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qPCR confirmation of CNV: Lessons learned from small CNVs and false positives

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I. Introduction

Genome wide microarray analysis continues to be the preferred method for the identification and classification of copy number variants (CNVs). While the ability of microarray analysis to detect small CNVs is limited by a number of factors including the total number of probes, the location of probes, DNA sequence, analysis software, and the quality of sample DNA, we can in many instances detect deletions as small as 10 kb. In the past, fluorescence in situ hybridization using BAC probes was widely accepted as the method of choice for confirmation of CNVs identified by microarray analysis. However, FISH analysis is time consuming, costly, and does not always have the appropriate resolution for the detection of smaller CNVs. For these reasons, we added qPCR analysis to our comprehensive microarray testing protocol which allows for the rapid confirmation of CNVs at a fraction of the cost of traditional FISH analysis. Currently we confirm possible CNVs below 50 kb that meet our diagnostic criteria as well as CNV calls considered questionable by a director.

II. Materials and Methods

Microarray analysis was performed using the Cytoscan™ HD (Applied Biosystems™). This array contains approximately 2.695 million markers across the entire human genome. There are approximately 743,000 SNPs and 1,953,000 structural non-polymorphic probes (NPCNs).

qPCR analysis was performed using the QuantStudio™ 7 Flex Real-Time PCR system (Applied Biosystems™) in conjunction with the VeriQuest Fast SYBR Green qPCR Master Mix (2X) (Thermo Scientific™). All samples were run in triplicate. To determine copy number, primers specific to an amplicon within a CNV identified by microarray analysis were compared to amplicons specific to two housekeeping genes (*RNase1*, *TERT*, or *TBP*). Two normal controls, one female and one male were run for all cases with validated primers. For cases with unvalidated primers, a positive control was included. Genomic copy number determinations for specific CNVs are made as follows: qPCR value of 0=0 copies, from .06 to 1.4=1 copy, 1.6 to 2.4=2 copies, 2.6 to 3.4=3 copies and 3.6 to 4.4=4 copies.

III. Results

Table 1. To date we have run 343 qPCR analyses of possible CNVs identified by microarray analysis. 53.6% of confirmations involved autosomal recessive genes, 38.8% of cases involved autosomal dominant genes, and the remaining 7.6% of cases involved X-linked dominant genes, X-linked recessive genes and genes associated with larger microdeletions, duplications and deletions. Approximately 75.5% of the 343 possible CNVs identified by microarray analysis, were confirmed by qPCR analysis.

CNVS	Total	Confirmed	Not Confirmed
Deletions	249	176	73
Duplications	86	76	10
Triplications	8	5	3
AD genes	133	74	59
AR genes	184	161	24
XLR genes	15	15	0
XLD genes	5	4	1
Other	6	6	0
All CNVs	343	257 (75.5%)	86 (24.5%)

Figure 1. qPCR analysis can help differentiate between false positive and true positive CNVs observed by microarray analysis. **A and C)** False positive common ~30kb interstitial *GALC* deletion observed by microarray analysis. qPCR reveals a copy number of 2x. **B and D)** True positive common ~30 kb interstitial *GALC* deletion. Red circle differentiates probe inclusion observed in true positives. qPCR analysis reveals a copy number of 1x.

