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Investigation of a Patient with a Partial Trisomy 16q Including the Fat mass and Obesity Associated gene (*FTO*): Fine Mapping and *FTO* Gene Expression Study

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Abstract

A female patient with a partial trisomy 16q was described previously. Her clinical characteristics included obesity, severe anisomastia, moderate to severe mental retardation, attention deficit hyperactivity disorder, dysmorphic facies, and contractions of the small joints. In this paper, we describe a more detailed analysis of the genetic anomaly in this patient. We were particularly interested in the involvement of the fat mass and obesity associated gene (*FTO*) in her duplication. Single nucleotide polymorphisms in *FTO* have recently been associated with obesity. The breakpoints of the duplication were precisely mapped using high resolution oligonucleotide array comparative genomic hybridization (CGH). We found that the duplication spans 11.45 Mb on 16q11.2 to 16q13 and it includes *FTO*. The increased copy number of *FTO* was confirmed with a qPCR on genomic DNA of the patient. We investigated the influence of the increased *FTO* copy number on *FTO* gene expression in immortalized lymphocytes from the patient using qPCR. No evidence of increased *FTO* expression was detected in the patient's lymphocytes. We discuss these findings and shared phenotypic features of patients with overlapping 16q duplications, as well as candidate genes for some of the clinical manifestations.

Keywords

16q duplication; obesity; *FTO*; CGH; qPCR; maternal imprinting; *SLC6A2*; ADHD; *BBS2*

INTRODUCTION

In 2000, Stratakis et al. described a 22-year-old female patient with a partial trisomy for the long arm of chromosome 16. The patient suffered from obesity, severe anisomastia, moderate to severe mental retardation, and several developmental abnormalities. Her genetic

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anomaly was investigated with the technologies that were available at that time: high resolution karyotype analysis, densitometric analysis of genetic markers, and fluorescence *in situ* hybridization (*FISH*). The high resolution karyotype revealed additional chromosomal material in the middle of the long arm of chromosome 16. This anomaly was not present in the karyotype of the patient's mother or that of her sister. The father was not available for karyotype analysis. Densitometric analysis of polymorphic markers showed that the duplication was on the maternally derived chromosome 16, resulting in two maternal copies and one paternal copy of the region. The location of the duplication was confirmed by *FISH* using as probes bacterial artificial chromosomes containing markers that map to 16q.

This patient is of special interest for the field of obesity research because her duplicated region is close to the location of the fat mass and obesity associated gene (*FTO*). Common variants in this gene are associated with obesity in various populations [Dina et al., 2007; Frayling et al., 2007; Wing et al., 2009]. Loss of *fto* in mice has recently been shown to lead to postnatal growth retardation and a significant reduction in adipose tissue and lean body mass [Fisher et al., 2009]. At present, the functional role of *FTO* in human energy homeostasis is unclear. Patients with a duplication of the gene may shed some light on this issue. The results that were described in the year 2000 do not have sufficient resolution to determine if *FTO* is included in the duplication of the patient. In the present study, we performed fine mapping of the genetic anomaly using oligonucleotide array comparative genomic hybridization (CGH). This analysis enabled us to confirm the inclusion of *FTO* in the duplication. This was confirmed with quantitative real-time PCR (qPCR) at the level of genomic DNA. Remarkably, the increased *FTO* copy number in the patient's genomic DNA did not give rise to an increased copy number of *FTO* mRNA molecules in her lymphocytes as measured with qPCR. We speculate that this may be the result of maternal imprinting of the gene. In addition, we review clinical findings in patients with overlapping 16q duplications to determine shared phenotypic features.

