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Discipline Sciences de la vie

TITRE

BASES MOLECULAIRES ET PHYSIOPATHOLOGIQUES DE SYNDROMES AVEC ANOMALIES DU DEVELOPPEMENT ET DEFICIENCE INTELLECTUELLE

THESE

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INTRODUCTION

La déficience intellectuelle (DI) est un groupe de pathologies cliniquement et génétiquement hétérogène. Différents cadres ont pu être regroupés en discernant les étiologies acquises et constitutionnelles. Parmi les étiologies constitutionnelles, la cytogénétique et la dysmorphologie ont permis la définition d'une grande diversité de syndromes avec DI. Depuis la généralisation de l'usage de la CGH-array de nombreux nouveaux syndromes ont pu être décrit et un grand nombre de patients ont pu bénéficier d'un diagnostic génétique. Cette technique a démontré son impact dans l'identification de gènes candidats de DI syndromique ou isolée dans des cas sporadiques. Le travail de corrélation génotype-phénotype de ces anomalies doit comprendre un phénotypage pluridisciplinaire des patients. Cette problématique est multipliée par la mise en place en diagnostique du séquençage haut débit (SHD) et en particulier du SHD d'exome (SHD-E).

I. LA DEFICIENCE INTELLECTUELLE

A. Définitions

Dans la classification internationale des maladies actuelle (CIM10) de 1996, l'Organisation Mondiale de la Santé (OMS) définit le retard mental (RM) comme «un arrêt ou développement incomplet du fonctionnement mental, caractérisé essentiellement par une altération, durant la période du développement, des facultés qui déterminent le niveau global d'intelligence, c'est-à-dire des fonctions cognitives, du langage, de la motricité et des capacités sociales. Le retard mental peut accompagner un autre trouble mental ou physique, ou survenir isolément». Le terme de RM a depuis été remplacé par déficience intellectuelle (DI) afin de souligner qu'une déficience peut bénéficier d'une réhabilitation. D'autres définitions du RM, qui mettent un accent plus franc sur les capacités adaptatives, ont été proposées par l'American Association On Intellectual and Developmental Disabilities (AAIDD, http://www.aaidd.org). Les

définitions se basent sur 2 mesures: le fonctionnement intellectuel («l'intelligence théorique»), évalué par la mesure du quotient intellectuel (QI), et les compétences adaptatives («l'intelligence pratique»). Ces principes seront repris pour constituer la future CIM11 de l'OMS.

Le QI peut être évalué par divers tests d'intelligence générale faisant appel à un ensemble d'aptitudes verbales et non verbales. C'est un score normalisé obéissant à une distribution gaussienne, dont la moyenne est à 100 et la déviation standard à 15. L'erreur sur la mesure du QI est de l'ordre de 5. Par définition, il est anormalement faible au-delà de -2DS, soit inférieur à 70 (entre 70 et 75 si l'on tient compte de l'erreur sur la mesure: cf. classifications de l'AAID). Mathématiquement, cela définit que 2 à 3 % de la population présente une DI.

Les compétences adaptatives sont représentées par des domaines de la vie courante, nécessaires à l'intégration sociale. Dans la définition, 2 domaines doivent être limités parmi la communication, les soins personnels, les compétences domestiques, les habiletés sociales, l'utilisation des ressources communautaires, l'autonomie, la santé et la sécurité, les aptitudes scolaires fonctionnelles, les loisirs et le travail.

Actuellement, la classification nosologique de la DI répartie les personnes en 3 groupes de gravité croissante selon les chiffres de QI : DI légère entre 70 et 50, DI modérée entre 50 et 35 ; DI sévère entre 35 et 20; et DI profonde si le chiffre de QI est inférieur à 20. Lorsque la déficience est sévère/profonde, il est parfois nécessaire d'utiliser des échelles standardisées évaluant un quotient de développement (QD). L'AAIDD et d'autres groupes de réflexion encouragent l'usage du score de QI comme indicateur global de la gravité de la DI en terme de moyens nécessaires à la prise en charge (structures d'accueil, thérapeutiques psychotropes, etc.). Cependant, ils encouragent fortement la considération des scores des sub-tests afin d'adapter, de personnaliser la prise en charge. Ces scores permettent également des regroupements nosologiques cliniques.

B. Epidémiologie

La DI est un problème de santé publique concernant 2 à 3% de la population. Il a pu être estimé que 85% des personnes sont atteintes de DI légère, 10% de DI modérée, 4% de DI sévère et 2% de DI profonde (1). Cela pourrait représenter parmi les 800 000 naissances françaises annuelles 16 000 à 24 000 patients par an. En l'absence de malformation associée, la durée de vie des personnes est semblable à

celle de la population générale ou faiblement diminuée (2,3). Cependant, la présence d'une DI semble prédisposer au développement de pathologies cardiovasculaires, respiratoires et de démences cortico-sous corticales (4). En France, on peut estimer que 1 300 000 personnes sont affectées. Le coût global de la DI a été estimé à partir d'une cohorte de 2000 patients par le Center for Disease Control (CDC). En 2003, elle représentait aux Etats-Unis une dépense annuelle de 730 millions de dollars (URL : http://www.cdc.gov/mmwr/preview/mmwrhtml/mm5303a4.htm).

Les statistiques pédiatriques et adultes disponibles par l'INSEE sur les structures d'accueil du handicap montrent que 6 à 7 patients accueillis sur 10 ont comme motif d'entrée principal une DI. La moitié d'entre eux présente une DI modérée, 25% une DI légère et 25% une DI sévère ou profonde.

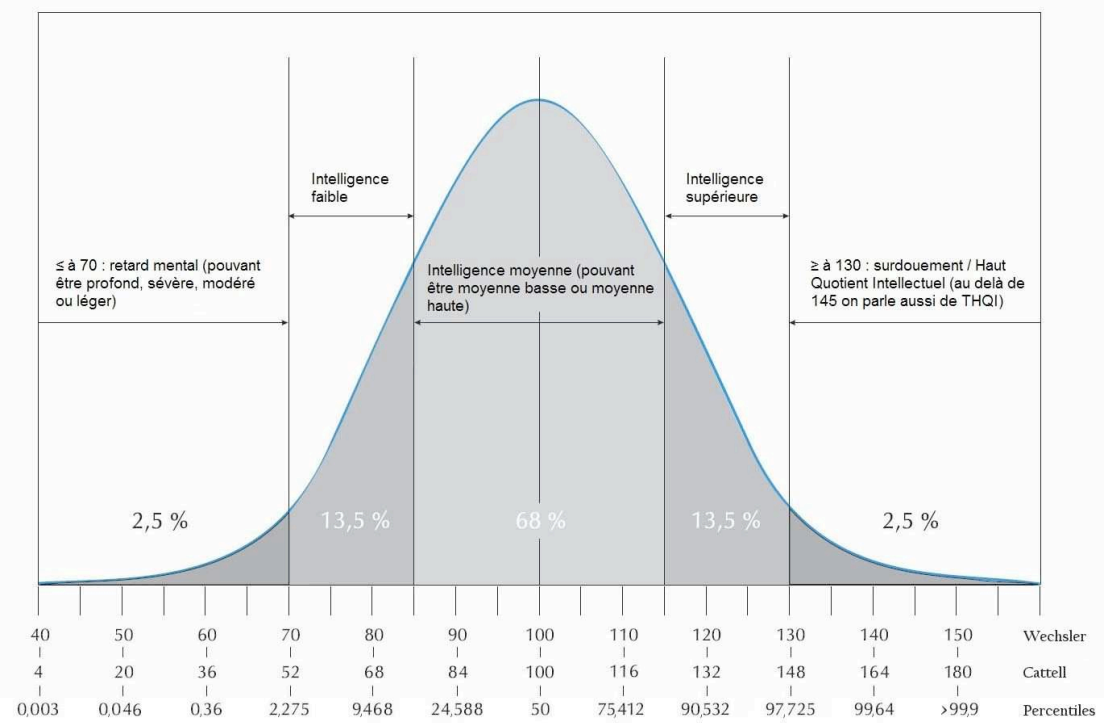


Figure 1 : Distribution gaussienne des chiffres de QI dans la population générale évaluée par les échelles de Weschler.

Environ 60% d'entre eux bénéficient d'emploi en Centre d'Aide pour le Travail (CAT), 20% participent à l'activité d'un foyer occupationnel et 20% en incapacité totale (http://www.insee.fr).

Cette classification par gravité continue permet une description globale de la population de patients avec DI. Cependant, elle ne permet pas de différencier les étiologies, orienter la prise en charge, préciser l'évolution clinique d'un individu ou d'un sous groupe de patients. Les groupes de gravité continue sont donc une population

d'étude très hétérogène et des indicateurs complémentaires individuels doivent être considérés.

C. Progrès dans les cadres étiologiques de la DI

Les étiologies rencontrées peuvent être acquises ou génétiques. Entre 2006 et 2007, 2 séries ont démontré qu'environ 40% des patients avec DI bénéficiaient d'un diagnostic étiologique. Strømme et ses collaborateurs ont rapporté le suivi d'une cohorte de 178 patients avec DI issus d'un recrutement initial de plus de 30 000 enfants (5). Dans cette cohorte, ils n'ont pas déterminé la cause de la DI dans 34% des cas. Parmi les 66% de cas diagnostiqués, 59% avait une origine étiquetée comme congénitale, incluant 35% de cause génétique étiquetée, 5% de cause acquise et 19% de cause syndromique non étiquetée. Les auteurs détaillent également 4,5% de causes périnatales, 3% de causes postnatales.

Le chiffre de 35% de cause génétique identifiée dans la DI était cohérent avec la littérature. Il correspond à différents cadres diagnostiques pouvant être regroupés selon : i) les causes chromosomiques, car le caryotype, puis les techniques de FISH, étaient l'examen paraclinique de référence ; ii) les causes syndromiques, diagnostiquées ou non étiquetées dès l'examen clinique devant l'association de plusieurs signes malformatifs ou d'une dysmorphie évocatrice ; iii) la DI isolée, où le caryotype, l'examen clinique et les différents examens paracliniques sont normaux.
Une série antérieure s'était attachée à détailler les étiologies génétiques identifiées chez environ 600 patients (6).

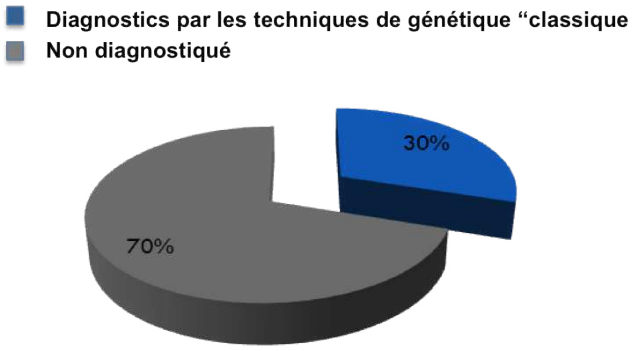


Figure 2 : Environ 30% des diagnostics de DI étaient réalisés grâce à l'examen clinique et les technique de cytogénétique (Caryotype et FISH ciblée).

1. Les causes chromosomiques

Au sein de leur cohorte, les anomalies chromosomiques représentaient la majorité des diagnostics réalisés (15-18%). Parmi les anomalies de nombre, une Trisomie 21 était diagnostiquée chez 9,2% des patients, 2/600 trisomies 13, 1 patient avec trisomie 18. Six patients présentaient des anomalies de nombre des gonosomes (1%). Les anomalies de structures représentaient 3,2% des diagnostics. En utilisant des sondes de FISH spécifiques, 1,3% des patients ont bénéficié d'un diagnostic de syndrome microdélétionnel.

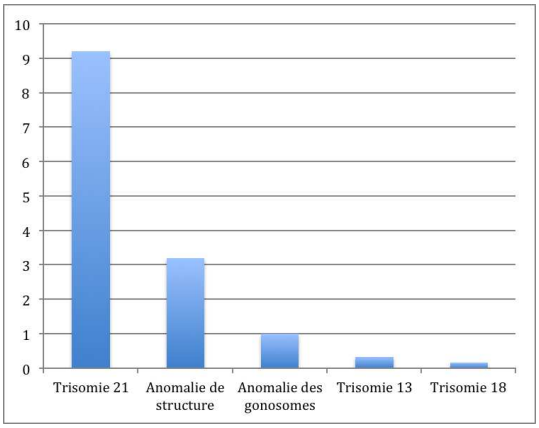


Figure 3 : Répartition étiologique des 15% de diagnostic cytogénétiques (série de Rauch et al., 2006). Ordonnée : pourcentage de cas.

2. Les causes syndromiques

Dans le groupe des pathologies syndromiques, on distingue les pathologies monogéniques des syndromes microdélétionnels. Dans cette étude, 15% des patients ont pu avoir un diagnostic : 5% des patients ont bénéficié d'un diagnostic clinique de syndrome monogénique et 4,7% d'un diagnostic de syndrome microdélétionnel par des techniques de cytogénétique ciblées (FISH). Considérant l'histoire familiale, 4% des patients ont pu recevoir un conseil génétique approprié sans diagnostic étiologique disponible.

3. Les causes de DI isolée

Dans ce groupe, le diagnostic ne peut pas aboutir par des examens orientés et il bénéficie des avancées technologiques actuelles de diagnostics pan-génomique détaillés ci dessous.

II. NOUVEAUX OUTILS DIAGNOSTIQUES DANS LA DEFICIENCE INTELLECTUELLE

Le bilan d'une DI peut être initié en amont de sa réelle expression, lors de la découverte d'un décalage des acquisitions psychomotrices. Cependant, il est fréquent que le diagnostic soit suspecté devant un échec scolaire faisant suite à un développement psychomoteur normal. Il est alors primordial de différencier une DI vraie d'un trouble spécifique des apprentissages et de faire le diagnostic de troubles du comportement associés qui pourront bénéficier d'une remédiation dédiée.

A. L'apport de la génétique

Avant l'ère des techniques de screening génome entier, une revue de la littérature estimait que tous degrés de DI confondus le taux diagnostic de la totalité des techniques utilisées expliquaient moins de la moitié des cas (7).

1. La CGH array

Depuis 2005, la CGH array permet un screening génome entier à la recherche de microréarrangements structurels provoquant des variations du nombre de copies (CNV). Entre 2005 et 2010, les meilleures résolutions des puces utilisées, la généralisation de cette technique et l'accumulation de données dans des bases de données publiques comme Decipher ou DGV ont permis une accélération des diagnostiques possibles. Des dizaines de syndromes microdélétionnels récurrents et de nombreux gènes candidats ont pu être mis en évidence. Ainsi le rendement diagnostic dans la DI a pu être amélioré d'environ 15 à 20%.

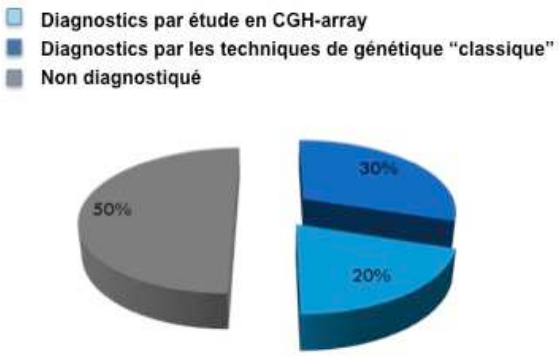
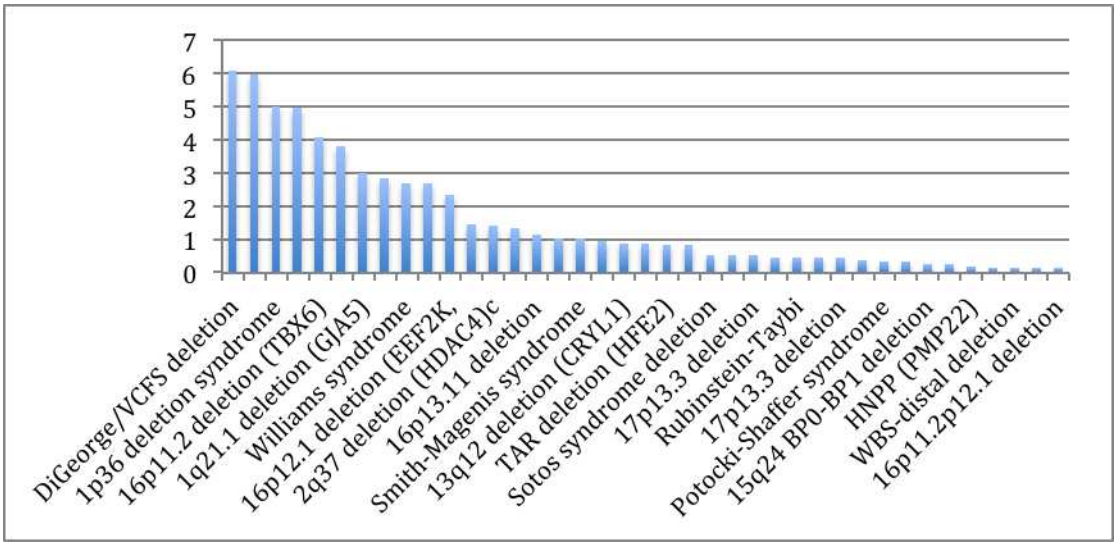


Figure 4 : 15-20% de diagnostics supplémentaires ont été possible grâce à la généralisation de la CGH-array.

