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Identification of ectodysplasin-A receptor gene deletion at 2q12.2 and a potential autosomal MR locus

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Mental retardation (MR) is not a common feature observed in patients with classical ectodermal dysplasias (EDs). Several genes responsible for EDs and MR have been identified. However, the causation has yet to be identified in a significant number of patients with either ED or MR. Here, we have molecularly characterized a *de novo* balanced translocation t(1;6)(p22.1;p22.1) in a female patient who had mild features of ED with hypodontia, microcephaly, and cognitive impairment. Mapping of the translocation breakpoints in the patient revealed no obvious causative gene for either ED or MR. Whole genome array CGH analysis unveiled two novel submicroscopic deletions at 2q12.2 and 6q22.3, unrelated to the translocation in the patient. The 2q12.2 deletion contains a known ED gene, ectodysplasin-A receptor (EDAR), and the loss of one copy of this gene is considered to be responsible for the ectodermal phenotype in the patient. It is plausible that a potential autosomal MR gene deleted at 2q12.2 or 6q22.3 is likely the cause of the neurodevelopmental defects in the patient.

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Introduction

Mental retardation (MR) is the most common developmental disability, affecting intellectual and adaptive functions in approximately 1–3% of the population. Yet, the underlying cause of MR is established in less than half the cases.^{1,2} The finding of MR among almost all patients with autosomal microdeletions and the association of MR with submicroscopic alterations in the subtelomeric region

of the autosomes indicates the ubiquitous distribution of autosomal genes that influence intelligence.^{3,4} Thus far, a limited number of patients have been reported who have both MR and classical ectodermal dysplasia (ED). EDs are a heterogeneous group of developmental disorders that affect the secondary differentiation of ectodermally derived structures. The classical EDs include a combination of defects in hair, missing or abnormally shaped teeth, and a reduction in the number of sweat glands. An early diagnosis of ED is essential for the management of the disease in patients and is a prerequisite for reducing mortality that primarily results from hyperthermia and respiratory tract infections.

Several genes involved in ED have been identified in recent years. The most common form of ED, the X-linked EDA, is caused by defects in ectodysplasin-1.^{5,6} This soluble tumor necrosis factor ligand binds to the EDA receptor,

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triggering a signaling cascade through NF- κ B to activate target gene expression.^{7–9} The EDA signaling pathway has been well established, but work is now just beginning to identify genes that are the targets of this pathway.^{10–15} The genes responsible for a large number of EDs and associated disorders have yet to be identified.

Chromosomal abnormalities including microduplications and microdeletions have been found in a significant number (10–15%) of individuals with MR and developmental disabilities (MR/DD).¹⁶ It is plausible that the microdeletions and microduplications harbor gene(s) responsible for the observed phenotypes in these patients. Furthermore, analysis of patients with submicroscopic deletions/duplications has facilitated identification of small regions of overlap linked to specific phenotypic characteristics and subsequent isolation of the causative genes. Recently, disruption of the *Eu-HMTase1* gene has been shown to be associated with the 9q34 subtelomeric deletion syndrome, and a duplication of the *HSD17B10* and *HUWE1* genes have been found in association with X-linked MR.^{17–18}

We have analyzed a patient who has mild ectodermal defects and a (1;6) (p22.1;p22.1) balanced translocation.¹⁹ Although it was speculated that one of the gene(s) affected by the translocation results in the patient's clinical features, no known ED gene was found at either translocation breakpoint. However, utilizing array CGH we identified two submicroscopic deletions at 2q12.2 and 6q22.3, which included the deletion of the ectodysplasin-A receptor (*EDAR*) gene at 2q12.2.

Materials and methods

Case report

CMS8770 has previously been reported.¹⁹ In brief, this female patient was born at 37 weeks to non-consanguineous parents after an uncomplicated pregnancy. She had speech delay, not saying any words at 24 months of age. Examination at 6.5 years of age revealed mild MR with a full scale intelligence quotient (IQ) of 73, a verbal IQ of 57, and a performance IQ of 62 on the WISC-III.¹⁹ Her head circumference was below the 2nd centile and height and weight were at the 25th centile. She showed esotropia and hyperopia. Ectodermal features included absent of lower incisors and some permanent teeth, thin sparse, straight, and blond scalp hair, sparse eye lashes, poor nail growth, and dry skin with decreased sweating. The patient's karyotype was determined to be 46,XX,t(1;6) (p22.1;p22.1). Parents were reported to be clinically normal and had normal karyotypes.