MATERIALS AND METHODS

Clinical Presentation of the Patient

Detailed clinical characteristics of the patient were described in a previous publication [Stratakis et al., 2000]. In short, the patient was a 22-year-old female with several developmental defects, obesity, and severe anisomastia. She had a normal birth and early infancy with developmental delay becoming evident after the first year of life. Developmental defects included moderate to severe mental retardation, dysmorphic facies (including a beaked nose, small mouth, and anteverted nares), dental hypoplasia with small teeth and large interdental spaces, an enlarged left atrium, an intensely hoarse voice due to vocal cord soft tissue nodules, a growing umbilical hernia, gastroesophageal reflux, clinodactyly, contractions of the small joints (fingers and toes), and hyperextensibility of large joints. Other chronic problems included attention deficit disorder, recurrent urinary tract infections, constipation, and severe hyperopia requiring bifocal lenses. The patient had a stocky body habitus. She was overweight during childhood (BMI ~95th%). Growth charts of the patient are provided in Supplementary information 1. At the age of 32, she had a BMI of 35.2 kg/m², blood pressure was 133/86, fasting plasma glucose 78 mg/dl, insulin 23.8 microunits/ml, c-peptide 3.8 ng/ml, leptin 20.8 ng/ml, total cholesterol 205 mg/dl, triglycerides 467 mg/dl, HDL 53 mg/dl, and LDL 90 mg/dl. Resting Metabolic Rate as measured by indirect calorimetry at the age of 34 was 1730 +/- 80 kcal/day, which is not significantly different from the value predicted by Harris and Benedict [1919] and Fleisch [1951] (1780 kcal/day). RQ was 0.85. Breast development was asymmetric, which led to left breast surgical reduction at age 15 yr. Both parents and an older sister were essentially healthy, although the father died of complications of liver cirrhosis due to chronic alcoholism.

Array Comparative Genomic Hybridization

Genomic DNA was extracted from peripheral blood as previously described [Stratakis et al., 1998]. We used a pool of genomic DNA from 18 healthy female VUmc locals as a reference. The hybridization was performed on a human genome CGH Agilent 244K custom array. The array contained 199,000 *insitu* synthesized 60-mer oligonucleotide probes representing unique locations for chromosome 16 and 42,193 unique oligonucleotides of the standard human genome CGH 4x44K Agilent array (Agilent Technologies, Palo Alto, USA). The average coverage for chromosome 16 was one probe every 500 bp. The array contained 1,416 probes in the *FTO* locus. The standard human genome probes span both coding and non-coding regions, with emphasis on well-characterized genes, particularly cancer-relevant genes. Resolution of this probe set is approximately one probe every 35 kb. Hybridization and image analysis were performed as described previously [Buffart et al., 2008]. The Agilent CGH-v4_95_Feb07 protocol was applied using default settings. The CGH data have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE12741).

CGH data were analyzed using CGH Analytics 3.5.14 with the annotation build 18 of the human genome. A filter with a minimum of three probes in a region and a minimum average absolute log₂ ratio for the region of 0.3 was used. We checked whether the chromosomal regions showing gain or loss in the patient overlapped with genomic imbalances reported in healthy individuals in the database of genomic variants (<http://projects.tcag.ca/variation/cgi-bin/gbrowse/hg18>; using human genome build 36). Presence of the CNV in one of these databases suggests that the aberration is not pathogenic because the information in the database of genomic variants is derived from healthy individuals [Lee et al., 2007]. We used NCBI's Map Viewer (<http://www.ncbi.nlm.nih.gov/projects/mapview/>) to inspect the genes in the chromosomal regions showing gain or loss.

Quantitative Real-time PCR to Confirm the Increased Copy Number of *FTO*

We used qPCR for *FTO* copy number quantification according to the procedure of Hoebeeck *et al.* [2007]. Primers were designed to amplify a part of *FTO* (Table I). The genes encoding syndecan-4 precursor (*SDC4*) and zinc finger protein 80 (*ZNF80*) were used as reference genes (Table I). Real-time PCR amplification mixtures (15 µl) contained 20 ng genomic DNA, 7.5 µl *POWER SYBR*[®] Green PCR master mix (Applied Biosystems), and 4.5 pmol of each primer. Reactions were run on a 7900HT Sequence detection system (Applied Biosystems). The thermocycler profile was: 2 minutes at 50°C, 10 minutes at 95°C, 40 cycles at 95°C for 15s and 60°C for 1 minute. A melting curve was run after PCR amplification, to confirm the specificity of the products: 15s at 95°C followed by 15s at 60°C. The assay contained a no-template control and genomic DNA from the patient and three normal controls from the Centre d'Étude du Polymorphisme Humain (CEPH), <http://www.cephb.fr/>. Reactions were run in duplicate. Ct values of duplicate measurements were averaged. These average Ct values were transformed into normalized relative copy numbers as described in Hoebeeck *et al.* [2007].