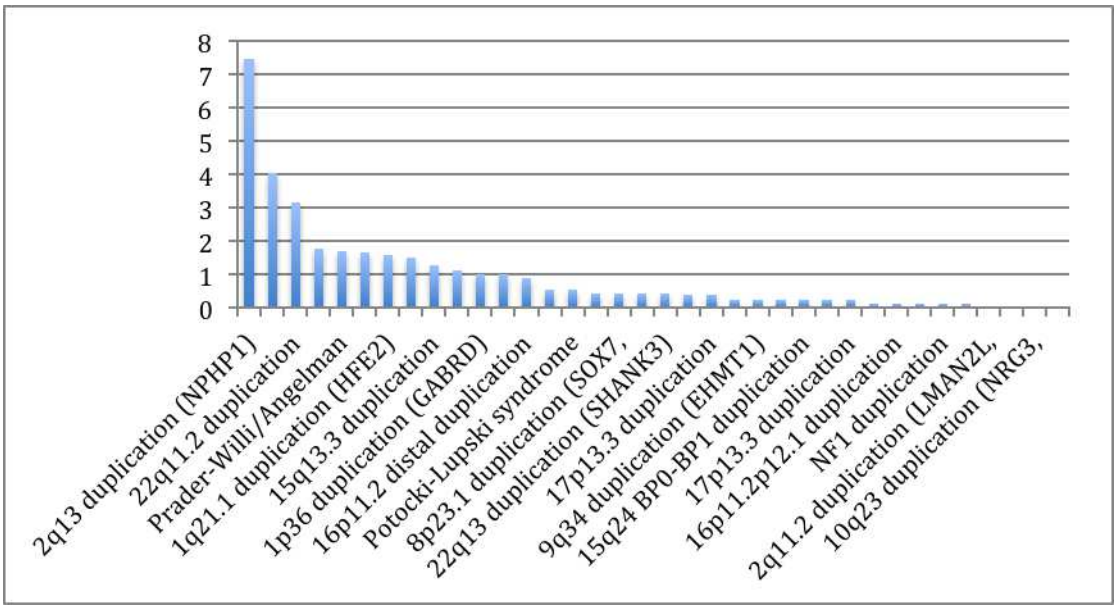
Un grand nombre de microréarrangements ont progressivement été rapportés. Une analyse de 15767 CGH-array réalisée devant des retards de développement a permis la définition d'une cartographie cytogénétique des loci impliqués de manière récurrente lorsqu'ils sont délétés (Figure 1a) ou dupliqués (Figure 1b)(8). Ces données sont un guide à l'interprétation des anomalies récurrentes mais ne sont que peu informatives sur les anomalies cytogénétiques rares.

Figure 5: Fréquence des microréarra`geme`ts récurre`ts ide`tifiés e` CGH array. Do``ées issues de Cooper et al (8). L'axe vertical représe`te la fréque`ce des a`omalies rapportée à 1000 exame`s.

A. Représentation des syndromes microdélétionnels.



B. Représentation des syndromes microduplicationnels.



De larges études statistiques ont permis de démontrer l'intérêt de considérer une taille de CNV supérieure à 400kb (8), contenant plus de 18 gènes (9,10), *de novo*. Ces données statistiques s'opposent naturellement à la description de CNV rares ou uniques, petit, pointant un gène candidat. Des recommandations pour l'interprétation des CGH array ont été proposées en 2011 (11,12). La stratégie adoptée dans cette étude pour déterminer la pathogénicité d'un CNV est argumentée dans la section matériel et méthodes.

2. *Le Séquençage Haut Débit (SHD)*

Depuis 2010, le séquençage haut débit (SHD) fait irruption dans le paysage diagnostique de la génétique médicale. Cette nouvelle technique permet d'accumuler une grande quantité de données de séquençage en un temps très réduit, rendant possible le séquençage d'un génome entier en moins d'une semaine. Une étude de concept a défini que le paradigme *de novo* était le plus représenté dans les causes de DI sporadique (13). Suite à cette démonstration, 2 études pilotes centrées sur la DI sévère sporadique non syndromique ont évalué le taux diagnostique de la stratégie de capture d'exome complet.

La première étude, a porté sur 100 patients atteints de DI sévère sporadique, isolée (14). Les investigations diagnostiques habituelles n'avaient pas permis de diagnostic chez ces patients. Dans ce contexte, le SHD-E a permis 13% de diagnostic certain lors de la publication des résultats. Lors d'une communication orale au congrès européen de génétique humaine (ESHG) 2013 à Paris, les auteurs annonçaient que l'accumulation des données de SHD avait permis de nouveaux diagnostics en confirmant l'implication de gènes candidats. Le rendement diagnostique actuel de cette étude est estimé en 2013 à 25% minimum (15). Il est probable que de nombreuses mutations de signification inconnue ce jour se révèleront pathogène dans quelques temps.

Dans cette première étude, les cas index étaient analysés seuls. Cette stratégie explique notamment l'amélioration des résultats diagnostiques avec l'accumulation des données statistiques de variants rares dans la population étudiée.

La seconde étude s'est affranchie de cette limitation en utilisant une stratégie d'étude par trio (16). L'analyse de trio permet dès la première analyse de filtrer les variants rares hérités des variants rares *de novo.* Ainsi, 45 patients ont bénéficié d'une SHD-E en trio. Avec cette stratégie d'analyse *de novo*, environ 50% des patients ont

pu bénéficier d'un diagnostic étiologique. Les données d'exome de ces 2 articles cumulées soulignent l'implication de 4 gènes de DI: *SCN2A, STXBP1, SYNGAP1* et *TCF4.* Par ailleurs, 39 gènes sont pointés, sans avoir pu être répliqués dans les cohortes rapportées. De nombreuses autres données de SHD devront être générées afin de déterminer l'implication de ces gènes et les spectres cliniques associés.

La stratégie d'exome permet une ré analyse des données à mesure de l'évolution de la connaissance scientifique. Cependant, sans aborder les nombreux problèmes éthiques que soulève la propriété de l'information générée par le SHD-E, le coût du SHD-E limite son utilisation en routine diagnostique. L'alternative choisie par plusieurs groupes est le choix d'un grand nombre de gènes impliqués dans la pathologie étudiée, qui seront séquencés par SHD ciblé. En France, le groupe du Professeur Mandel (Strasbourg) a choisi cette stratégie dans la DI non syndromique et les résultats des 200 premiers patients montrent un rendement diagnostique équivalent au SHD-E de cas index de 20%, avec un coût bien inférieur et en se limitant à la capture des gènes connus de DI.

Au total, à partir des 35% de diagnostics réalisés par une étude clinique, on peut ajouter 15-20% de diagnostic grâce à la CGH-array, puis 20 à 40% de diagnostics supplémentaires grâce au SHD. Théoriquement, ces stratégies cumulées devraient résoudre 75% des cas de DI d'origine génétique. De plus, il est techniquement possible que le SHD-E puisse remplacer toutes les techniques précédentes.

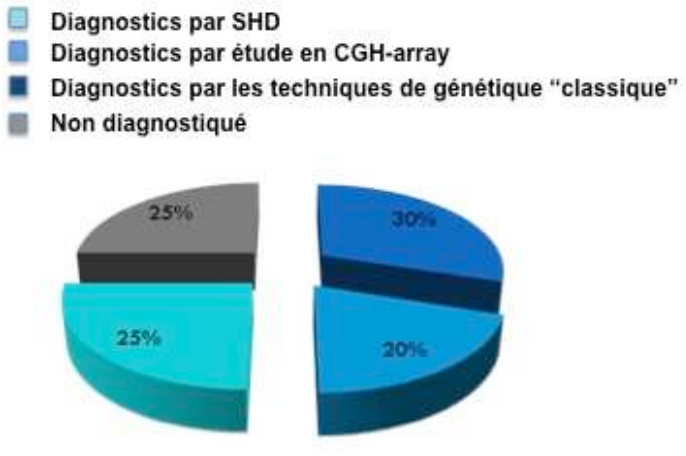


Figure 6 : 25% de diagnostics supplémentaires sont réalisés grâce au SHD. Il est probable que ce chiffre soit sous-estimé à l'heure actuelle, et pourrait atteindre 40 à 50% dans les années à venir.

B. L'apport des sciences cognitives

Il existe de nombreuses échelles et exercices permettant d'évaluer la cognition. Des échelles développementales, cognitives, comportementales sont disponibles. Selon l'orientation diagnostique initiale, le parcours de soin pourra emprunter une évaluation dans un centre de génétique, un centre ressource autisme, un centre spécialisé dans les troubles du langage…

Les tests psychométriques diagnostiques se concentrent sur l'utilisation des échelles de Weschler. Ces échelles sont normées par âge, contrôlées sur des populations variées permettant de limiter les biais socio-économiques ou ethniques. La WISC (Wechsler Intelligence Scale for Children) est la plus utilisée dans la DI légère à modérée car elle permet l'évaluation d'enfants âgés de 6 à 16 ans. La WISC IV développée en 2003 permet l'évaluation de 4 domaines cognitifs par différents subtests : i) un index de compréhension verbal (VCI), ii) index de raisonnement perceptuel (PRI), iii) index de vitesse de traitement (PSI) et iv) index de mémoire de travail (WMI). Ces scores composites permettent un calcul de QI total et remplacent le QI verbal et le QI performance des précédentes WISC tout en autorisant une sémiologie plus détaillée.

Tableau 1 : Echelles psychométriques couramment utilisées dans le diagnostic de pathologies neurodévelopmentales (Repris de Verloes et al (1)).

Type	Nom	Âges
Tests cognitifs généraux	Wechsler préscolaire (WPPSI-III : *Wechsler preschool and primary scale of intelligence*)	3 ans–7 ans
	Weschler enfant (WISC-IV : *Wechsler intelligence scale for children*)	6 ans–16 11/12
	Weschler adulte (WAIS-III : *Wechsler adult intelligence scale*)	> 16 ans
	NEPSY (*developmental neuropsycological assessment*)	3 ans–16 11/12
Tests de développement	Brunet-Lezine révisé	1 mois–30 mois
	PEP-R (*psycho educative profile, revised*)	6 mois–7 ans
Échelles d'adaptation	VABS (Vineland adaptive behavior scales)	Naissance–18 ans 11/12
Tests comportementaux	CBCL (*Child behavioural check list*)	2 ans–16 ans
	ADI-R (*Autism diagnostic interview-revised*)	2 ans–adulte
	Conners (plusieurs versions : parent, enfant, enseignant)	3 ans–17 ans

En cas de mise en évidence d'un profil cognitif hétérogène, le calcul de QI total devient non relevant et des tests complémentaires sont fréquemment nécessaires. Il existe pour chaque index des évaluations complémentaires validées en routine diagnostique ainsi que des tests générés par des équipes de recherche et demandant à être validés sur des patients après avoir été étalonnés sur des populations contrôles.
Il est assez fréquent de retrouver intriqués chez un patient ou dans une famille des phénotypes différents ou partiellement chevauchants. Cette constatation clinique est

renforcée par les larges spectres phénotypiques rattachés aux causes génétiques identifiées ces dernières années. D'ailleurs, la version 5 du Diagnostic and Statistical Manual of Mental Disorders (DSM V) à paraître en 2013 prendra en compte les avancées de la génétique moléculaire pour revisiter les spectres et les chevauchements de pathologies psychiatriques, notamment dans les troubles psychotiques tels que les troubles bipolaires et la schizophrénie (17).

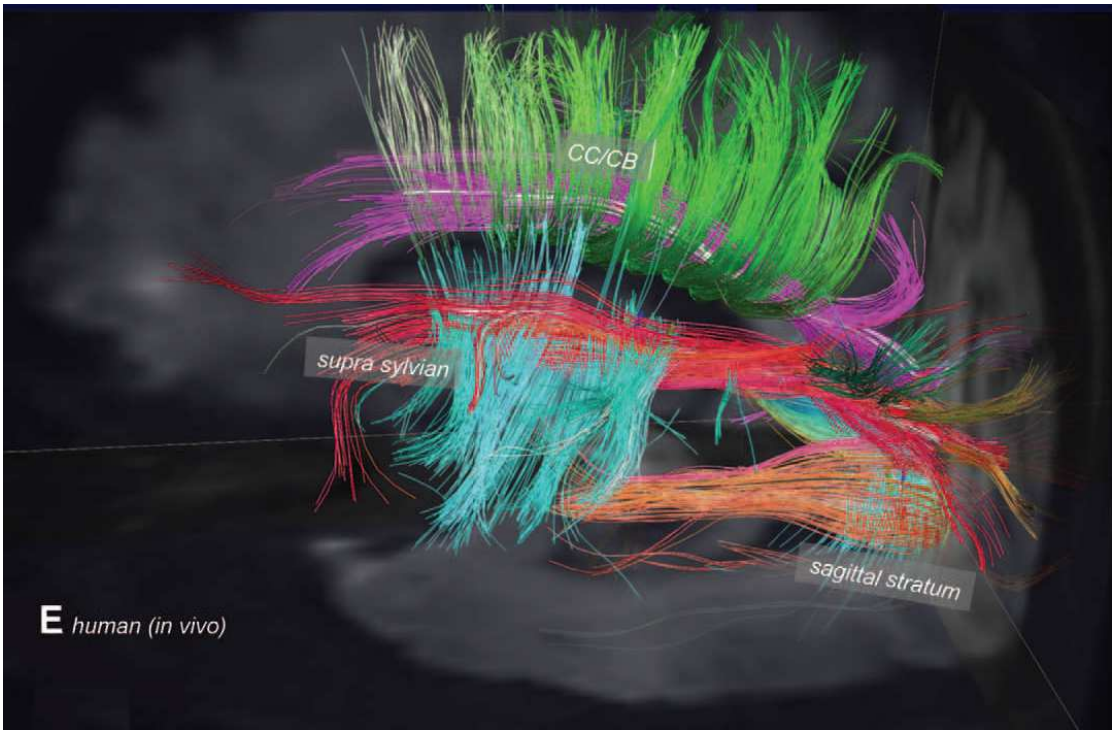
La généralisation des neurosciences a permis de mettre en évidence des profils cognitifs récurrents chez des patients a priori atteints de pathologies différentes. Par exemple, un déficit de mémoire de travail a été noté de manière récurrente chez des patients avec des patients avec des troubles du spectre autistique, des schizophrénies. Ces constations cliniques convergent vers les spectres pathologiques associés aux nouvelles causes génétiques de DI identifiées grâce aux nouvelles technologies disponibles.

C. L'apport de l'imagerie

L'imagerie cérébrale a une place importante dans le diagnostic de la DI. Il est recommandé de réaliser une imagerie devant une anomalie de mensuration du périmètre crânien, une épilepsie, un signe neurologique focal à l'examen clinique, un retard moteur sévère. Suivant ces critères, cet examen est anormal dans 30 à 40% des cas (7). Cependant, permettent d'orienter le diagnostic dans moins de 5% des cas environ (18). A notre connaissance, il n'existe pas de série de cas avec DI ayant bénéficié d'imagerie cérébrale fonctionnelle (19). En revanche, cette approche a été largement envisagée dans les troubles du spectre autistique et les pathologies psychiatriques. Cela a notamment permis la généralisation des IRM avec spectroscopie visant à l'étude de métabolites présentes en quantité anormale telles que le lactate ou la créatine.

Le domaine de la corrélation clinique des imageries cérébrales, anatomiques et fonctionnelles, a été fondé par les pathologies acquises lésionnelles. Depuis la description de l'aire de Broca, de nombreux travaux rapportant des patients avec des lésions focales cérébrales associées à des déficits sélectifs de fonctions cognitives ont été réalisés. Ainsi, une cartographie de corrélation clinico-imagerique a été progressivement constituée. L'imagerie fonctionnelle cérébrale permet de confirmer par une approche indépendante les conclusions des examens neuropsychologiques (20).

Figure 7 : Représentation de la structure géométrique des ''fibres cérébrales'' humaines d'après Wedeen et al. (21).

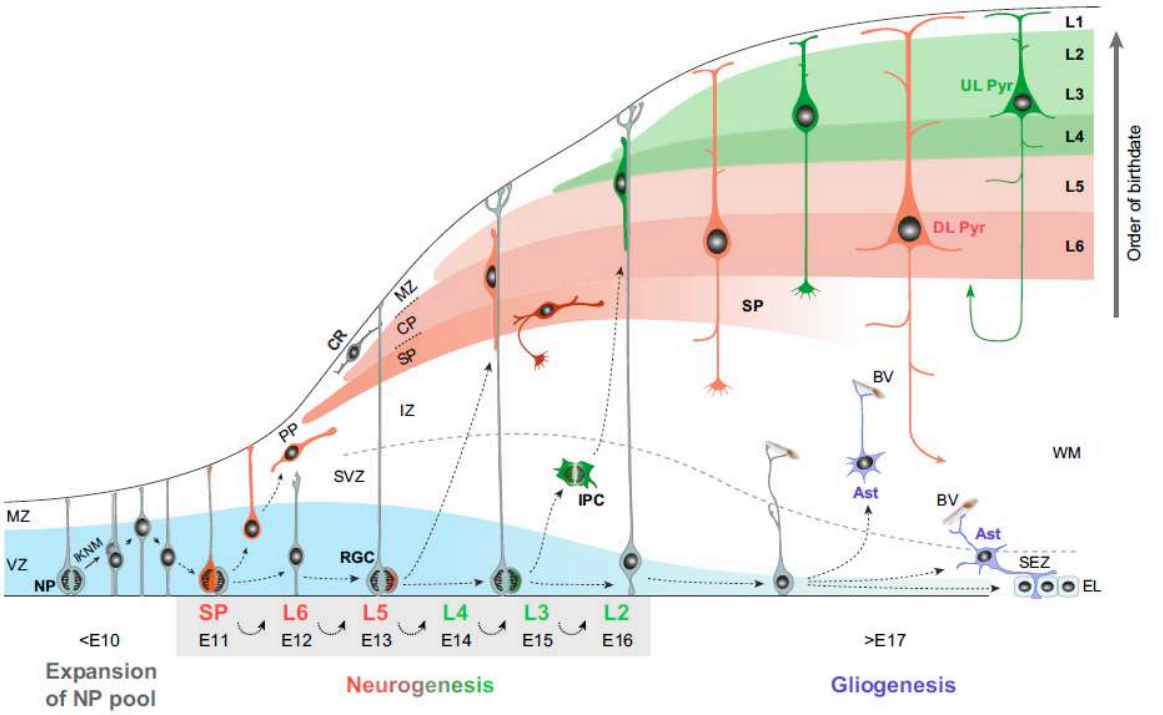


Avec l'amélioration de la puissance des aimants utilisés, les nouvelles générations d'IRM de recherche permettent des études fibres à fibres (fibre tracking) des réseaux neuronaux (21). Il est possible qu'une utilisation plus large de l'IRM fonctionnelle renseigne sur l'architecture cérébrale des pathologies développementales avec DI. Dans l'attente d'une puissance statistique suffisante, les rapports de cas comprenant de l'imagerie fonctionnelle se concentrent sur l'utilisation de tomographie par émission de positon (PET scanner) au 18 fluoro-désoxy-glucose (18-FDG). Une fois normalisé sur des individus contrôles, cet examen renseigne sur l'activité métabolique de régions cérébrale et permet une première approche encourageante de la physiologie cérébrale (22,23).