Molecular cytogenetics

Genomic contigs, clones, markers, mapping, and sequence information were obtained from the NCBI databases (<http://www.ncbi.nlm.nih.gov/genome/guide/human>). BAC

and PAC clones were obtained from commercial resources. DNA was isolated using Qiagen Mini-Prep columns and was labeled by incorporation of digoxigenin-11-dUTP or biotin-16-dUTP (Boehringer Mannheim) by nick translation using DNA polymerase (Life Technologies). For *in situ* hybridization, metaphase chromosome spreads were obtained from lymphoblastoid cells using conventional methods, and fluorescence *in situ* hybridization (FISH) was performed essentially as described previously.^{20,21} Commercially available chromosome-specific labeled centromeric alphoid probes were used to identify specific chromosomes.

Quantitative real-time RT-PCR

Total RNA was extracted from lymphoblastoid cell lines using the GenElute™ Mammalian Total RNA Purification Kit (Sigma), according to the manufacturer's protocols. Samples were treated with TURBO™ DNase (Ambion Inc.) using 2 units/50 µg RNA at 37°C for 30 min. RNA was subsequently purified using the RNeasy Mini Kit (Qiagen), following the manufacturer's protocols.

Quantitative Real-Time PCR was performed for two genes, ABCD3 and ZNF184, using the iScript™ One-Step RT-PCR Kit with SYBR® Green (BioRad) on an iCycler iQ™ Real-Time PCR detection System (BioRad). The primer pair sequences are provided upon request.

Whole genome array CGH and quantitative PCR

Genomic DNA was purified using the Zymo DNA Clean & Concentrator™ kit (ZymoResearch, Orange, CA, USA). DNA concentration and purity was determined with an ND-1000 Spectrophotometer (NanoDrop Technologies, Delaware). DNA was labeled, hybridized to the Affymetrix GeneChip® Human Mapping 250K Nsp Array, and washed according to the manufacturer's commercial protocol (Affymetrix). Data quality assessment was generated using the DM (Dynamic Modeling) calling algorithm within the Affymetrix GTTYPE software package. Intensity information and copy number status for each probe set was extracted using Affymetrix Genotyping Console™ Version 2.0 software.

Genomic quantitative PCR was performed using iQ SYBR Green Supermix (Bio-Rad) on an iCycler iQ real-time PCR detection system (Bio-Rad), as described previously.²¹

Results

Delineation of translocation breakpoint regions at 1p22.1 and 6p22.1 and analysis of candidate genes for ED and MR

Using FISH, we localized the 1p22.1 breakpoint to within the clone RP11–366L18 (Figure 1a and c). Clones RP11–86H7, RP11–465K1, RP11–57H12, and RP11–146P11 mapped proximal and clone RP11–148B18 mapped distal to the 1p22.1 breakpoint. Subsequent analysis using the clone RP11–6J23 (Figure 1a and d), narrowed the 1p22 breakpoint to within a 60 kb region of overlap between

Figure 1 Delineation of translocation breakpoint regions and candidate genes. Physically mapped BAC clones used in FISH analysis of chromosome 1 (a) and chromosome 6 (b) are indicated. Clones spanning the breakpoint are shown in red. Candidate genes from the region flanking the breakpoint regions are noted with arrows showing direction of transcription. FISH analysis of the 1p22.1 breakpoint spanning clones RP11-366L18 (green) (c) and clone RP11-6J23 (green) (d). Chromosome 1 centromeric control probes are shown in red. FISH analysis of the 6p22.1 breakpoint spanning clone RP11-106D17 (green) (e). Chromosome 6 centromeric control probes are shown in red.

clones RP11-6J23 and RP11-366L18 (Figure 1a). We mapped the 6p22.1 translocation breakpoint to a 140 kb region within clone RP11-106D17 (Figure 1b and e). Clones RP3-425P12, RP1-45P21, RP1-153G14 mapped distal and clones RP1-97D6, RP1-29K1, RP11-999A17, and RP11-150A6 mapped proximal to the 6p22 breakpoint.