FTO Gene Expression Study

Lymphocytes of the patient and controls were immortalized and grown in RPMI medium with 10–15% fetal bovine serum for optimal growth. We used cell lines from four healthy Dutch individuals as controls. Total RNA was isolated from the cell lines using RNA-Bee (Tel-Test Inc, Friendswood, Texas). RNA was treated with RNase-free DNase (Invitrogen) according to the manufacturer's instructions. Two independent cDNA samples were synthesized from the same RNA sample using random hexamers and SuperScript[™] III

reverse transcriptase according to the manufacturer's instructions (Invitrogen). For the patient, we generated three cDNA samples from two independent cell lines.

Two sets of primers were used to amplify parts of *FTO*: one in exon 2 and 3 (*FTO*₂₋₃) and one in exon 7 and 8 (*FTO*₇₋₈) (Table II). We used beta-2-microglobulin precursor (*B2M*) and 60S ribosomal protein L13a (*RPL13a*) as reference genes (see Supplementary information 2). Measurements were performed in duplicate in an assay that contained two cDNAs for all healthy controls, three cDNAs for the patient, and a no-template control. Real-time PCR amplification mixtures (10 µl) contained 1 µl template cDNA, 5 µl *POWER SYBR*[®] Green PCR master mix (Applied Biosystems) and 3 pmol of each primer. PCR cycling was performed as described above. Average Ct values were converted to relative normalized expression levels using the formulae that are listed in Supplementary information 3. The resulting normalized relative expression levels of the different cDNA samples were averaged for each individual (i.e. average of two values for controls and of three values for the patient).

RESULTS

Comparative Genomic Hybridization and qPCR on genomic DNA

We were able to determine the extent of the duplicated region in the patient using array CGH. The duplication spanned 11.45 Mb on 16q11.2 to 16q13 (Figure 1). The proximal breakpoint was close to the centromere, between physical position 45.026 and 45.058 Mb. This region does not contain an annotated gene. The distal breakpoint was located between position 56.506 and 56.507 Mb, in the gene encoding the cyclic nucleotide-gated channel, beta-1 (*CNGB1*). This gene is no obvious candidate for any of the phenotypes of the patient. There are 149 annotated genes in build 36.3 of the human genome in the region between 45 and 57 Mb on chromosome 16. These include *FTO*, which is located at 52.3–52.7 Mb. The increased copy number of *FTO* was confirmed with qPCR targeted at *FTO* using genomic DNA as a template. *FTO* copy number in the patient was 1.5 times higher than the copy number in a CEPH control (Figure 2). The duplicated region contains at least two additional genes that may be of interest for the clinical features of the patient: the norepinephrine transporter (*SLC6A2*) and the *BBS2* gene. Both will be described in the discussion section of this paper. In addition to the large duplication, we detected several smaller aberrations, six of which were not listed in the database of genomic variants (Supplementary information 4).

FTO Gene Expression Study

We measured *FTO* expression in immortalized lymphocytes of the patient and four control cell lines using qPCR. Average Ct values for *FTO* were approximately seven cycles higher than the average Ct values for the reference genes. In other words, *FTO* expression was approximately seven-fold lower than expression of the reference genes in the investigated cells. Expression levels measured with the primers in exon 2 and 3 were similar to those measured with exon 7 and 8. *FTO* expression in immortalized lymphocytes of the patient was within the range of what we observed in normal controls (Figure 3; results for the individual cDNA samples are presented in Supplementary information 5). It seems that the expression of *FTO* in lymphocytes of the patient is not affected by the increased copy number at the level of genomic DNA.