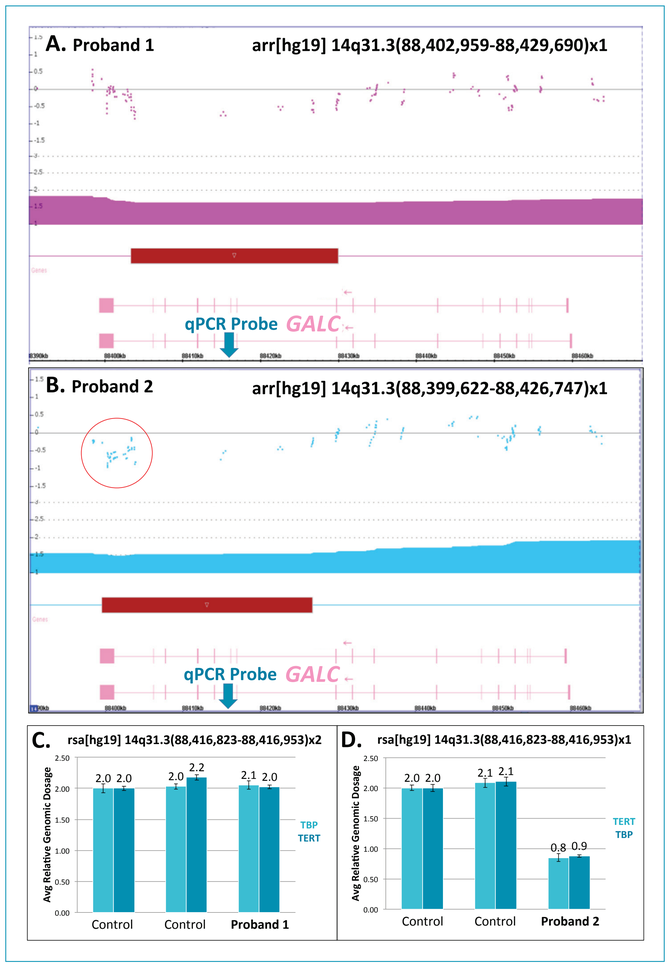

Figure 2. PCR analysis is helpful in confirming triplications observed by microarray analysis. **A and C)** False positive common ~30 kb interstitial *SLC3A1* triplication. qPCR reveals a copy number of 3x. **B and D)** True positive common ~30 kb interstitial *SLC3A1* triplication. qPCR reveals a copy number of 4x.

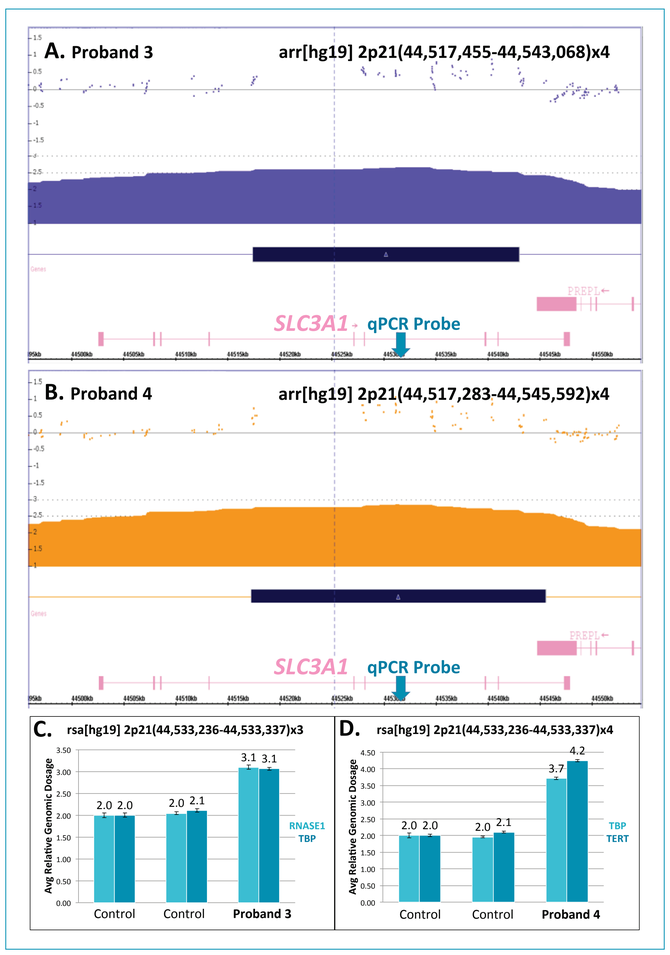

Table 2. 24.5% of the possible CNVs identified by microarray analysis did not confirm by qPCR. Many CNVs calls that failed to confirm were recurring artifacts identified in a number of well known genes.