Five known annotated genes, *F3*, *SLC44A3*, *ABCD3*, *ABCA4*, and *ARHGAP29*, were identified within 800 kb of the 1p22.1 translocation breakpoint region (Figure 1a). The chromosome 6 breakpoint region is in a relatively gene poor region. There are two known Zinc-finger genes, *ZNF184* and *ZNF204* near the breakpoint region (Figure 1b). A hypothetical gene *LOC645950* and the

PRSS16 gene are located telomeric to the *ZNF204* (Figure 1b and data not shown). Attempts to amplify the putative *LOC645950* gene using primers designed from the EST databases were unsuccessful.

Candidate gene expression was measured by real-time RT-PCR. Four candidate genes could not be checked by this method because of no (*ABCA4*, *ARHGAP29*, and *ZNF204*) or limited (*SLC44A3*) expression in lymphoblastoid cell lines. The two genes that could be checked through real-time RT-PCR were *ZNF184*, the closest candidate gene to the chromosome 6 breakpoint region, and *ABCD3*, which partially overlaps with the telomeric side of the chromosome 1 breakpoint region. Real-time RT-PCR results

Figure 2 Normalized relative expression for *ABCD3* and *ZNF184*. No significant difference in expression was seen between the patient, CMS8770, and four control individuals (CMS4633, CMS6265, CMS5865, and CMS5863) for either gene. Real-time RT-PCR was performed in triplicate. Data were analyzed using the iCycler iQ™ software to generate a standard curve for each gene and calculate the fluorescence generated from the experimental primer pairs relative to the reference gene *RPII* using the comparative C_t method.

for these genes are shown in Figure 2. Neither gene appears to have an altered transcript level in the patient as compared to controls.

To test for the presence of a chimeric transcript, we performed northern analysis on the patient's RNA using a probe spanning exons 1–3 on *ABCD3*. The northern blot showed only the 4 kb band size of a normal *ABCD3* transcript (data not shown).

Identification of two submicroscopic deletions, unrelated to the translocation, and deletion of the *EDAR* gene

We analyzed DNA from cell lines for CMS8770 using an Human Mapping 250K Nsp Array and identified two

submicroscopic deletions at 2q12.2q14.1 (106.5–117.3 Mb) and 6q22.3 (127.5–130 Mb) (Figure 3). We looked at the log2 ratio and corresponding negative Log10 *P*-values and determined the extent of the deletion in each case. We verified the deletions at 2q12.2q14.1 and 6q22.3 by real-time genomic PCR using DNA isolated from patient's blood (Figure 3 and data not shown). The deletion regions at 2q12.2 and 6q22.3 contain multiple annotated genes (Figure 3a and b). The 2q12.2 deletion contains a known ectodermal dysplasia gene, *EDAR*, and is therefore considered responsible for the ectodermal phenotype in this patient (Figure 3b).²² Furthermore, we confirmed the deletion of the *EDAR* gene using real-time genomic PCR (data not shown). The 6q22.3 deleted region contains

Figure 3 Array CGH profiles of chromosome 2 (a) and chromosome 6 (b) in patient CMS8770 are shown. The location of deletion at 2q12.2 and 6q22.3 are indicated by gray bars. The log₂ ratio of about -0.5 indicates the deletions are heterozygous. A representative gene content of the deletion is shown in each case. For a complete list of genes, see the gene content map of the corresponding region (NCBI). The deletions at 2q12.2q14.1 and 6q22.3 were further confirmed by real-time genomic PCR using DNA isolated from patient's blood. Genes used for real-time genomic PCR are indicated in bold types. The location of the *EDAR* gene on chromosome 2q12.2 is indicated by the gray arrow.

several genes and could possibly include a gene responsible for the neurodevelopmental defects in the patient (Figure 3b).