DISCUSSION

Experimental Findings

We used oligonucleotide array CGH to study the genomic anomaly of our patient in detail. Her large duplication was mapped to the physical position of 45.1 Mb to 56.5 Mb. The

duplication clearly involved *FTO*, which was confirmed by qPCR on genomic DNA of the patient. The increased *FTO* copy number did not seem to give rise to increased expression of the gene in immortalized lymphocytes of the patient, as measured with qPCR. There are several possible explanations for this apparent discrepancy. First, immortalized lymphocytes are not the optimal cell type for our study. *FTO* expression in the hypothalamus, adipose tissue, and in pancreatic islets is more interesting from the energy balance point of view. The expression in lymphocytes may not be representative of expression in these more relevant tissues. We were not able to study *FTO* expression in these tissues in the patient. In addition, immortalized lymphocytes are also suboptimal because the transformation may alter expression patterns. Second, our results demonstrate that there is substantial variation in *FTO* expression between normal healthy controls. This may indicate that regulatory elements that are located outside of the duplicated region are important in controlling *FTO* expression. Third, we speculate that the results could be explained by maternal imprinting of *FTO*. The microsatellite marker analysis that we described in our previous paper showed that the duplication of the patient was on the maternally derived chromosome 16. We would not expect to observe elevated *FTO* expression in the patient compared to controls if maternal copies of *FTO* would be silenced due to maternal imprinting. *FTO* is indeed listed as a candidate maternally imprinted gene in mice in the Expression-based Imprint Candidate Organizer (<http://fantom2.gsc.riken.jp/EICODB/>). This database contains candidate imprinted genes identified by the RIKEN full-length mouse cDNA microarray study [Nikaido et al., 2004]. In this study, genes were identified as potentially imprinted by comparing mRNA expression profiles between parthenogenetic and androgenetic embryos [Mizuno et al., 2002]. Imprinting may have important implications for the interpretation of the genetic association studies that have appeared in the past few years, including a lower-than-expected power.

Involvement of Increased *FTO* Copy Number in Obesity

Phenotypic abnormalities in patients with chromosomal duplications can be the result of the disruption of genes localized at the boundaries of the duplicated region or of increased dosage of the involved genetic material. The first often gives rise to unique phenotypes because the boundaries of the lesions usually differ between patients. The latter is expected to result in phenotypes that are shared by patients with duplications in the same region. Proximal duplication of 16q has been reported in a small number of patients [Barber et al., 2006; Engelen et al., 1999; Fryns, 1990; Gustavsson et al., 2007; Mascarello and Hubbard, 1991; Ren et al., 2005; Romain et al., 1984; Trimbom et al., 2006; Verma et al., 1997]. Obesity is a consistent finding in these patients (Figure 4). In addition, obesity cosegregated with the chromosomal abnormality in family 1 in the study of Barber et al. [2006]. It is therefore tempting to conclude that increased copy number of *FTO* is associated with obesity in humans. This would be in line with the recent finding of reduced obesity in *fto*-deficient mice [Fisher et al., 2009]. However, the results should be treated with caution for several reasons. First, obesity is a very common observation in patients with chromosomal abnormalities. Environmental factors may play a large role in this because these patients often live in institutions and because their clinical problems may prevent them from exercising enough. Second, as outlined above, it is unclear whether the increased *FTO* copy number of the patient results in altered expression of *FTO* in relevant tissues. Third, the proximal part of the long arm of chromosome 16 contains two obesity candidate genes in addition to *FTO*. The gene *RPGRIP1L* (which is adjacent to *FTO*) has been shown to have an expression pattern identical to *FTO* and it may play a role in energy homeostasis as well [Stratigopoulos et al., 2008]. Loss-of-function mutations in the *BBS2* gene (position 55.08 to 55.11 Mb) have been found in families with Bardet-Biedl syndrome [Nishimura et al., 2001]. Bardet-Biedl syndrome is a genetically heterogeneous autosomal recessive disorder with clinical features including obesity, pigmented retinopathy, polydactyly,

hypogonitalism, mental retardation, and renal anomalies (OMIM #209900). The effect of an increased copy number of this gene is unknown.