Gene	Mode	Size	CNV	ISCN
CHD7	AD	28 kb	DEL	arr[hg19] 8q12.1q12.2(61,585,541-61,613,375)x1
CNTNAP2	AR	28 kb	DEL	arr[hg19] 7q36.1(148,057,572-148,097,501)x1
COL3A1	AD	66 kb	DEL	arr[hg19] 2q32.2(189,824,312-189,889,823)x1
FOXP1	AD	27 kb	DEL	arr[hg19] 3p13(71,350,880-71,378,187)x1
GALC	AR	27 kb	DEL	arr[hg19] 14q31.3(88,402,959-88,429,690)x1
MYCN	AD	32 kb	DEL	arr[hg19] 2p24.3(16,063,186-16,095,671)x1
PIXT2	AD	35 kb	DEL	arr[hg19] 4q25(111,531,210-111,566,224)x1
PTEN	AD	66 kb	DEL	arr[hg19] 10q23.31(89,613,196-89,679,026)x1
RUNX2	AD	35 kb	DEL	arr[hg19] 6p21.1(45,290,633-45,325,579)x1
SHANK3	AD	92 kb	DUP	arr[hg19] 22q13.33(51,092,248-51,183,871)x3
TPO	AD	28 kb	DEL	arr[hg19] 2p25.3(1,460,139-1,488,103)x1
UBE3A	AD	29 kb	DUP	arr[hg19] 15q11.2(25,637,157-25,666,281)x3

Table 3. Familial inheritance can be helpful in determining the pathogenicity of a small CNV. A number of small CNVs resulting in deletion of one or more exons in genes associated with autosomal dominant disorders were shown to be inherited. Unfortunately, parental follow-up was limited with only 14.2% of families resulting in informative parental follow-up once a CNV was confirmed in the proband.

Parental Follow-up	Inherited	Maternal	Paternal	De Novo
Total = 49/343 (14%)	45 (92%)	24 (49%)	21 (43%)	4 (8%)

Gene	Mode	Size	CNV	ISCN
ANKRD1	AD	29 kb	DEL	arr[hg19 16q24.3(89,481,006-89,509,853)x1 mat
AUTS2	AD	48 kb	DEL	arr[hg19] 7q11.22(70,188,374-70,236,724)x1 mat
DMD	XL	210 kb	DEL	arr[hg19] Xp21.1(31,795,363-32,004,910)x1 pat
SDHB	AD	12 kb	DEL	arr[hg19] 1p36.13(17,378,934-17,391,108)x1 pat
SNRPN	AD	14 kb	DEL	arr[hg19] 15q11.2(25,070,846-25,084,944)x1 mat
ZFPM2	AD	20 kb	DEL	arr[hg19] 8q23.1(106,634,384-106,659,140)x1 pat

IV. Conclusion

qPCR is an effective method for confirmation of CNVs identified by microarray analysis. In total, 24.5% of CNVs called by microarray did not confirm by qPCR analysis. Many possible CNVs that failed to confirm were within the same genes, suggesting common underlying DNA elements that contribute to false positive results. Our analysis of *GALC* deletions called by microarray analysis demonstrates the utility of qPCR in differentiating between overlapping common artifacts and well characterized deletions associated with disease. In addition, our analysis was helpful in differentiating triplications from duplications. This is important since triplications of dosage sensitive genes can have significant phenotypic consequences, while triplications due to intragenic duplications of autosomal recessive genes such as *SLC3A1* can lead to disease when carried in trans.

Parental follow-up studies add to the interpretation of CNV pathogenicity. Unfortunately, parental follow-up was limited to just 49 of 343 (14%) cases reported. 45 of 49 (92%) CNVs followed up by qPCR analysis were inherited from a presumably normal parent and no trend was observed in either the type of abnormality or parent of origin. However, a number of small CNVs involving one or more exons within autosomal dominant genes were inherited, suggesting that not all exonic CNVs within haploinsufficient genes are deleterious.

V. References

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