Discussion

This study has identified the translocation breakpoint critical regions in a patient with ED, mild MR, and a *de novo* t(1;6)(p22.1;p22.1) translocation. The closest gene to the chromosome 1 breakpoint region is *ABCD3*. Real-time RT-PCR did not indicate changes in expression of this gene in lymphoblastoid cells from the patient and northern analysis failed to show the presence of a chimeric transcript resulting from the translocation. Taken together, these results suggest *ABCD3* is not physically disrupted by the translocation.

There is no known gene present in the chromosome 6 translocation breakpoint region. This suggests that any deleterious effect of the translocation would likely be the result of a disruption in a distant transcriptional control site for genes flanking the translocation breakpoint region. As a result, expression of the target gene can be either upregulated or downregulated.

The two strong candidate genes that could be screened using mRNA derived from lymphoblastoid cell lines, *ZNF184* and *ABCD3*, showed no change in expression compared to controls. The ABC family of transporters have been linked to such human diseases as retinopathies, cystic fibrosis, and cardiomyopathies.²³ *ABCD3* is a peroxisomal membrane protein involved in the biogenesis of peroxisomes and the transfer of long-chain acyl-CoA across peroxisomal membranes.²⁴ The *ABCD3* gene was found to be mutated in the biochemical disorder Zellweger syndrome; however, this association remains to be confirmed.²⁵ Mutations in *ABCA4* have been associated with a wide phenotypic spectrum.²⁶ *ABCA4* mutations are linked to Stargardt disease, the most common juvenile macular dystrophy, autosomal recessive cone-rod dystrophy, and retinitis pigmentosa.^{26,27}

There is no known ED gene located in either breakpoint region. Of all of the candidate genes identified in this study, *ARHGAP29* is possibly the best candidate gene for the neurological findings in the patient if it is indeed affected by the translocation.²⁸ *ARHGAP29* is a GTPase which has been shown to regulate Rho activity.²⁹ Rho proteins act as directors of cytoskeleton organization in

dendrites, dentritic spines, and axons controlling the growth and development of these structures.²⁸ Several members of this family of proteins have been implicated in MR.²⁸ The *PRSS16* gene encodes a serine protease similar to the *PRSS12* autosomal MR gene³⁰ and thus appears to be a good candidate. However, it has been reported to be expressed exclusively in the thymus.³¹

F3 is known to be integral member of the clotting pathway and is considered a poor candidate gene. Little is known about the other three candidate genes, *ZNF184*, *ZNF204*, and *SLC44A3*. Initial studies of *ZNF184* expression show no difference in the patient and control, suggesting it is not affected by the translocation. *SLC44A3* is a membrane protein that is predicted to act as a solute carrier and expressed at low levels in both skin and brain (UniGene Hs.483423).

We also examined the possibility that the ED or the MR seen in the patient is not the result of the translocation and is, in fact, the result of a separate cause. In several patients with apparently balanced translocations, additional chromosomal aberration(s) have been previously reported either at the breakpoint or at a remote location unrelated to the translocation.³² Whole genome array CGH analysis in this patient indeed revealed two unique submicroscopic deletions. The deletion at 2q12.2 included the EDAR gene. The EDAR gene has been previously reported to cause both dominant and recessive forms of EDs and thus is likely responsible for the ectodermal defects observed in patient CMS8770. We anticipate that the mild neurodevelopmental problems in the patient are likely due to a deletion of a second gene. A review of the deleted genes revealed several strong candidates for MR such as *TLL* and *ACTR3* at 2q12.2 and *ARHGAP18* at 6q22.3. However, the mild neurodevelopmental problems due to the combined haploinsufficiency of a number of genes remain a possibility. Identification of mutations or other overlapping deletions in additional MR patients affecting the candidate genes will help pinpoint the molecular cause of the neurodevelopment phenotype seen in this patient. Our finding also signifies the utility of whole genome scan in cases where no genes are found to be affected by the *de novo* translocation breakpoints.

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