Shared Phenotypic Features of Patients with Proximal 16q Duplications

Complete trisomy of 16q is a relatively well-described condition [Chen et al., 2004; Masuno et al., 2000; Ridler and McKeown 1979]. Clinical findings in these patients include pre- and postnatal growth retardation, failure to thrive, psychomotor retardation, congenital heart disease, anorectal and urogenital abnormalities, flexion deformities of joints, craniofacial dysmorphism (including a high forehead, downward slanting palpebral fissures, malformed low set ears, and micrognathia), and early death. Most patients with distal duplication of 16q share many of these clinical findings, suggesting that more distally located genes are responsible for these phenotypes [Barber et al., 2006; Brisset et al., 2002; Chen et al., 2005; Hatanaka et al., 1984; Houlston et al., 1994]. The phenotype of patients with proximal duplication of 16q is less specific [Barber et al., 2006; Engelen et al., 1999; Fryns, 1990; Gustavsson et al., 2007; Mascarello and Hubbard, 1991; Ren et al., 2005; Romain et al., 1984; Trimborn et al., 2006; Verma et al., 1997]. In addition to obesity, most of these patients presented with short stature, developmental and speech delay, learning difficulties, and behavioral problems (Supplementary information 6). The patients do not seem to share facial characteristics or joint contractures (Supplementary information 6). Patients with proximal duplication of 16q frequently have behavioral problems including aggression and attention deficit hyperactivity disorder (ADHD) [Barber et al., 2006]. Our patient suffered from ADHD. The duplicated region of our patient contains a strong candidate gene for these behavioral problems: the gene encoding the norepinephrine transporter (*SLC6A2*, position 54.25 to 54.30 Mb). This transporter terminates noradrenergic signaling by re-uptake of released norepinephrine into presynaptic terminals. Abnormal norepinephrine transmission has been hypothesized to contribute to ADHD [Biederman and Spencer, 1999]. The norepinephrine transporter is a target of drugs used to treat psychiatric disorders including attention deficit hyperactivity disorder (ADHD) and eating disorders [Spraggs et al., 2005]. It will be very interesting to further study the relationship between increased copy number of *SLC6A2* and behavioral problems in patients with proximal 16q duplications.

Anisomastia

The most prominent phenotype in our patient was anisomastia. Her anisomastia was very severe, suggesting a genetic cause. Breast abnormalities have not been reported in any other patient with chromosome 16 abnormalities. This suggests that it may be caused by gene disruption at the borders of the duplication. Our analysis did not enable us to determine if genes are disrupted by the duplication. Our karyotype analysis revealed that the additional chromosomal material is located in the middle of the long arm of chromosome 16 [Stratakis et al., 2000]. There are no obvious candidate genes for anisomastia in this region. In addition to the large duplication, we detected several smaller aberrations. Most of these were considered genomic variants without any clinical significance because they were also found in healthy individuals. Six aberrations were not listed in the database of genomic variants. Future studies will address the possible involvement of these aberrations in clinical problems that are unique to this patient, such as anisomastia. Risk assessments for the smaller copy number variable regions should involve testing these regions in the healthy mother and sister of the patient.