III. CONCEPTS CELLULAIRES DE LA COGNITION

Les hypothèses physiopathologiques actuelles de la DI suggèrent que ce groupe hétérogène de pathologies est secondaire à des anomalies de la mise en place et de l'entretien de la connectivité neuronale. Le système nerveux central dérive du tissu embryonnaire neuro-ectodermique, qui va former le tube neural. Au niveau rostral, les neuroblastes en contact avec la lumière du tube neural vont proliférer. Une partie de ce pool cellulaire restera en prolifération dans les régions sub-ventriculaires et constituera une réserve neuronale tout au long de la vie. Plusieurs étapes se succèdent pour mettre en place l'architecture cérébrale mature. On peut grossièrement séparer 4 processus : la prolifération de neuroblastes, la migration, la différenciation et la synaptogenèse (24).

Figure 8 : Etapes de la `euroge`èse séparа`t les phases de proliféra`tio`, migra`tio` et différe`ciatio` d'après Kwa` et al (24). Les étiologies de la DI isolée se retrouve`t plus particulièreme`t da`s les phases de différe`ciatio` `euro`ale et de sy`aptoge`èse.



A. Prolifération

Dans les régions sub-ventriculaires, les neuroblastes vont proliférer et constituer un pool de croissance neuronal. Après une phase d'expansion de la population, des mitoses asymétriques vont permettre la sortie du cycle cellulaire de certaines cellules filles. Ces dernières entameront une migration corticale. En pathologie humaine, les troubles de la prolifération neuronale seront responsables de syndromes avec microcéphalie pouvant être sévère, fréquemment associée à une DI (25).

B. Migration

On distinguera 2 types de migration : i) la migration radiaire, empruntée par les neurones excitateurs peuplant les futures couches corticales, ii) la migration tangentielle, empruntée par les interneurones inhibiteurs.

Une fois sorti du cycle cellulaire, les neuroblastes vont migrer le long de la glie radiaire. Cette étape est donc sous la dépendance de l'intégrité de la matrice extra cellulaire, de la glie radiaire et des molécules de signalisation permettant la progression et l'arrêt de la migration neuronale. Ainsi, les neuroblastes vont peupler différentes strates corticales et constituer les 6 couches du cortex mature (24). Les pathologies de la migration seront représentées cliniquement par une DI syndromique pouvant être associée à une microcéphalie, une épilepsie… La neuro-imagerie est clé dans le diagnostic, et permettra d'identifier des anomalies de la gyration, des hétérotopies, témoin de la migration neuronale anormale (26).

Les inter-neurones sont issus d'un pool de prolifération cellulaire de la région sub-ventriculaire préfrontale. Les neuroblastes sortant du cycle cellulaire vont emprunter une migration tangentielle pour peupler les différentes couches corticales. Leur migration est sous dépendance de chémokines, et indépendante de la glie radiaire. Les pathologies humaines causées par des anomalies des interneurones sont essentiellement représentées par la DI avec des troubles psychiatrique et la schizophrénie en particulier (27,28).

C. Différenciation

L'étape terminale de la formation de la substance grise mature repose sur la différenciation des neuroblastes et la mise en place des connexions synaptiques. Il existe différents types cellulaires neuronaux et on sépare les neurones Glutamatergiques excitateurs des neurones Gabbaergiques inhibiteurs.

Les neurones excitateurs sont essentiellement issus de la migration radiaire et projettent des axones et dendrites entre les couches corticales. Au sein de la population de neurones excitateurs, différents sous groupes vont se différencier en neurones pyramidaux, stellaires... La différenciation neuronale est régie par une cascade de régulation transcriptionnelle. Il a été possible d'identifier des pattern d'expression géniques caractéristiques de chaque sous type de population neuronale (29). Au cours de la différenciation, les neurones vont projeter des connexions entre les différentes couches corticales, entre les hémisphères cérébraux, vers les noyaux gris centraux et la moelle épinière. Ces projections ont un pattern récurrent et précis qui a pu être étudié avec l'amélioration de l'imagerie fonctionnelle cérébrale (21).

Les neurones inhibiteurs Gabbaergiques, ou interneurones, ont des fonctions particulières dans le neurodéveloppement et leur défaut semble être plus particulièrement associé aux troubles psychotiques et à la schizophrénie (27).

D. Mise en place du réseau synaptique

Les neurones en cours de différenciation et différenciés vont établir des connexions synaptiques. Une fois établies, ces connexions restent plastiques et seront renforcées ou supprimées selon leur degré de stimulation. La structure d'un connexion synaptique est très complexe et des dizaines de protéines sont impliquées dans sont fonctionnement physiologique (30). Schématiquement, on sépare le compartiment pré-synaptique du compartiment post-synaptique. Les hypothèses actuelles suspectent que les anomalies de la structure et/ou de la fonction synaptique sont responsables de phénotypes plus isolés. Ce postulat a été renforcé par l'identification de nombreuses causes d'épilepsies par le séquençage de canaux ioniques localisés à la jonction synaptique (31).

Ces observations suggéraient donc que des phénotypes « cognitifs purs» pouvaient être secondaire à des anomalies de gènes codant pour des protéines d'expression synaptique (30). Des données de génomique évolutive ont renforcé cette

hypothèse en pointant plus particulièrement l'implication du compartiment post synaptique dans les fonctions cognitives supérieures. En effet, les dizaines de protéines qui composent ce compartiment subissent dans l'espèce humaine la plus grosse pression de sélection du génome (32).

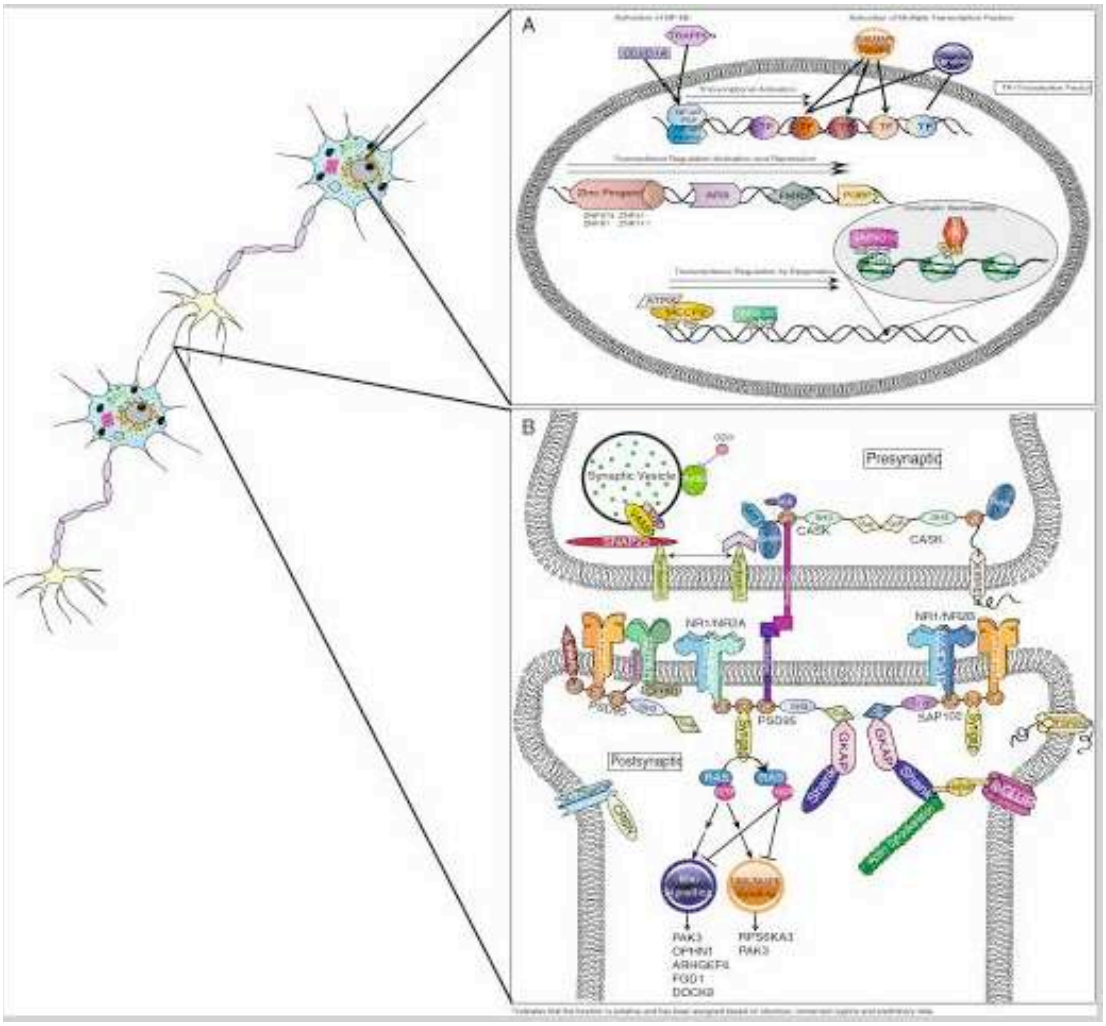
Indépendamment du type de DI, isolée ou syndromique, les revues disponibles regroupent les gènes candidats à la DI en 2 parties :

- les gènes codant pour des facteurs de transcription limitant la prolifération neuroblastique et promouvant la différenciation cellulaire neuronale.
- les gènes codant pour les protéines de la jonction synaptique.

Ces regroupements fonctionnels permettent de donner des arguments pour considérer un gène candidat. Cependant, l'implication certaine d'un nouveau gène de DI est démontrée par la récurrence des cas porteurs de mutations.

L'identification des bases moléculaires de la DI permettra d'améliorer la compréhension physiopathologique des processus en cause, mais également d'envisager des cibles thérapeutiques futures. Le phénotypage pluridisciplinaire et transversal des patients ouvre le champ d'une nosologie plus fine de la description sémiologique de la DI. La mise en évidence d'endophénotypes de DI est une première étape à la considération de stratégies de remédiation adaptées.

Figure 9 : les gènes identifiés dans la DI non syndromique peuvent être séparés en 2 groupes fonctionnels : les facteurs de transcription promoteurs de la différenciation neuronale et les composants des compartiments synaptiques.



OBJECTIF DE LA THESE

L'objectif de cette thèse de sciences est l'identification de nouveaux gènes de déficience intellectuelle, syndromique ou non et de caractériser au mieux les manifestations cliniques associées.

PATIENTS ET METHODES

I. ETUDE DE COHORTES HOMOGENES DE PATIENTS PORTEURS DE MICROREARRANGEMENT CHROMOSOMIQUE DIAGNOSTIQUE PAR CGH-ARRAY

Cet axe de thèse s'est concentré sur un recrutement de patients issus de la consultation du centre de référence du Grand Est « Anomalies du développement et syndromes malformatifs» coordonné par le Professeur Laurence Olivier-Faivre. Cette phase ne permettait que le recrutement de cas index, et la poursuite de l'étude reposait sur l'existence d'autres cas recrutés à l'aide d'un réseau de collaborations nationales et internationales. Les critères d'inclusions des patients étaient la mise en évidence d'une anomalie de CGH-array impliquant un gène d'expression neuronale, intragénique ou non, portée par plusieurs patients présentant une DI. Nous avons exclu les réarrangements déjà rapportés dans la littérature.

A. Interprétation de la CGH-array

Nous avons considéré les microréarrangements chromosomiques intragéniques et multigéniques. Une analyse systématique des résultats de CGH a été développée à partir de recommandations existantes et en ajoutant des annotations informatives à partir de bases de données publiques (11,12). L'expression neuronale a été évaluée grâce aux sites UCSC (www.genome.ucsc.edu) et Genatlas (www.genatlas.medecine.univ-paris5.fr). Les gènes étaient annotés par différentes bases de données en utilisant le pipeline de Genedistiller (www.genedistiller.org).

Une anomalie était considérée pathogène lorsque les critères suivants, classés par importance décroissante, étaient remplis :

- Absence de l'anomalie dans la Database of Genomic Variant

(dgv : http://dgv.tcag.ca/dgv/app/home)

- Ségrégation familiale concordante
- Présence de micro réarrangements similaires dans les bases de données publiques

  (Decipher : http://decipher.sanger.ac.uk)
- Microréarrangement chromosomique intragénique
- Absence de mutations tronquantes dans la base de données d'exome du NHLBI

  (Exome Variant Server : http://evs.gs.washington.edu/EVS/)
- Expression cérébrale ou neuronale

  (UCSC, Genatlas)
- Phénotype murin compatible

  (MGI : http://www.informatics.jax.org/phenotypes.shtml)
- Implication dans la physiologie neuronale.

  (KEGG pathways : www.genome.jp/kegg/pathway.html et
  GO terms : www.geneontology.org/)

B. Evaluation globale du cas index

Lorsqu'une anomalie non rapportée dans la littérature était retenue pathogène, une évaluation multidisciplinaire était proposée aux patients.

Cette évaluation comprenait :

- une évaluation neuropsychologique par l'échelle de Weschler adaptée à l'âge
- une évaluation orthophonique
- un examen neuropédiatrique
- une évaluation dysmorphologique
- une imagerie cérébrale anatomique (IRM)
- +/- d'autres examens (malformatifs), évaluation ergothérapique, etc…

A l'issue de ces évaluations, une synthèse était réalisée et permettait d'orienter d'éventuelles investigations complémentaires des secteurs déficitaires.

- les évaluations neuropsychologiques et/ou orthophoniques complémentaires pouvaient être complétée par des taches expérimentales en cours d'évaluation par le Laboratoire d'Etude des troubles Apprentissages et du Développement (LEAD-CNRS, université de Bourgogne, Dijon)
- une imagerie fonctionnelle par PET scanner pouvait être proposée.

C. Recrutement d'autres patients : DECIPHER/ ACPA

Afin de recruter d'autres patients avec des anomalies cytogénétiques comparables, des appels à collaboration ont systématiquement été réalisés. Le réseau Français Achropuce (www.renapa.univ-montp1.fr/) et Européen Decipher (http://decipher.sanger.ac.uk) ont été les sources de nos recrutements secondaires. Des fiches cliniques détaillées ont été envoyées aux cliniciens référents de chaque centre concerné.

II. ETUDE D'UNE COHORTE HOMOGENE DE PATIENTS PAR SHD-E : IDENTIFICATION DE LA CAUSE MOLECULAIRE DU SYNDROME DE SHPRINTZEN-GOLDBERG

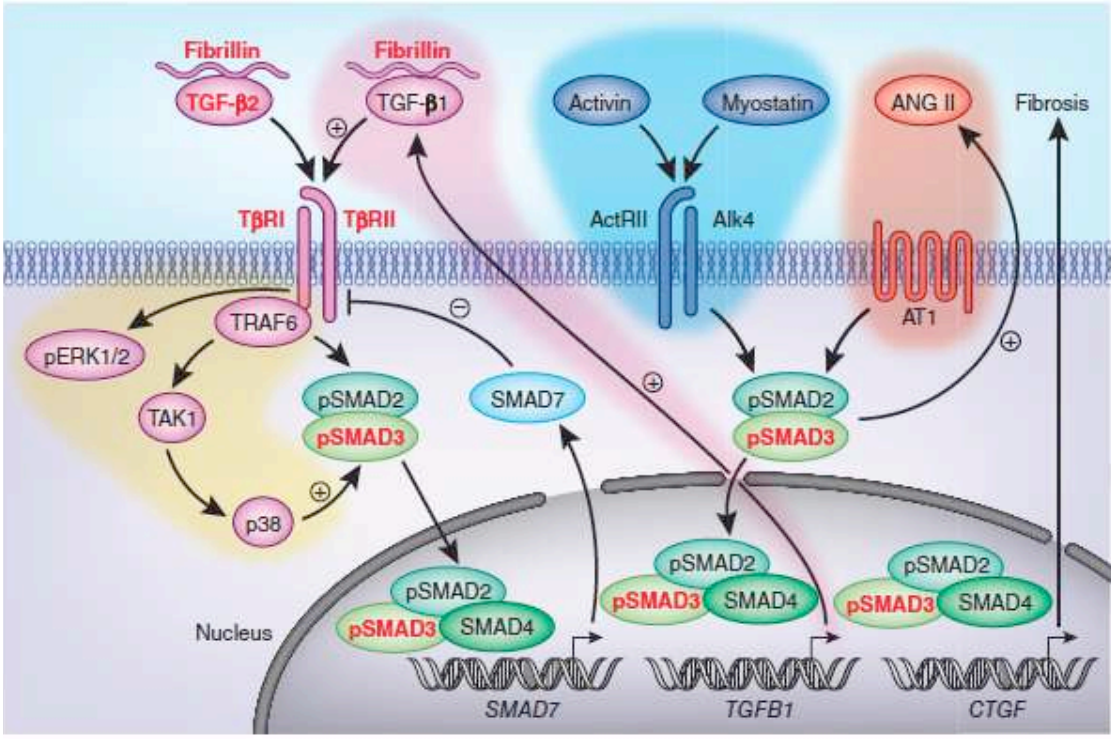
Dans le cadre d'un travail collaboratif international, le Professeur Olivier-Faivre a pu constituer une cohorte de patients atteints de syndromes marfanoïdes avec DI. Cette entité clinique est hétérogène, mais il existe des syndromes cliniquement identifiés dont la cause génétique est inconnue. Le syndrome de Shprintzen-Goldberg est un syndrome marfanoïde avec classiquement reconnaissable, caractérisé par l'association à une craniosténose, des rétractions articulaires, une dysmorphie faciale caractéristique. Bien que chevauchante avec le syndrome de Loeys-Dietz, le SGS est une entité clinique indépendante et reconnaissable.

