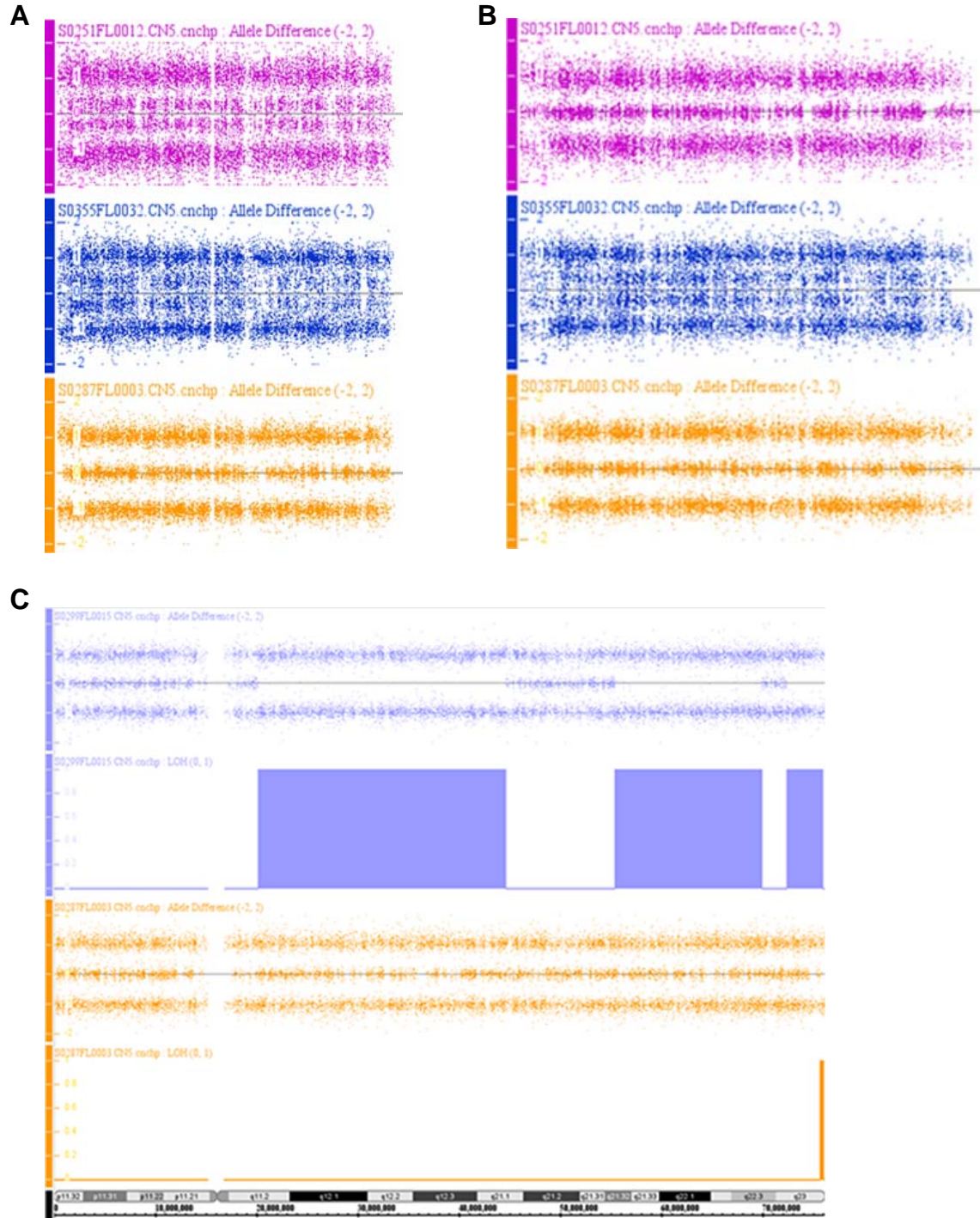


Figure 5