Conclusion

In conclusion, we have precisely mapped the breakpoints of the duplication of a patient that was first described in 2000 using CGH. Our results confirm the inclusion of *FTO* in the duplication. We found no evidence of increased *FTO* expression in the patient. We speculate

that these results can be explained by maternal imprinting of the *FTO* locus. Future studies will have to confirm this hypothesis. In addition, we provide a review of clinical findings in patients with 16q duplications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Graphical summary of results of oligonucleotide array comparative genomic hybridization for chromosome 16. The horizontal axis represents the test/reference intensity ratio after normalization and $\log_2$ transformation. Black dots correspond to probes with $\log_2$ ratios near zero; red dots correspond to probes with positive ratios (i.e. increased copy number); and green dots correspond to probes with negative ratios (i.e. decreased copy number). The blue bars on the extreme left and right side of the figure mark the aberrant regions. The patient has a large duplication of 16q11.2 to 16q13. In addition, she has 25 smaller aberrations (17 gains; 8 losses) dispersed over chromosome 16. The smallest aberrations are not visible in this figure. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com].

Figure 2.

FTO copy number in genomic DNA of the patient and three healthy controls from the Centre d'Étude du Polymorphisme Humain (CEPH). Copy numbers are relative to CEPH 1331-01, which is an arbitrary choice. Relative *FTO* quantities were normalized to the relative quantity of reference genes *ZNF80* and *SDC4*. Error bars represent standard deviations.

Figure 3.

FTO expression level in immortalized lymphocytes of the patient (p) and four controls (c1-4) determined by qPCR. Gene quantities were calculated relative to the average Ct. Data were normalized to transcript levels of *RPL13a* and *B2M*. *FTO* expression was measured with primers in exon 2 and 3 of the gene (light bars) and with primers in exon 7 and 8 (dark bars). Expression levels of controls are based on duplicate measurements of two independent cDNAs. Expression level of the patient is based on duplicate measurements of three independent cDNAs. Error bars represent standard errors of the mean of two (controls) and three (patient) cDNA samples.

Figure 4.

Obesity in patients with 16q duplications that overlap with the duplication of our patient. An ideogram and a ruler with positions in megabases are included at the top of the figure. *FTO* is located at position 52.3–52.7 Mb. Shades of grey represent the precision of the method that was used to determine the extent of the duplication (see legend). Further details about the patients are provided in Supplementary information 6. No clinical details were provided for the patients of Mascarello and Hubbard [1991] and Ren et al. [2005], so these were excluded from the figure.

TABLE I

Primer pairs used for amplification of genomic DNA

Gene	Sequence (5'-3')	Position ^a	Fragment size (bp)
<i>FTO</i>	AGGTGCCAGTCACGAATTG	IVS8-2 to 1381	92
	AGAGGCATCGAAGCATCATC	1435 to 1454	
<i>SDC4</i>	ACCGAACCCAAGAACTAGAA	301–320	102
	GTGCTGGACATTGACACCT	383–402	
<i>ZNF80</i>	CAGGGACCAAGTAAAGACAC	103–122	105
	CTGCTGATGTCGAACAAGGA	188–207	

^aWe used the nomenclature recommended by den Dunnen and Antonarakis [2001]: the A of the ATG start codon is designated number 1; the nucleotide 5' to this A is numbered -1; and the nucleotide 3' of the translation termination codon is *1. IVS= intervening sequence. Positions in introns refer to the nearest exon. Positions are based on ensembl transcripts ENST00000268349 (*FTO*), ENST00000372733 (*SDC4*), and ENST00000308095 (*ZNF80*).

TABLE II

Primer pairs used for amplification of cDNA

Gene	Sequence (5'-3')	Position (exon) ^a	Fragment size (bp)
<i>FTO</i>	ACACTTGGCTCCCTTATCTG	2	100
	GATACACTGCTGGCTTCTC	3	
<i>FTO</i>	TGGAAGCACTGTGGAAGAAG	7	113
	GGCAAGGATGGCAGTCAAG	8	
<i>RPL13A</i>	CCTGGAGGAGAAGAGGAAAGAGA	7	126
	TTGAGGACCTCTGTGTATTGTCAA	8	
<i>B2M</i>	CGTGTGAACCATGTGACTTTG	2	81
	TTCAAACCTCCATGATGCTG	3&4	

^aExons numbers are based on ensembl transcripts ENST00000268349 (*FTO*), ENST00000270634 (*RPL13a*), and ENST00000349264 (*B2M*).