Au moment de notre étude, les causes moléculaires des pathologies suivantes étaient connues:

- la maladie de Marfan secondaire à des mutations de la fibrilline 1 (*FBN1*)
- le syndrome de Loeys-Dietz, lié à des mutations des récepteurs au TGF béta (*TGFBR1* et *TGFBR2*)
- le syndrome Congenital Contracture Arachnodactyly, avec mutations de la fibrilline 2 (*FBN2*)

Toutes ces pathologies ont en commun le développement d'un habitus marfanoïde chez les patients, vraisemblablement secondaire à une hyperactivation de la voie TGF béta (33).

Figure 10 : Protéines composant la voie de signalisation du TGF béta publiée par Akhurst et al. (33).



Une stratégie de trio a été décidée devant l'hypothèse d'une pathologie sporadique dont l'origine suspectée est la survenue de mutations *de novo*. Une famille suspecte de mosaïcisme germinal (ou somatique) a également été étudiée.

Les variants ont été annotés avec le pipeline de Genedistiller en suivant les mêmes critères que l'interprétation des CGH-array. Un filtre biologique de la voie du TGF béta a été mis au point à partir des données de l'EMBL-EBI (www.ebi.ac.uk).

Les variants candidats ont été vérifiés en séquençage Sanger. Une ségrégation familiale était systématiquement réalisée.

RESULTATS

I. ETUDE D'UNE COHORTE HOMOGENE DE PATIENTS PORTEURS D'UN MICROREARRANGEMENT DIAGNOSTIQUE PAR CGH ARRAY.

Notre approche a permis d'achever la caractérisation de plusieurs syndromes microdélétionnels pointant des gènes candidats impliqués dans la physiologie neuronale (délétion 3p14.1p13, délétion 12p13.33, délétion 3q27.3) et d'identifier deux nouveaux gènes de DI (*CAMTA1*, *SALM1*). La présentation des résultats sera organisée selon les regroupements fonctionnels auxquels appartiennent les nouveaux gènes de DI ou les gènes candidats du syndrome microdélétionnel rapporté

A. Facteurs de transcription impliqués dans la différenciation neuronale

Intragenic CAMTA1 rearrangements cause non-progressive congenital ataxia with or without intellectual disability.

1. *Résumé de l'article*

Background : L'ataxie congénitale non progressive (NPCA) avec ou sans DI sont des présentations cliniques et génétiques hétérogènes. En conséquence, l'identification de gènes responsables de ces phénotypes restait limitée.

Méthodes : suite à la mise en évidence de trois cas familiaux ou sporadiques porteurs de réarrangements intragéniques du gène calmodulin-binding transcription activator 1 (*CAMTA1*) identifié par CGH-array et recrutés par une collaboration nationale, nous avons défini les conséquences cliniques et moléculaires de ces réarrangements. Une cohorte de patients avec DI plus ou moins NPCA a été constituée pour rechercher des mutations ponctuelles de *CAMTA1* par séquençage Sanger direct.

Résultats : Les réarrangements intragéniques de *CAMTA1* étaient localisés dans le domaine de liaison à l'ADN nommé CG-1. Les réarrangements ségrégeraient avec une DI avec une NPCA dans 2 familles indépendantes et était identifiée *de novo* chez une enfant présentant une NPCA. Dans les familles présentant une DI, les réarrangements aboutissaient à la production d'une protéine tronquée alors que la délétion de la patiente avec NPCA isolée respectait le cadre de lecture. L'IRM cérébrale des patients a révélée un pattern d'atrophie progressive affectant les lobes moyens cérébelleux, le vermis supérieur, les lobes pariétaux et les hippocampes. Le séquençage du domaine CG-1 de 197 patients avec une DI non syndromique sporadique ou familiale, le séquençage complet du gène chez 50 patients avec DI et 47 patients avec NPCA n'a pas permis d'identifier de patient supplémentaire.

Conclusion : Nous avons identifié des mutations perte de fonction du gène *CAMTA1* responsable de NPCA avec ou sans DI.

2. *Discussion et perspectives*

Nous avons rapporté les premières descriptions cliniques de patients porteurs de réarrangements intragéniques de *CAMTA1.* Une patiente porteuse d'un réarrangement intragénique de *CAMTA1* avait été rapportée dans une large série de CGH array (34). Cette patiente présentait un phénotype plus sévère que les patients

rapportés ici et avait été adressée pour une épilepsie sévère avec un retard de développement profond. Depuis la publication de cette étude, un autre patient a été rapporté avec une délétion intragénique emportant le promoteur de *CAMTA1*. Ce patient présentait une DI associée à une démarche ataxique (35). Par ailleurs, une nouvelle famille a été diagnostiquée en France par le Dr Journel (Vannes). Cette famille composée de 3 individus semble compatible avec les données cliniques que nous avons rapportées dans cet article.

Le gène *CAMTA1* était connu comme étant un gène suppresseur de tumeur d'expression cérébrale spécifique. Cependant, nous avons récemment été contacté par l'équipe du Professeur Loffing de l'université de Zurich. Leur objectif est d'étudier le rôle dans la physiologie rénale de *CAMTA1.* Cette équipe a pu produire les premières souris KO conditionnelles pour *Camta1.* Leurs premiers résultats montrent que les souris KO présentent une ataxie et des études phénotypiques sont toujours en cours. Un travail collaboratif est envisagé avec cette équipe afin d'étudier in vivo les fonctions cérébrales de *CAMTA1.*

3. *Article*

ORIGINAL ARTICLE

Intragenic *CAMTA1* rearrangements cause non-progressive congenital ataxia with or without intellectual disability

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ABSTRACT

Background Non-progressive congenital ataxias (NPCA) with or without intellectual disability (ID) are clinically and genetically heterogeneous conditions. As a consequence, the identification of the genes responsible for these phenotypes remained limited.

Objective Identification of a new gene responsible for NPCA and ID.

Methods Following the discovery of three familial or sporadic cases with an intragenic calmodulin-binding transcription activator 1 (*CAMTA1*) rearrangement identified by an array-CGH and recruited from a national collaboration, the authors defined the clinical and molecular characteristics of such rearrangements, and searched for patients with point mutations by direct sequencing.

Results Intragenic copy number variations of *CAMTA1* were all located in the CG-1 domain of the gene. It segregated with autosomal dominant ID with non-progressive congenital cerebellar ataxia (NPCA) in two unrelated families, and was de novo deletion located in the same domain in a child presenting with NPCA. In the patients with ID, the deletion led to a frameshift, producing a truncated protein, while this was not the case for the patient with isolated childhood ataxia. Brain MRI of the patients revealed a pattern of progressive atrophy of cerebellum medium lobes and superior vermis, parietal lobes and hippocampi. DNA sequencing of the CG-1 domain in 197 patients with sporadic or familial non-syndromic intellectual deficiency, extended to full DNA sequencing in 50 patients with ID and 47 additional patients with childhood ataxia, identified no pathogenic mutation.

Conclusion The authors have evidence that loss-of-function of *CAMTA1*, a brain-specific calcium responsive transcription factor, is responsible for NPCA with or without ID.

Accession numbers CAMTA1 reference sequence used was ENST00000303635. Protein sequence was ENSP00000306522.

INTRODUCTION

Clinically and genetically heterogeneous conditions such as childhood ataxia or intellectual disability (ID) hindered the elucidation of those groups of pathologies. Childhood ataxias comprise a group of neurological signs secondary to a dysfunction of the cerebellum and its connections (cerebellar ataxia) and/or to problems in the posterior column of the spinal cord or peripheral nerves (sensory ataxia). Non-progressive congenital cerebellar ataxia (NPCA) manifests itself in infancy as hypotonia, delayed motor development and ataxia with or without dysarthria.[1] ID and spasticity can also be associated with NPCA. NPCA is presumably monogenic in over half of the cases with a mainly autosomal recessive mode of inheritance. Structural abnormalities of the cerebellum are not always present on neuroimaging.[2]

ID is the most prevalent severe handicap in children, affecting 1%–3% of the population.[3] ID is grouped into syndromic ID (S-ID) and non-syndromic ID (NS-ID). ID can be caused by environmental and/or genetic factors. The high degree of heritability of ID has been highlighted, in particular with the estimation that up to 50% of severe cases are caused by genetic abnormalities.[4] Genetic causes include various chromosomal rearrangements diagnosed by conventional or array-based techniques, with a broad range of prevalence,[5–7] as well as mutations of numerous autosomal or X linked genes.[8] It has recently been shown that de novo mutations or intragenic rearrangements were a major cause of sporadic ID.[9] To date, 15 genes have been identified in autosomal dominant NS-ID by candidate genes strategies (*SYNGAP1, KIF1A, GRIN1, EPB41L1* and *CACNG2*),[10 11] by mapping breakpoints of chromosomal rearrangement (*DOCK8, CDH15, KIRREL3* and *ARID1B*)[12 13], by microarrays indicating de novo copy number variations (CNVs) (*MBD5* and *SHANK2*)[14 15] and by exome sequencing (*DYNC1H1, YY1, DEAF1, CIC*).[9]

Their encoded proteins exert highly specific functions in neuronal connectivity and plasticity.[8 16]

The *calmodulin-binding transcription activator 1* (*CAMTA1* gene) is a member of the family of calmodulin-binding transcriptional activators (CAMTAs), and has been shown to be conserved in all organisms from multicellular eukaryotes to humans. The *CAMTA1* gene, first identified in a human brain-specific cDNA cloning study, belongs to the human CAMTA family[17] defined by four conserved protein domains, including the family-specific DNA-binding domain termed CG-1, which contains a bipartite nuclear localisation signal with transcriptional regulation properties, a transcription factor immunoglobulin-like DNA-binding domain (TIG domain), calmodulin-binding IQ motifs (IQ domain) and ankyrin repeats necessary for oligomerisation (ANK domain).[18–23]

In this article, we report on three independent families with NPCA with or without ID, secondary to intragenic *CAMTA1* rearrangements disrupting the CG-1 domain.

SUBJECTS

Based on the identification of an intragenic rearrangement of the *CAMTA1* gene in family 1 identified by an array-CGH, a national call for collaboration was performed to reinforce the hypothesis of the implication of this gene in ID. Among the 23 000 array-CGH analyses performed in France for the work-up of developmental delay and/or intellectual deficiency, two additional families were recruited. Family pedigrees and patients' pictures are presented in figure 1. The patients presented with dysmorphic facial features that are detailed in figure 1. Major clinical and neurological findings are summarised in table 1. A specific consent for research investigations was accepted by each patient or legal representative. The research protocol was approved by the local ethic committee.

Family 1

Patient III-6 was referred to the genetics centre for delayed psychomotor development. She was the second child of non-consanguineous parents with a family history of ID. She had speech delay with normal social interactions. Standard chromosomal analysis revealed 47,XXX aneuploidy. The brain CT-scan showed no abnormality. Her four-and-a-half-year-old sister (III-7) presented with right eye strabismus, unsteady gait and behavioural difficulties. She was unable to speak. Her 2-year-old brother (III-8) presented with neonatal global hypotonia followed by developmental delay. He could walk unaided but with an ataxic gait at 22 months of age. Upper arm myoclonic epilepsy was noticed by the mother. The EEG and brain CT-scan were normal.

The mother (II-6) also presented with ID without behavioural abnormalities and went to schools for students with special needs. She currently works in a job reserved for the disabled. Clinical examination revealed an ataxic gait and mild intentional tremor. There were no lateralised falls, Romberg signs or nystagmus. Neuropsychological evaluation revealed mild ID with homogeneous performances in verbal and non-verbal processes, and poor abstract verbal reasoning. The PRI score was 66–73 revealing borderline intellectual abilities (poor spatial perception, visual abstract processing, non-verbal abstract problem solving and non-verbal reasoning). Low-speed processing and problems with selective attention were noticed (speed processing index =61; normal =100) with an impact on concentration when mental mathematical problems were considered. There was dissociation between memory processes in the auditory–verbal and visual modality, indicating a lack of rehearsal processing in episodic visual memory. Results showed a working memory index at 50, indicating short-term memory disabilities predominantly on auditory memory modality. Brain MRI showed mild hippocampus atrophy, simplified gyration of the dentate gyri, and bilateral posterior cortical atrophy including the parietal lobes and the precuneus and cuneus. There was a cerebellar atrophy of the medium lobes and of the superior vermis (figure 2A). An 18FDG PET scan revealed a hypometabolism of the left temporal region and temporal medial lobe, biparietal lobe and the precuneus/cuneus (figure 2B).

Patient II-4, a half-sister of patient II-6, attended a special needs school and worked as a non-qualified operator. The clinical

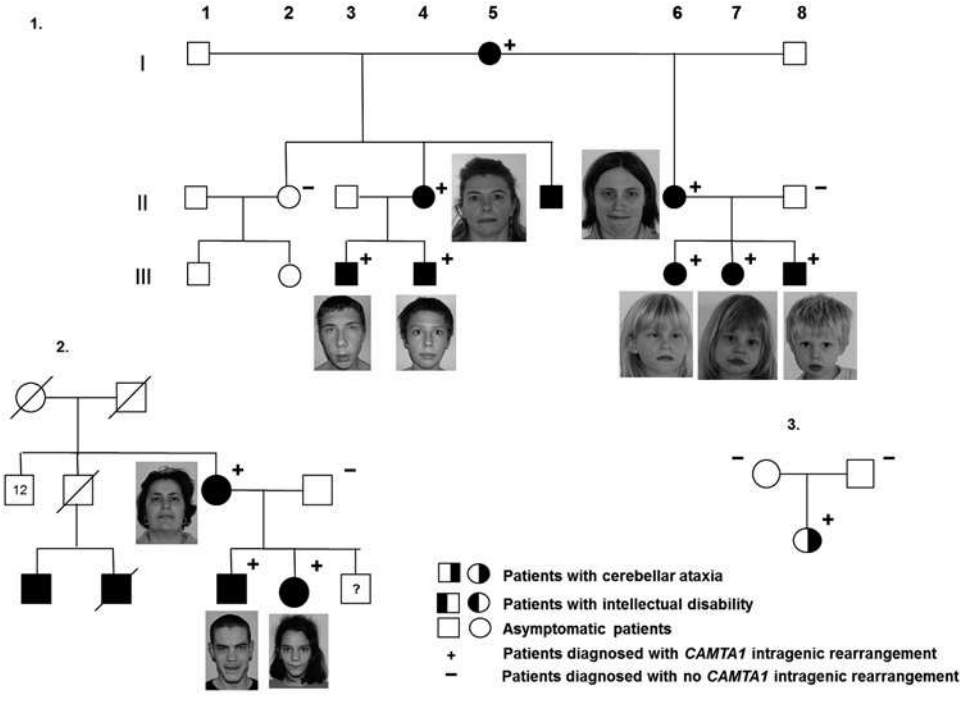

Figure 1 Pedigrees and photographs of families 1–3. Intellectual disability is represented by black boxes, and cosegregates with the family. Note some common facial features including a long face with a pointed chin, bulbous nose with anteverted nostrils, a long philtrum and a thick lower lip with down-turned corners of the mouth.

Table 1 Detailed clinical features and neurological explorations in patients with *CAMTA1* rearrangements

	Family 1							Family 2			
	III-6	III-7	III-8	II-6	II-4	III-3	III-4	II-1	II-2	I-1	Family 3
Development history											
Term of gestation of birth	38	40	39	NR	NR	Term	36+5	38	38	NR	
Measurements at birth	N	N	N	NR	N	N	N	N	N	NR	IUGR
Neonatal hypotonia	No	No	Yes	NR	NR	No	No	N	Yes	NR	Yes
Sitting age (months)	9	11	10	NR	NR	10	NR	NR	NR	NR	9
Walking age (months)	18	24	20 (with assistance)	NR	NR	18	22	24	18	Delayed	21
First word association (months)	48	Absent	Absent	NR	NR	Delayed	Delayed	36	36	NR	Delayed
Clinical examination											
Age at clinical examination (years old)	6	4	2	41	38	18	14	16	12	42	4
Growth parameters	N	N	N	N	N	N	N	Macrocephaly	N	N	N
Facial dysmorphism	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Neurological examination											
Cerebellar symptoms											
Ataxic gait	Yes	No	Yes	No	No	Yes	No	Yes	No	Yes	Yes
Dysmetry	Mild	No	Yes	No	No	Mild	No	Mild	No	Mild	Yes
Instability	Yes	Yes	Yes	Mild	Mild	Mild	Mild	Yes	Mild	Yes	Yes
Dysarthria/abnormal pronunciation	Yes	NR	NR	Yes	Yes	No	Yes	No	No	No	Yes
SARA score (/56)	9	9	9	6	6	8	4	9	8	6	9
Standard examination											
Nerve sensitivity	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Tendon reflexes	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Polykinetic	Normal	Normal	Normal
Pyramidal symptoms	No	No	No	No	No	No	No	Yes	No	No	No
Tremor	No	No	No	No	No	No	No	No	No	No	No
Vestibular symptoms											
Nystagmus	No	No	No	No	No	No	No	No	No	No	No
Romberg sign	No	No	No	No	No	No	No	No	No	No	No
Convulsions	No	No	2 febrile crisis	No	No	No	No	No	No	No	No
Strabismus	Yes	No	No	No	No	No	No	No	No	No	No
Neuropsychological evaluation (IQ, VIQ, PIQ)	58, 52, 57	67, 78, 61	NR	58, 59, 58	65, 66, 68	51	61, 76, 65	40, 50, 50	53, 55, 56	54, 52, 60	Dissociation between verbal and non-verbal skills
Scholarship/job	Special needs	Special needs	NR	Job reserved for disabled	Not working	Job reserved for disabled	Special needs	Special needs	Special needs	Not working	Special needs
Behavioural troubles	Stereotypies, aggressiveness	No	No	No	No	Solitary	No	ASD, aggressiveness	No	No	No
Brain MRI	NR	NR	NR	Pathological	Pathological	NR	Abnormal	NR	NR	NR	Normal

IQ, VIQ, PIQ were assessed using the Wechsler standardised evaluation scales.[24 25]
Cerebellar symptoms were described using the Scale for the Assessment and Rating of Ataxia (SARA).[26]
ASD, autism spectrum disorder; IUGR, intrauterine growth retardation; N, normal; NR, not referenced; PIQ, performance intellectual quotient; VIQ, verbal intellectual quotient.

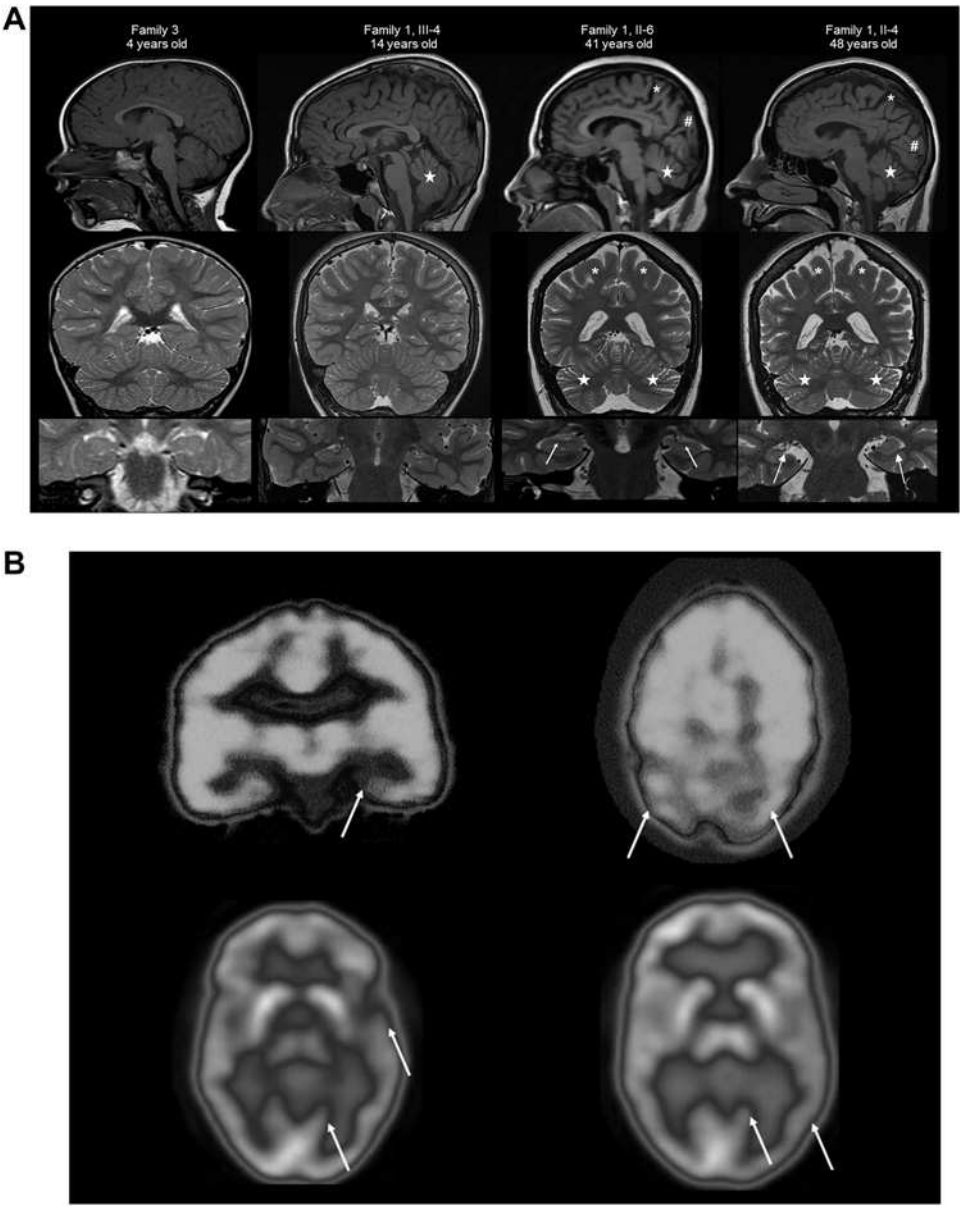

Figure 2 Brain imaging. (A) MRI examination of patient II-4 (48-years-old), II-6 (41-years-old), III-4 (14-years-old) from family 1 and patient from family 3 (4-years-old). A progressive pattern could be identified showing mild progressive cerebellar atrophy of the superior vermis and medium cerebellar lobes (star), marked bilateral posterior cortical atrophy including the parietal lobes (*), precuneus and cuneus (#). Mild bilateral hippocampal atrophy (Scheltens's medial temporal lobe atrophy scale: grade 2) and simplified gyration of the dentate gyri were noticed (arrows). No white matter abnormality was identified. (B) PET scan (radiotracer: FDG) showing the left temporal area including the left temporal medial lobe the biparietal lobe and the precuneus/cuneus hypometabolism (arrows).

examination revealed static instability associated with mild ataxic gait, with no evidence of vestibular dysfunction. Brain MRI showed a similar pattern of mild hippocampus atrophy, simplified gyration of the dentate gyri, and bilateral posterior cortical atrophy including the parietal lobes and the precuneus and cuneus. Cerebellum showed atrophy of the medium lobes and of the superior vermis (figure 2A). Her elder son (patient III-3), a 23-year-old boy, was described as hypotonic from birth with psychomotor delay but no behavioural troubles although he is rather solitary. He attended a special needs school and now works at a supported employment. He acquired reading, writing and lives independently. He presented with left eye strabismus and an ataxic gait. Her younger son (patient III-4) also presented with psychomotor delay. He attended a special needs school from 10 years of age. He followed speech and language therapy for delayed receptive language. Brain MRI showed no supratentorial atrophy, but a mild atrophy of the superior vermis (figure 2A).

The maternal grandmother (patient I-5) had limited schooling and took up early employment in manual jobs.

Family 2

The proband (II-1), a 16-year-old male, was the first child of non-consanguineous parents (figure 1). He had delayed motor skills (walked at 24 months) and speech delay (first words at 36 months). He attended a special needs school, learnt to write and read simple sentences. He is now following a vocational course to work in a job for people with disabilities. He shows neuropsychological diagnosed mild ID, associated with autistic spectrum manifestations, poor interactive skills and bursts of aggressiveness. The clinical examination noted a large forehead, palpebral oedema, enophtalmia, a wide flat nose, short ears, a small mouth and abnormally implanted teeth. His younger sister (II-2) presented with neonatal global hypotonia and mild psychomotor delay. She walked at 14 months with an ataxic gait and has normal behaviour. Speech was delayed, with the first words at 24 months and poor elocution. Neuropsychological evaluation revealed mild ID. She attended a special educational institution. She had similar facial features to her brother.

Their mother (I-1) also presented with mild ID, attended a special needs school and is currently unemployed. The clinical

examination revealed an ataxic gait with static instability and mild dysmetry. No vestibular signs were noticed. Neuropsychological examination revealed a mild ID. She comes from a large family of 14 brothers and sisters with no other case of ID. Her parents were deceased.

Family 3

The proband was the first child of healthy unrelated parents. Hypotonia and motor delay were noted from birth. Ataxia was observed at 17 months; she walked independently at 21 months. The clinical evaluation at 22 months showed an interactive child with good understanding abilities. She was able to build a stack of three cubes, scribbled, played symbolic games and spoke about 10 words. She had neither dynamic cerebellar signs nor motor deficit but walked with a wide-based gait with occasional falls. The ataxia improved and became visible only during tandem walking at 4 years. She was able to draw a square, presented mild speech difficulties but spoke well-structured sentences. This was confirmed by the neuropsychological evaluations performed at 4 years and 2 months: (1) WPPSI–III testing showed normal non-verbal skills (blocks design 10:19, matrix reasoning 12/19, pictures concept 10:19) and deficient verbal skills due to defective articulation and fluency and (2) Nepsy testing showed normal sensorimotor but mildly altered visuospatial (design copying) functions.

Cohorts of patients with sporadic or autosomal dominant ID/childhood ataxia

Two cohorts of patients were assembled. Group 1 included 150 male or female patients with NS-ID, including 25 (17%) with a family history of ID compatible with an autosomal dominant mode of inheritance. All patients had normal standard chromosomal analysis and half of the series had a normal array-CGH. When performed, cerebral MRI as well as plasma amino acids and urinary organic acid chromatography showed no abnormalities. Group 2 included 47 patients with non-progressive and early-onset childhood ataxia, with (30 patients) or without ID (17 patients). Informed consent was obtained for all tested patients.

METHODS

Array-CGH/SNP

DNA was extracted from peripheral blood lymphocytes. Patients issued from families 1 and 2 were studied using the Human Genome CGH Microarray 105K (Agilent Technologies, Santa Clara, California, USA). Data were processed with feature extraction (V.9.1) software and the results were analysed with CGH analytics (V.4.0) software (Agilent®) in the Hg19 genome assembly. The CNVs identified through an array-CGH were confirmed by quantitative PCR or FISH analysis. For family 3, chromosome microarray analysis was performed on the proband and her parents using Illumina HumanCytoSNP-12 v2.1 chips. All of the procedures were carried out according to the manufacturer's protocol. Raw data were analysed using Illumina GenomeStudio v2011.1 software. To determine the log ratios and the B allele frequencies of every SNP, we generated our reference by using the GeneTrain 2.0 cluster algorithm with 95 other samples processed in the same run. CNVs were called with the cnvPartition v2.4.4 algorithm (Genecall Threshold of 35), and also by visual analysis on the GenomeStudio Genome Viewer. Mapping data were analysed on the human genome sequence using ensemble website, with Hg18 rebuild. CNVs were assessed in the Database of Genomic Variants.

Molecular impact of the *CAMTA1* deletion

Skin fibroblasts

After informed consent was obtained, primary fibroblasts were harvested from skin biopsy from the patient II-6 (family 1) and two healthy controls. The cells were cultured in DMEM (HyClone) containing 10% fetal bovine serum (HyClone) and 1% penicillin/streptavidin (PromoCell). For each sample, total RNA was extracted from three independent culture wells using the RNeasy mini kit (Qiagen) following the manufacturer's recommendations and stored at −80°C.

CAMTA1 cDNA sequencing analysis and quantification

From the extracted mRNA, two experiments were realised. Total cDNA was synthetised from 1 µg of RNA using the Superscript III RT-PCR kit (Invitrogen) using usual procedures.

First, a reverse transcription with specific *CAMTA1* primers was performed to produce cDNA. PCR primers were chosen in exons 3 and 7 using PrimerBlast online software (PCR primers available on request). A standard Touchdown PCR protocol was used. The different amplified cDNA were sequenced to evidence the frameshift secondary to the exon 4 deletion.

The latter experiment was realised in order to quantify the transcription of the mutated allele. *CAMTA1* primers were designed using PrimerBlast online software (PCR primers and conditions available on request). *GAPDH* was used as the reference gene. Quantitative reverse transcriptase PCR (qRT-PCR) data were run on a LightCycler 480 (Roche) using the QuantiTect SYBR green PCR kit (Qiagen). Relative quantification was calculated using the Light Cycler 480 SW1.5 software.

In silico analysis

In patients with unavailable fibroblast cultures, in silico mRNA prediction was used. Deleted exons diagnosed by an array-CGH were removed from the reference sequence. Translation prediction was performed with a prediction tool. Alignments were performed with the clustalw2 program.

Genomic *CAMTA1* sequencing analysis

Group 1

Twenty-three coding exons and intronic flanking regions of the *CAMTA1* gene were amplified using a touchdown protocol in 50 patients from group 1 (n=150). Exons encoding the CG-1 region (exons 2–7) were sequenced for 100 additional patients (PCR primers and conditions available on request). PCR fragments were purified using the multiscreen Vacuum Manifold system (Millipore). Sequencing was performed using an ABI BigDye Terminator Cycle Sequencing kit (v3.1) (Applied Biosystems) in an ABI 3130 sequencer. Sequence data were analysed with SeqScape v2.7 (Applied Biosystems) and Sequencer v4.1.

Group 2

Thirty-six amplicons were designed using the GS FLX Titanium Fusion Primers tool from Integrated DNA Technology (primers available on request) (n=47). The library was prepared according to the Roche Amplicon Library Preparation Manual. GC Rich buffer 1X from the Roche Fastart DNA Polymerase kit was added for amplicon 1. Amplicons were pooled and sequenced on the Roche GS Junior in a single run, according to the Roche emPCR Amplification Method protocol followed by the GS Junior Sequencing Method protocol. The resulting sequences were analysed using GS AVA software.

Cerebral expression of CAMTA1 in mouse embryos

Cerebral expression of CAMTA1 was obtained from the brain map of the developing mouse referenced on the Allen institute

website, and provided as free open resources for the global scientific community.[27]

RESULTS

Array-CGH/SNP results

All of the rearrangements are reported in Hg18 and represented in figure 3. In family 2, the rearrangement was proved to be a tandem duplication by FISH analysis (probe RP11-334N17).

Molecular impact of the *CAMTA1* rearrangements

In family 1, mRNAs obtained from cultured skin fibroblasts allowed a direct analysis of the transcriptional consequences of the intragenic rearrangement. Standard PCR amplification allowed the identification of four PCR products and cDNA fragments were sequenced separately (figure 3B). They included: (i) the native cDNA (named CAMTA1), (ii) an alternative spliced cDNA lacking exon 6 (named CAMTA1Δ6), (iii) a cDNA lacking exon 4 (named CAMTA1Δ4) and (iv) a cDNA lacking exons 4 and 6 (named CAMTA1Δ4Δ6). The absence of exon 4 led to a frameshift (+1) and a subsequent premature stop codon at position 256 (figure 3B,C). In silico prediction of the cDNA sequences revealed that in family 3, the deletion of exons 2 and 3 was predicted not to alter the frame (figure 3C).

Using the fibroblasts from the *CAMTA1* patient II-6 (family 1), we were able to quantify the normal, unmutated mRNA only by qRT-PCR. The quantity of mutated mRNA was lower than the sensitivity of the experiment could quantify (data not shown).

Genomic CAMTA1 sequencing analysis in the NS-ID/childhood ataxia cohort

No pathogenic *CAMTA1* mutation was identified in the coding sequence. One exonic missense sequence variation was identified. Prediction tools were in favour of a benign sequence variation. All of the other variants were intronic. All of the identified variations were previously known polymorphisms listed in control databases or synonymous mutations. No mutations in the exon–intron boundaries were found.

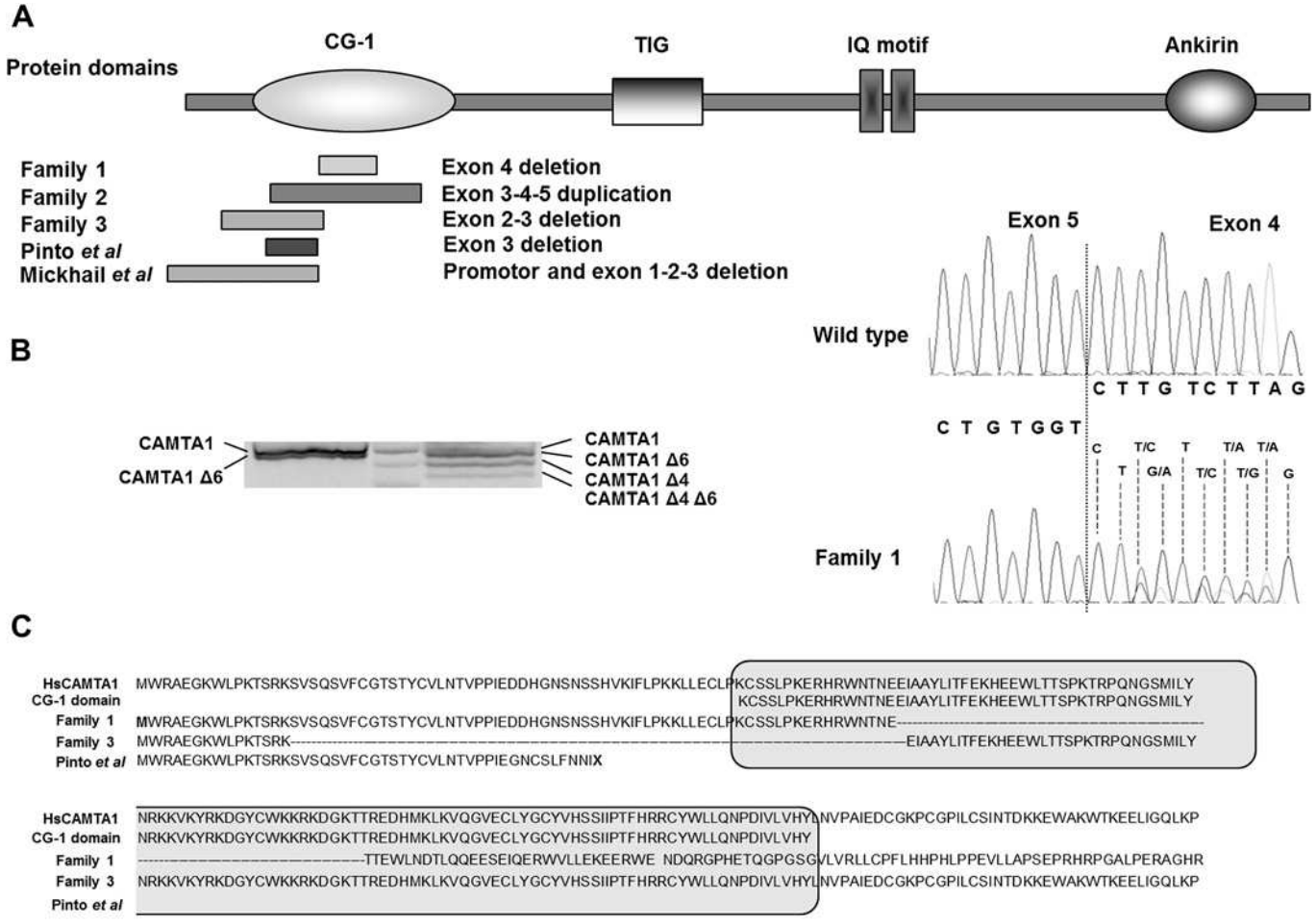

Figure 3 Molecular impact of the intragenic *CAMTA1* rearrangements in reported patients. (A) All the reported rearrangements implicated the CG-1 domain. An 81 Kb deletion at 1p36.31p36.23 encompassing exon 4 (arrCGH 1p36.31p36.23 (7119268–7200395)x1) was identified in family 1 and cosegregated with the disease. A 539 kb duplication at 1p36.31p36.23 (arrCGH 1p36.31p36.23 (6882372–7422115)x3) was identified in the two affected siblings of family 2, inherited from the mother (also affected). The rest of the family was not accessible for study. (A) A de novo 49 kb deletion at the 1p36.31 locus (arrSNP 1p36.31 (6777038–6826186)x1 dn) encompassing exons 2 and 3 was diagnosed in the proband of family 3 (figure 3A). (B) Analysis of the cDNA in fibroblasts revealed that the deletion of exon 4 caused a frameshift. (C) Protein products from the intragenic rearrangements of *CAMTA1* demonstrating a truncation (families 1 and 2), contrasting with the patient with childhood ataxia (family 3). The deletion of exons 3 and 4 (patient from Pinto *et al*[7]; family 1) both led to the production of a truncated protein and a premature termination codon respectively in positions 48 and 256.

Cerebral expression of CAMTA1 during mouse development

Embryonic mice show a global brain expression of the CAMTA1 protein that became restricted to the cerebellum, hippocampi and olfactive bulbs later on in development (figure 4). At E15.5, CAMTA1 is expressed in the whole brain. In the forebrain, positivity is strong in the ventricular zone of the cerebral hemisphere and the olfactory recessus, fulfilled at this stage with migrating neuroblasts. In the midbrain, positivity is stronger in the dorsal wall. In the hindbrain, the first rhombomere shows positivity as well as the ventral neuroepithelium (figure 4A). At E18.5, positivity is marked in the cortical plate and reduced in the ventricular zone. The olfactory system and the cerebellar anlage express also positivity (figure 4B). At P4, positivity is found in the olfactory system and is weakly present in the superficial layer of the cortical plate and in the gyrus dentate of the hippocampus. In the cerebellum, the vermian external granular layer shows positivity (figure 4C). At P14, positivity is restricted to the olfactory system and also found in the internal granular layer of the vermis (figure 4C).

DISCUSSION

We report on three independent NPCA ± ID families with an intragenic rearrangement affecting the CG-1 domain of the *CAMTA1* gene, arising de novo or segregating with the disease making *CAMTA1* a novel gene for S-ID. DNA sequencing analysis of CAMTA1 in a cohort of 197 patients with ID and/or NPCA failed to identify further mutations, indicating that micro-rearrangements are the most frequent mutations encountered in this gene.

All of the rearrangements affected the CG-1 domain of the protein and two-thirds were predicted to create a frameshift and a truncated protein (figure 3). There was no truncating mutation identified in more than 7000 exomes realised on patients without ID (NHLB1 Exome Sequencing Project data). By interrogating the Database of Genomics Variants, we found one intragenic CNV affecting the *CAMTA1* gene identified in a healthy individual.[28] The Q-PCR control of the deletion finally defined that the CNV was purely intronic (S Scherer, personal communication). Apart from the identification of intragenic *CAMTA1* deletions/duplication, other evidence supports the disease-causing role of the gene as: (i) the *CAMTA1* gene appears to be predominantly expressed in the human brain[17] and its integrity is necessary for visual response in the fruit fly, which suggests that it has a superior function in mammalians;[22 23 29] (ii) the cellular functions of genes being under CAMTA1 transcriptional control suggest a putative role of CAMTA1 in promoting neuronal differentiation and are responsible for ID ± ASD when genes such as *SHANK3, CHRNA7, NRG1* and *2, CAMK4* and *CASK* are mutated;[30 31] and (iii) a replicated association between a CAMTA1 allele (rs4908449; p=0.0002) and differential performance in normal episodic memory, which adds *CAMTA1* to the list of calcium-responsive proteins responsible for memory processes in humans.[32 33]

Physiopathological mechanisms by which the loss of function of CAMTA1 leads to NPCA and ID remain to be determined. The function of the CAMTA proteins has been studied in various models.[19] In *Arabidopsis*, CAMTAs located in the nucleus and interacted with double-stranded DNA and calmodulin and activate transcription in response to various cellular stresses.[20 21] The CG-1 domain of *CAMTA1* is crucial for nuclear localisation and further transcription regulation in neuronal cells.[23 31] The analysis of the CAMTA1 cDNA from cultured fibroblasts (family 1, patient II-6) allowed the identification of different transcriptional products (figure 3). The detection of the mutated cDNA by sequencing does not exclude the possibility of a residual truncated protein. However, in the patient's fibroblasts, the qRT-PCR was unable to quantify the mutated cDNA. This non-quantifiable cDNA suggested that nonsense-mediated mRNA decay could be activated and is in favour of haploinsufficiency causing the phenotype. This argument is reinforced by a transcriptomic analysis suggesting that haploinsufficiency of the *CAMTA1* gene could lead to a transcriptional misregulation of known genes implicated in neuronal differentiation and proliferation.[30] A dominant

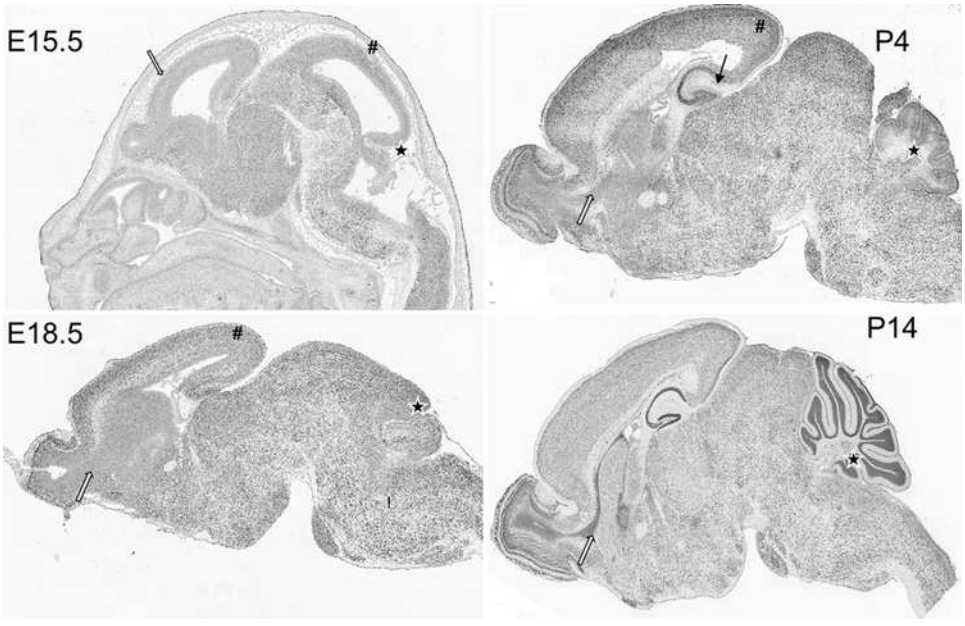

Figure 4 CAMTA1 expression during mouse brain development. The inset shows the expression of CAMTA1 in the murine brain as detected by in situ hybridisation. Embryonic mice show a global brain expression of the CAMTA1 protein that became restricted to the cerebellum, hippocampi and olfactive bulbs later on in development. At E15.5, CAMTA1 is expressed in the forebrain (prosencephalon), midbrain (mesencephalon) and hindbrain (rhombencephalon). In the forebrain, positivity is strong in the ventricular zone of the cerebral hemisphere and the olfactory recessus (white arrow), fulfilled at this stage with migrating neuroblasts. In the midbrain, positivity is stronger in the dorsal wall (#). In the hindbrain, the first rhombomere shows positivity as well as the ventral neuroepithelium (star). At E18.5, marked positivity in the cortical plate, and reduced in the ventricular zone. The olfactory system (white arrow) and the cerebellar anlage (star) also express positivity. At P4, positivity is found in the olfactory system (white arrow) and is weakly present in the superficial layer of the cortical plate and in the gyrus dentate of the hippocampus (black arrow). In the cerebellum, the vermian external granular layer shows positivity (star). At P14, positivity is restricted to the olfactory system (white arrow) and also found in the internal granular layer of the vermis (star).

negative model could not be ruled out since oligomerisation of CAMTA1 is needed for the proper function of nuclear localisation and transcriptional regulation.[22 23 29]

Although ID was the leading feature in the majority of patients of our cohort, ataxia appeared as a key feature in the cases with *CAMTA1* rearrangements. From the patients we report, ataxia is consistent and of early onset although it can be mild and easily missed. Interestingly, one patient with a rearrangement of the *CAMTA1* gene including the promoter and the first three exons has been reported in a series of patients with ID and ASD screened for intragenic rearrangements by high resolution array-CGH. The patient presented with developmental delay, ID, attention deficit hyperactivity disorder and pervasive developmental disorder associated with an ataxic gait and speech disorders such as velopharyngeal deficiency.[34] One further patient with ID and autism was identified in the context of a genome wide CNV study in autism, but no clinical data are provided.[7] *CAMTA1* mutations/rearrangements are likely to be extremely rare, since we found none in a series of 196 cases and the patient above was the only one in a cohort of 996 patients with ASD (both with or without ID) detected by an array-CGH and found to harbour an intragenic *CAMTA1* rearrangement.[7] Congenital non-progressive ataxia appeared isolated in the family with the inframe deletion (family 3) but associated with ID in families with truncating rearrangements (family 1). Thus, both ID and ataxia will be associated with CAMTA1 loss of function. Cerebellar symptoms, such as ataxia, are compatible with the cerebellar expression of CAMTA1 in mouse at late stages of development (figure 4).

As expected, considering the functions of CAMTA1 in learning and memory processes we identified a number of manifestations in the adult patients we investigated through careful neuropsychological evaluation in family 1. Members of the family demonstrated a lack of rehearsal processing in episodic visual memory. This clinical finding was found in association with the presence of mild bilateral hippocampal atrophy, simplified gyration of the dentate gyri and severe bilateral posterior cortical atrophy including the parietal lobes, the precuneus and the cuneus on neuroimaging (figure 2). A similar pattern of mild parietal atrophy was identified in the two adult patients, although not specific. These findings converge with the results of functional MRI of patients with differential episodic memory performance as described by Huentelman and colleagues.[32] In this study, there was an asymmetrical activation of the mediotemporal lobe in two comparison groups on functional brain imaging. Here, PET scan analysis revealed asymmetrical hypometabolism of regions involved in memory processes (figure 2). Those neuropsychological data support the role of *CAMTA1* in the visual and sound perception network in human left cerebrum. A bio computational analysis of STAT2 mediated transcriptional control in human versus chimpanzee study underlined the expression of *CAMTA1* in this network.[35]

In conclusion, the identification of three novel independent families with intragenic rearrangements combined with their specific cerebral expression and the functions of the calcium-dependent transcription factor adds *CAMTA1* to the list of genes responsible for NPCA with ID.

Web resources

Primer blast designing tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/), translating prediction tool (http://web.expasy.org/translate/), reference genomic sequence (http://www.ensembl.org), protein alignment sequences (http://www.genome.jp/tools/clustalw/ website), Database of Genomic Variants: (http://projects.tcag.ca/variation/), Decipher database (http://decipher.sanger.ac.uk/), Control DNA SNP databases on Hapmap (http://www.hapmap.org); dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/), NHLB1 Exome Sequencing Project (ESP) Exome Variant Server (http://evs.gs.washington.edu/EVS/), Polyphen 2 (http://genetics.bwh.harvard.edu/pph2/), SIFT (http://sift.jcvi.org/), OMIM (http://omim.org/). Allen brain institute, mouse brain CAMTA1 images: http://developingmouse.brain-map.org/data/compare/ivt.html?ispopup=true&include=100046547,100046440,100046759,100057301,100083105,100042096,73716734,69540333,100057147.

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The *FOXP1* hs1149 enhancer: good candidate for distal limb contractures in 3p14.1p13 microdeletion

1. *Résumé de l'article*

Connaissances antérieures: Les contractures articulaires distales (DLC) est une condition cliniquement et génétiquement hétérogène. Environ 25% des DLC bénéficient d'un diagnostic moléculaire par l'identification de mutations de gènes composant l'apparatus musculaire squelettique. De larges délétions interstitielles du bras court du chromosome 3 ont déjà été rapportées par analyse de caryotypes standard, mais aucun phénotype spécifique n'avait été rapporté.

Méthodes et Résultats: Nous rapportons 4 patients atteints de DLC syndromiques et porteurs d'une microdélétion 3p14.1p13 *de novo*. Le phénotype clinique des patients porteurs d'une microdélétion 3p14.1p13 semble comporter des contractures multiples, des troubles alimentaires, un retard de développement et une déficience intellectuelle. La dysmorphie faciale était constante et associait un blépharophimosis, des oreilles basses implantées en rotation postérieure. Une revue des cas précédemment publiés avec un localisation précise des points de cassure a permis de réduire la plus petite région chevauchante associée au phénotype de DLC à 250kb. Cette région comprenait le gène *EI4E3*, les 3 premiers exons de *FOXP1* et des régions introniques dont l'enhancer hs1149. Afin de déterminer l'implication de ces candidats dans les DLC, une cohorte de 11 patients français porteurs de DLC isolées ou syndromique sans diagnostique a été étudiée. Le séquençage Sanger de hs1149, *EIF4E3* and *FOXP1*, ainsi qu'une quantification au locus est revenu normal. Une étude phénotypique de la souris KO hétérozygote pour *Foxp1* a été étudiée mais n'a pas révélé de manifestation de type DLC.

Conclusion: Nous rapportons un nouveau syndrome microdélétionnel comprenant des DLC et impliquant le locus 3p14.1p13. Ces données sont consistantes avec les patients rapportés précédemment et pointent une région minimale pour la présence de DLC. Les données de la littérature et l'étude de la souris KO *Foxp1* soulignent l'importance l'enhancer intronique hs1149 comme candidat aux DLC dans le syndrome microdélétionnel 3p14.1p13. Ces résultats renforcent l'importance des régions introniques régulatrices et leur implication en pathologie développementale.

2. Discussion et Perspectives

L'intervalle d'étude comprenait 2 gènes dont *EIF4E3*. L'expression cérébrale et musculaire de la protéine EIF4E3 nous a fait considérer ce gène comme candidat. Cependant, le séquençage négatif de ce gène dans notre cohorte nous a fait considérer les autres candidats de la région minimale d'intérêt. Il existait dans la littérature de nombreux patients avec des mutations ponctuelles, des réarrangements intragéniques ou multigéniques affectant *FOXP1*. Ces anomalies génétiques étaient associées à des phénotypes de DI avec troubles productifs du langage et la présence de troubles du comportement autistique.

Cependant, *FOXP1* a d'abord été caractérisé en physiologie murine comme un facteur de transcription promoteur de la différenciation neuronale. L'expression de FOXP1 permettait la différenciation d'un pool de neurones progéniteurs des motoneurones de la chaine latéro-médiale, spécifiques des membres.

Les données d'expression embryonnaires de Foxp1 (www.emouseatlas.org) suggèrent 3 localisations principales : cardiaque, cérébrale (en particulier dans le lobe frontal) et médullaire. Il existe des enhancer introniques spécifiques de l'expression de FOXP1 dans chacun de ces sites anatomique (http://pipeline.lbl.gov). La souris KO rapportée est léthale à cause de malformations cardiaques. Les patients atteints de DI ou troubles autistiques ont des réarrangements chromosomiques comprenant l'enhancer de *FOXP1* spécifique de son expression cérébrale.

La description de ce syndrome microdélétionnel 3p14.1p13 suggère une nouvelle implication de *FOXP1* en pathologie humaine, en se basant sur les données de la littérature disponible sur les études de physiologie murine.

A la suite de ce travail, nous avons intégré l'annotation de régions introniques conservées à nos interprétations de CGH.

3. Article

The hs1149 enhancer: good candidate for distal limb contractures in 3p14.1p13 microdeletion

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ABSTRACT

Distal limb contractures (DLC) is a heterogeneous clinical and genetic condition. Overall, 20 to 25% of the DLC are caused by mutations in genes encoding the muscle contractile apparatus. Large interstitial deletions of the 3p have already been diagnosed by standard chromosomal analysis, but not associated with a specific phenotype. We report on four patients with syndromic DLC presenting with a *de novo* 3p14.1p13 microdeletion. The clinical features associated multiple contractures, feeding problems, developmental delay and mental retardation. Facial dysmorphism was constant with low-set posteriorly rotated ears and blepharophimosis. Reviewing previously reported cases with a precise mapping of the deletions: a 250kb smallest region of overlap (SRO) necessary for DLC was narrowed. This region contained 1 gene, *EIF4E3*, the first 3 exons of the *FOXP1* gene, and an intronic enhancer of *FOXP1* named hs1149. Sanger sequencing and locus quantification of hs1149, *EIF4E3* and *FOXP1* in a cohort of 11 French patients affected by DLC appeared normal. We report on a new microdeletional syndrome for DLC involving the 3p14.1p13 locus. Together with previously reported patients, a SRO necessary for DLC was highlighted. A parallel with the *Foxp1* null mouse pointed the hs1149 enhancer as candidate for the pathophysiology of the DLC.

INTRODUCTION

Arthrogryposis Multiplex Congenita (AMC) is not a specific diagnosis, but rather a clinical finding. It characterises more than 300 different disorders from amyoplasia to distal limb contractures (DLC), which may be syndromic or isolated. The overall prevalence of AMC is one in 3000 live births[1]. Among them, DLC are characterized by congenital contractures of two or more different body areas affecting primarily the distal extremities. A classification was proposed and referenced at least ten different isolated DLC, that have been described and classified hierarchically according to the proportion of features they share. In this classification, type 1 Distal Arthrogryposis (DA1) would correspond to the pure clinical forms of dominant DLC[2].

Genetic aetiologies of DLC were mainly represented by mutations in genes encoding for the skeletal muscle contractile apparatus[3]. These genes include myosin heavy chain (*MYH3* and *MYH8;* MIM*160720 and 160741), troponin I (*TNNI2*; MIM*191043), troponin T (*TNNT3*; MIM*600692), tropomyosin (*TPM2*; MIM*190990) and an embryonic myosin binding protein (*MYBPC1*; MIM*160794). Apart from mutations in the muscular apparatus genes, DLC may be secondary to motor neuron dysfunction or premature death. Indeed, DLC can be present in neonatal forms of Spinal Motorneuron Amyotrophy (SMA) or in spinal CMT disease[4]. These pathophysiological processes with DLC as a clinical feature of a motor neuron disease infer that both motor neuron integrity and proper muscular innervation are necessary for a proper development of distal joint movements. Recently, two independent approaches identified *ECEL1* (MIM*605896) as a major gene implicated in DLC, implicated in axonal migration and neuro-muscular junction integrity[5, 6].

During embryogenesis, limb specific motor neurons are clustered within the anterior horn of spinal chord in the latero-medial column (LMC) following a very precise developmental process[7]. Among the implicated genes, *FOXP1 is* a key player of the LMC progenitors migration and differentiation dependent from a spatially and temporally tightly tuned expression. The *FOXP1* gene encodes for a ubiquitous F-box transcription factor implicated in cellular differentiation notably in neuronal population during central nervous system development. In human disease,

FOXP1 was reported to be associated with intellectual disability, ASD and speech delay when deleted or mutated[8]. Although the phenotype of these patients was restricted to neurodevelopmental affection, large chromosomal deletions could be identified in the literature to be associated with a syndromic clinical presentation systematically associated with DLC[9-13, 16]. Recently, an embryological enhancer of the limb specific motor neuronal expression of FOXP1 has been reported and named hs1149[17].

Here, we present the delineation of a new microdeletional syndrome encompassing the 3p14.1p13 locus and associated with syndromic DLC. Reviewing the literature, we defined a SRO necessary for the DLC containing 2 genes and an intronic regulatory sequence named hs1149. Data from previous studies pointed hs1149 as a key enhancer of FOXP1 expression in limb specific motor neurons.

PATIENTS AND METHODS

Index case (Decipher patient 257047) (France)

The propositus was the first child from healthy non-consanguineous parents. The pregnancy was marked by calcification of the choroid plexus associated with intra-uterine growth retardation. Amniocentesis revealed a normal standard chromosomal analysis (46,XX). Fetal brain MRI was performed because of choroïd plexus calcification and showed no other malformations. The infant was born premature at 34+5 weeks of gestation. Birth measurements were below normal: birth weight 2250g (5th centile), length 44cm (3rd centile) and OFC 32.5cm (25-50th centile). The neonatal examination found axial hypotonia. Facial dysmorphism was noticed with hypertelorism, low-set backward rotated ears, mild microstomy and a high arched palate (Figures 1A and 1B). Examination of the limbs showed DLC with ulnar deviation of the wrists and the absence of palmar flexion creases on the fingers. Neonatal transfontanellar echography confirmed the choroid calcifications associated with pellucid cysts. Cardiac ultrasound revealed atrial septal defect with pulmonary hypertension. The ophthalmologic examination showed nystagmus and bilateral converging

strabismus. First months of life were marked by recurrent infections and low weight gain (<3rd centile at 9 months of age), leading to chronic nasogastric feeding and then gastrostomy. She presented complicated and fever-induced convulsive episodes, requiring chronic anticonvulsive treatment. Partial growth hormone deficiency was diagnosed and supplemented. Growth treatment was stopped after 1 year because of inefficacy. She presented with severe psychomotor delay: at 2 years of age, the axial hypotonia was severe with peripheral spastic hypertonia (Figure 1C); she was able to hold her head but was unable to sit. Passive finger movements were possible, but the DLC were very severe leading to no spontaneous prehension. She was not treated surgically but was helped by wrist and corset orthesis, active physiotherapy and occupational therapy. Brain tomodensitometry,electroencephalogram, electromyography and muscle biopsy were normal. A central or peripheral cause of limb contractures remained possible. The proband died at 3 years of age because of an underlying infectious event. No sequence variation could be identified in the *TNNT3*, *TPM2*, *TNNI2* and *MYH3* genes by sanger direct sequencing of all exon and intron boundaries.

Other patients

The European Decipher database (See URLs) was searched for 3p14.1p13 deletion, which allowed us to find three other cases. Additional clinical data for these patients were collected from their treating physicians (Table 1).

Patient 2 (Decipher patient 2059)

Patient 2 was reported as a 19-year-old woman presenting with syndromic DLC. The phenotype included hypotonia, club feet, bilateral hip dislocation, intellectual disability, sensorineural deafness, and constipation with intestinal malrotation. Because the patient died, it was not possible to collect additional clinical data.

Patient 3 (Decipher patient 4438)

Male patient 3 was the second child from healthy, unrelated parents from Danish origin. He

had a healthy older sister. After *in vitro* fertilization, the pregnancy was unremarkable. At birth at 38 weeks of gestation, weight was 2680g (<5th centile), length 49cm (10-25th centile) and OFC 34cm (25-50th centile). Syndromic limb contractures were diagnosed in the first months of life because of congenital contractures of the fingers and metatarsus varus (Figure 1D). He also presented facial dysmorphism including short palpebral fissures, hypertelorism, low-set dysplastic ears, very narrow meatus acousticus externus and microstomia. Follow-up was marked by recurrent respiratory tract infections, failure to thrive (weight 9100g at 3 years of age), severe feeding difficulties, and severe developmental delay with no language or ambulatory function. Cardiac ultrasound was normal. Electromyography, muscle biopsy and brain MRI have not been performed. No sequence variation could be identified in the *TNNT3*, *TPM2*, *TNNI2* and *MYH3* genes by sanger direct sequencing of all exon and intron boundaries.

Patient 4 (Decipher patient 252324)

The female patient 4 was born from healthy unrelated parents. Birth weight was 3.400g (10e-50e centile) at term. At 2 years of age her weight was 9.500g (3rd centile), with height at 81.5cm (10th centile) and OFC at 47cm (25-50th centile). Facial dysmorphism included ptosis, epicanthic folds, mild clinical micropthalmia, squared low-set backward rotated ears, and small mouth. Her hands appeared large compared with her size, with a hand length of 10cm (25th centile) and a middle finger length of 4.2cm (25-50th centile). She had no clinodactyly of her fingers. She had a slender build in keeping with her height to weight ratio. She had bilateral clinodactyly of toes III, IV and V. Electromyography, muscle biopsy and brain MRI were not performed.

Cohort of patients with distal limb contractures

A cohort of 11 patients with DLC was collected by the French reference centre for developmental abnormalities and malformative syndromes. DNA samples were extracted from lymphocytes after patient consent had been obtained from the referring clinicians. Given the genetic

heterogeneity of this clinical presentation, we defined the phenotypes more precisely with clinical examinations, electromyography and muscle biopsies when possible to exclude neurogenic or myopathic joint contractures. Limb contractures appeared isolated in 5/11 patients. The 8/11 patients with syndromic joint contractures presented facial dysmorphism (2/8 cases), oculo-motor disorders (2/8 cases), epilepsy (2/8 cases), hypospadias (1/8 cases), dyspraxia (1/8 cases) and developmental delay (6/8 cases). Electromyography was normal in 6/7 cases with evidence of myogenic disorders in 1/7 cases. Muscle biopsy was normal in 4/4 cases. Brain MRI showed cortico-sub cortical atrophy in 2/8 cases and abnormal subcortical signal in 1/8 case.

Standard chromosomal analysis was normal in 11/11 cases, as well as high-resolution array-CGH in 3/3 patients. Mutations in the *TNNI2*, *TNNT3, TPM2* and *ECEL1* genes were excluded in all cases by Sanger sequencing analysis. Mutations in the *MYH3* gene were excluded in 3/11 patients.

Array-CGH

The different platforms used either the Human Genome CGH Microarray 44K, 105K or 244K from Agilent, according to the manufacturer's protocol (Agilent Technologies, Santa Clara, CA). Data were processed with feature extraction (v. 9.1) software and the results were analysed with CGH Analytics (v. 4.0) software (Agilent). When a deletion was identified through array-CGH, the abnormality was confirmed either by fluorescence *in situ* hybridization (FISH) or by specific quantitative PCR. Results from the array CGH were converted from Hg18 to Hg 19 builds using the liftOver tool with standard parameters (See URLs). Genetic content of the smallest region of overlap was defined using Table Browser (See URLs).

Caracterisation of the smallest region of overlap (SRO)

Al the patients presenting with a interstitial deletion of the 3p, both from the literature and public databases were aligned, leading to the definition of a SRO region at the 3p14.1p13 locus

necessary for DLC. This SRO was refined using two references. First, Petek and collaborators reported a 22-month-old boy with developmental and psychomotor retardation as well as facial dysmorphism, including a cleft lip but no DLC. Standard karyotyping identified a *de novo* interstitial deletion of the short arm of chromosome 3, del 3p13p11. Mapping was refined by specific BAC clones and microsatellite studies, defining a 15 Mb deleted segment between the centromere and marker D3S3551[14]. Second, Carr and collaborators described a patient presenting with speech delay and mental retardation but no symptoms of multiple joint contractures. The deletion in this patient was mapped arr 3p14.1 (70425428-71536242)x1, *dn* [15]. The deletion included the *FOXP1* gene except for the first non-translated 2 exons[15]. The deletion reported by Petek et al. encompassed 15 Mb, starting at the D3S3551 (Chr 3:71,861,570) and the deletion reported by Carr et al. ended at the probe mapped located in 3p14.1 (Chr 3:71,618,932). The genomic coordinates of these articles were updated the Hg19 built using the lift-over tool (See URLs). The SRO (chr3:71536242-71778880, Hg19) contained the *EIF4E3* and *FOXP1* genes, and intronic regulatory sequences with the hs1149 enhancer.

EIF4E3, FOXP1and hs1149 enhancer sequencing and quantification analyses

The genes sequence were chosen on the ensembl.org website (See URLs). The coding sequence used were ENST00000318789 for *FOXP1* and ENST00000389826 for *EIF4E3*. DNA direct sequencing analysis of exon and intron-exon boundaries was performed to identify mutations in the coding sequences and flanking region of the *FOXP1* and *EIF4E3* genes (primer sequences and PCR conditions are available on request). All the exons of the *EIF4E3* gene were quantified to detect a heterozygous deletion or duplication (primer sequences are available on request). The hs1149 enhancer and the first exons of *FOXP1*, within the SRO were quantified, using the standard protocol of the Invitrogen *SybrGreen* kit for relative quantitative PCR, amplifications were carried out in a Roche LC4800 machine

Phenotypic study of Foxp1 KO mice

Previously published KO mice[18] were breeded to study moderate DLC in heterozygous KO mice. Both walking and bone morphology were studied for mild phenotypic criteria of DLC.

RESULTS

Array-CGH (Hg19)

For patient 1, array-CGH identified a *de novo* 2.33 Mb deletion on the 3p14.1p13 locus (arr 3p14.1p13 (71006071-73344433)x1 *dn*. Patient 2 was diagnosed with a *de novo* 11.18 Mb deletion (arr 3p14.1p12.2 (71540307-82716711)x1 *dn*. Array-CGH diagnosed 2 chromosomal aberrations in patient 3. The first one was a *de novo* 4.3 Mb deletion on chromosome 3 (arr 3p14.1p13 (68541944-72842054)x1 *dn* and a *de novo* 1.24 Mb deletion on chromosome 8 (arr 8q23.2q23.3 (111231630-112475547)x1 *dn*. In patient 4, array-CGH identified a *de novo* 3.01 Mb deletion (arr 3p14.1p12.3 (71366497-74373822)x1 *dn* (Table 1, Figure 2).

EIF4E3 and FOXP1 sequencing and quantification analyses

No mutation was identified by the Sanger sequencing analysis of the *FOXP1* and *EIF4E3* genes in 11 patients with DLC. This analysis has also been performed in our index case in order to search for a mutation in the second allele and was normal. No intragenic deletion was detected by quantitative PCR of all the exons of the gene. Sequencing of the hs1119 intronic regulatory region did not show sequence variation.

Intronic region study

Alignment of the SRO across 9 vertebrate organisms allowed the identification of clustered and conserved intronic sequences (Figure 3A). The ENCODE project data defined these conserved regions carrying transcriptionally active chromatin markers and DNAse hypersensitivity (Figure 3A). Searching for regulatory elements in the Enhancer DB pointed an enhancer (hs1149) (Figure 3A). The genomic sequence of the enhancer hs1149 was sequenced in the 11 patients, but did not

identified any pathogenic mutation.

Phenotypic study of Foxp1 KO mice

Heterozygous mice for FOXP1 KO were examined for DLC. No abnormal gait and no skeletal sign of DLC were noticed.

DISCUSSION

We report on 4 patients with microdeletions at the 3p14.1p13 locus and question the pathophysiological hypotheses to explain the implication of this new microdeletional syndrome in syndromic DLC.

Syndromic DLC had already been reported in several patients with large deletions or translocations implicating chromosome 3, diagnosed by standard chromosomal analysis [9-13, 16]. There was no precise delineation of the interstitial breakpoints in those patients. Hertz and collaborators reviewed six cases with 3p deletions identified by standard chromosomal analysis, but they did not manage to delineate a 3p deletion syndrome[11]. Only 4/6 cases were clinically described. All 4 patients presented with clinical manifestations of multiple contracture abnormalities such as ulnar deviation of the wrists or talus calcaneovalgus feet[11]. Additional sporadic case reports diagnosed with standard chromosomal analysis were consistent with the association of the 3p14.3p13 locus with syndromic DLC[12, 13]. Only one patient was reported with a 785kb 3p14.1p13 microdeletion associated with syndromic DLC[16].

Herein, the 4 presented new patients and others from the literature, together with the clinical homogeneous phenotypes suggest the definition of a new microdeletional 3p14.1p13 syndrome. Patients presented with distal abnormalities with multiple contractures (4/4 cases), gross psychomotor delay (4/4 cases) and feeding difficulties with low food intake (3/4 cases). Facial dysmorphism was constant (4/4 cases) with low-set and posteriorly rotated ears (3/4 cases), and blepharophimosis (1/4 cases) (Table 1). Because DLC was found in the 4 reported patients, a pathogenic mutation in the genes previously implicated in DLC (*TNNT3*, *TPM2*, *TNNI2* and *MYH3)*

was excluded in 2/4 of the reported patients. The presence of syndromic DLC in all the reported patients, together with the exclusion of DLC with other patients allowed the definition of a SRO necessary for DLC at the 3p14.1p13 locus between positions chr3:71536242 and chr3:71778880, Hg19 (Figure 2).

This 250kb critical region contains the *EIF4E3* gene and the first 2 untranslated exons of the *FOXP1* gene. These 2 genes were sequenced and quantified in a cohort of 7 patients, without identifying pathogenic mutation or deletion. *EIF4E3* is a member of Eukaryotic initiation factor 4E family (*eIF4E*) and a cap-binding protein. 5' cap-binding is necessary for translation initiation[19]. EIF proteins are components of the spliceosome and were found to be associated with a several human diseases with acrofacial dysostosis such as the Nager or Richieri-Costa-Pereira syndromes[20] (Favaro et al., personal communication). The EIF4E3 protein was mainly described in chordates and expressed specifically in brain, skeletal and cardiac muscle in mice[21, 22]. Although *EIF4E3* has not been implicated in human disease yet this gene ought to be implicated in some of the clinical features of the patients with 3p14.3p13 microdeletions.

In human pathology, truncating mutations or deletions of *FOXP1* are implicated in speech delay, ASD and intellectual disability[8]. Recently, patients with cardiac septal defects were reported mutated in *FOXP1,* thus expanding the clinical phenotype associated with this gene (Chang et al. 2013). In mice model, *Foxp1* was studied on haematological, cardiovascular and motor neuronal purpose. Null *Foxp1* mice died during early embryogenesis because of cardiac septal defect, but they do not seem to be affected by arthrogryposis[18]. This might be due to an extreme premature death, or a different phenotypic expression. Absence of arthrogrypotic phenotype may be explained by a phenotypic variability between human and mouse and by the location of the deletion distal to the SRO for DLC defined herein[18].

Previous reports demonstrated the critical role of Foxp1 induced migration of motor neurons from a progenitors pool to the limb specific LMC in a dose dependant manner[7]. The specific motor neuron differentiation role of *Foxp1* was studied using a motor neuron specific conditional KO

mice[23]. The conditional KO mice presented with abnormal reinnervation of their limb muscles by other motor neurons pools and phenotypic criteria could be suggestive for DLC[23]. Recently, the intronic hs1149 enhancer was specifically pointed as a binding site for limb specific HOX genes[17]. The activation of the hs1149 enhancer is responsible for the limb specific expression of FOXP1 and further differentiation and migration of motor neuron progenitors into limb specific motor-neurons of the Latero-Medial colums (LMC)[17]. Intronic deletions encompassing developmentally expressed enhancers have been implicated in a series of patients with isolated talipes equinovarus[24]. This reports highlights the role of copy-number variations encompassing intronic and developmentally active enhancers as a novel pathogeneous mechanism. Because of its developmental role regulating *FOXP1* spatio-temporal expression, deletion of the hs1149 enhancer could be suspected to induce DLC in patients with a 3p14.1p13 microdeletion.

Conclusion: These data suggest that 3p14.1p13 microdeletion is a new microdeletional syndrome associated with syndromic DLC. We defined a 250kb SRO associated with DLC encompassing *EIF4E3,* the 5' part of *FOXP1,* and a developmentally active enhancer named hs1149. The hs1149 enhancer is responsible for the expression of FOXP1 in the differentiating limb specific motor neurons. We suspected that the deletion of this enhancer might induce DLC in the reported patients with 3p14.p13 microdeletion. This report reinforces the possible impact of intronic deletions encompassing enhancers of pleiotropic transcription factors as pathogenic mechanisms for developmental anomalies and expands the neuromuscular aetiologies for DLC by affecting the sensori-motor connectivity.

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Competing interests

None

Patient consent

Obtained.

Provenance and peer review

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URLs

Decipher: http://decipher.sanger.ac.uk

UCSC browser: http://genome.ucsc.edu

Ensembl browser: http://www.ensembl.org

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LEGENDS

Figure 1: Pictures of the different patients 1 (A,B,C) and 3 (D). Note the facial dysmorphism with small, backward rotated ears, mild microstomia and variable blepharophimosis.

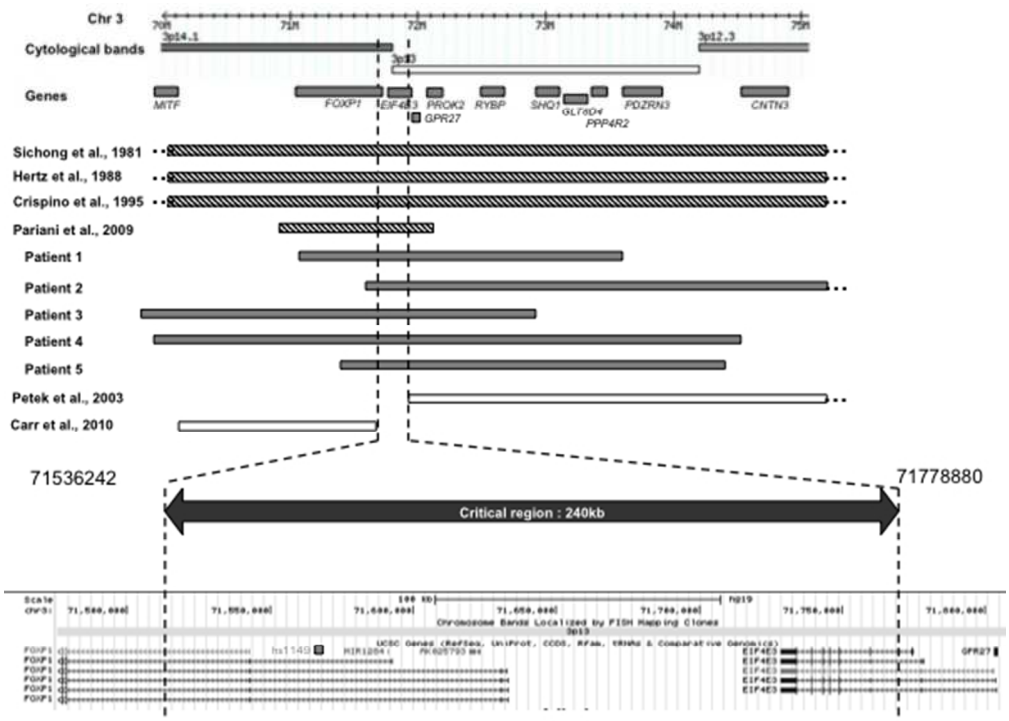
Figure 2: Alignment of the deletions identified in the 4 reported patients and the 4 patients from the literature with distal limb contractures (solid bars) with the 2 patients from the literature without distal limb contractures (hatched bars). The critical region for this condition is limited to a 250kb region containing the *EIF4E3* gene and the first 2 exons of the *FOXP1* gene.

Table 1. Clinical features of the patients with 3p14.1p13 deletion*

	Patient 1 257047**	Patient 2 2059**	Patient 3 4438**	Patient 4 252324**	Pariani et al.
Sex	F	F	M	F	M
Joint examination	ulnar deviation of wrists finger immobility	club feet bilateral hip dislocation	congenital contractures of fingers metatarsus varus	bilateral clinodactyly of toes III, IV and V	severe contracture of hands and feet
Neurological examination	severe developmental delay axial hypotonia convulsions nystagmus	mental retardation hypotonia	developmental delay	developmental delay	developmental delay (sitting around 15 months, absent speech at 24 months) hypertonia
Dysmorphism	hypertelorism low-set and backward rotated ears high arched palate	NA	hypertelorism short palpebral fissures low-set and dysplastic ears small mouth	hypotelorism, ptosis, epicanthic folds, mild clinical microphtalmia low-set and backward rotated ears small mouth	blepharophimosis, epicanthic folds small upturned alae nasi prominent cheeks
Nutrition	feeding difficulties requiring gastrostomy	constipation	severe feeding difficulties	feeding difficulties	feeding difficulties
Other features	recurrent infections bilateral converging strabismus atrioseptal defect	intestinal malrotation sensorineural deafness	recurrent respiratory infections failure to thrive	proportionate short stature	hypermetropia
Brain neonatal transfontanellar echography/MRI	choroid plexus calcification pellucid cysts	NA	NA	NA	mild asymmetric enlargement of the ventricles and sulci, consistent with minor atrophy
Electromyography	N	NA	NA	NA	N
Muscle biopsy	N	NA	NA	NA	NA
Deletion mapping (Hg19)	3: 71006071-73344433	3: 71540307-82716711	3: 68541944-72842054	3: 71366497-74373822	3:71,164,161-71,958,845
Size of the deletion	2.33 Mb	11.18Mb	4.3Mb	3.01Mb	0.8Mb

F: female; M: male; N: normal; NA: not available; *Patients with larger deletion identified by standard chromosomal analysis are excluded from the table; ** number in the DECIPHER database

Figure 1: Pictures of the different patients 1 (A,B,C) and 3 (D). Note the facial dysmorphism with small, backward rotated ears, mild microstomia and variable blepharophimosis.



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SUPPLEMENTAL MATERIAL

The hs1149 enhancer: good candidate for distal limb contractures in 3p14.p13 microdeletion

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Keywords: Array-CGH, 3p14.1p13 microdeletion, distal limb contractures, intronic regulatory sequence, *FOXP1*, *EIF4E3*

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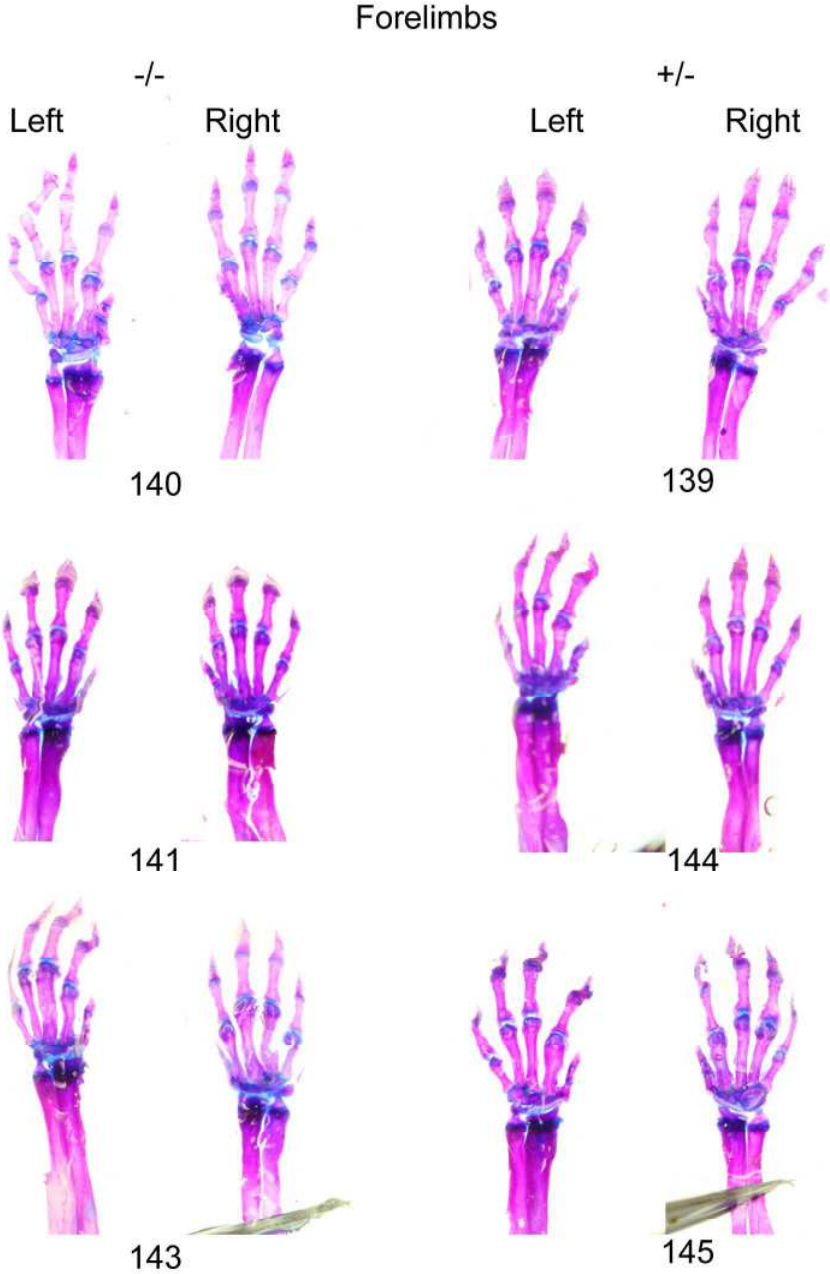
Supplemental figure 1 : Pictures of forelimbs and hind limbs of Foxp1 heterozygous null mice (+/-) compared to wild type mice (-/-). Skeletal preparations of Foxp1 heterozygous null mice (+/-) compared to wild type mice (-/-). No significant difference was identified between the 2 conditions.


Forelimbs
Hindlimbs
Left
Right
Left
Right
140-/-
141-/-
143-/-
139+/-
144+/-
145+/-


Forelimbs
-/-
+/-
Left
Right
Left
Right
140
139
141
144
143
145


Hindlimbs
-/-
+/-
Left
Right
Left
Right
140
139
141
144
143
145

A. Description d'anomalies génétiques impliquant des facteurs synaptiques

Hemizygous deletion of the *SALM1* gene is responsible for a selective working memory deficit.

1. *Résumé de l'article*

L'usage de la CGH-array dans les troubles des apprentissages (LD) a notablement accru le nombre de gènes impliqués dans ce groupe hétérogène de pathologies. Les gènes candidats ont renforcé l'implication de facteurs de transcription post-mitotiques et de facteurs synaptiques dans les troubles des fonctions cognitives.

Nous rapportons une famille unique diagnostiquée avec une LD co-ségrégeant de manière dominante avec une microdélétion de 870kb au locus 6p21. Ce réarrangement chromosomique comprend le gène *LRFN2/SALM1*, codant pour une protéine d'expression cérébrale de localisation post-synaptique.

L'évaluation neuropsychologique de cette famille a identifié un déficit sélectif de la mémoire de travail, sans DI associée. Des évaluations plus poussées ont caractérisé un déficit des fonctions exécutives et des processus auditoro-verbaux. Ces données étaient cohérentes avec l'imagerie cérébrale fonctionnelle montrant une atrophie modérée et un hypométabolisme de régions impliquées dans le réseau neuronal de la mémoire de travail. Une immuno-colocalisation a démontré l'étroite co-localisation de LRFN2/SALM1 avec la sous unité NR1 du récepteur NMDA (N-Methyl-D-Aspartate) dans les parties latérales de la densité post-synaptique de neurones hippocampiques et cérébelleux de rats.

Ces données issues d'approches combinées soulignent l'implication de LRFN2/SALM1 dans les troubles des apprentissages, et plus spécialement dans les processus de mémoire de travail et des fonctions exécutives. En conclusion, l'identification de cas familiaux porteurs d'endophénotypes homogènes de LD participera à l'amélioration de la prise en charge des patients.

2. *Discussion et Perspectives*

La mémoire de travail (WM) est un concept de neuroscience représentant la part de mémoire « active » ou « à court terme ». Ce type de mémoire permet De

traitement des informations visuelles ou auditives, mais conditionne également le stockage de données vers la mémoire à long terme. Le modèle de Baddeley, faisant toujours référence bien que remanié, compose la mémoire de travail de 4 éléments clés : le calepin visuo-spatial et le registre auditoro-verbal (ou boucle phonologique), gérant les informations affluentes, le ‘’buffer’’ épisodique permettant un stockage transitoire de l'information et l'administrateur central qui traite les données, limite l'impact des ‘’interférences’’ sur le traitement de la tâche débutée et relie le système de la WM aux autres types de mémoire.

Les données d'imageries fonctionnelles suggèrent l'existence d'un réseau cérébello-cérébral de la WM. Les données afférentes seraient traitées via les cortex sensoriels par le système limbique. Les données efférentes seraient initiée par le vermis supérieur et relayé par le cortex préfrontal vers les zones cérébrales impliquées dans la réponse demandées. Ces données proviennent de modèles lésionnels issus de pathologies acquises de l'adulte ou d'études IRM fonctionnelles avec des tâches de sollicitation de la mémoire de travail. Bien que l'IRM fonctionnelle avec tâches dédiées soit l'examen de choix pour étudier l'activation de la WM chez nos patients, cet examen nécessite un groupe de patients afin de normaliser les résultats obtenus.

La WM est une capacité cognitive fortement héritable. Cependant, aucune cause monogénique de déficit isolé de WM n'a été identifiée. En population générale, il est estimé qu'une part importante des échecs scolaires soit secondaire à des performances inférieures de WM. Les modèles de pathologies développementales avec déficit de la WM sont les troubles psychiatriques de la lignée psychotique, et en particulier l'autisme et la schizophrénie. Dans ces pathologies, le déficit de WM est bien sur associé à des symptômes psychiatriques au premier plan.

Une grande série de modèles murins de schizophrénie consiste en l'inactivation systématique de protéines de la jonction post synaptique appartenant à la famille des LRR (Leucine Rich Repeat). Au sein de cette grande famille de protéine, les LRFN constituent un groupe de 5 protéines impliquées dans la croissance dendritique et la synaptogenèse. L'équipe ayant découvert le gène de *SALM1/LRFN2* a produit un modèle murin KO. Ce modèle non publié a été présenté au dernier congrès de la Society for Neuroscience 2012, New Orleans, USA (Poster 45.16/D28). Les souris KO présentaient des troubles du comportement évoquant des troubles schizophréniques et autistiques, des vocalisations limitées. De manière intéressante, les souris avaient de bonnes performances de mémorisation et

d'apprentissage. L'étude des synapses hippocampiques des animaux montraient des synapses dysmorphiques avec une activité glutamatergique altérée.

Dans le cas de notre famille, il a pu être proposé une rééducation orientée pour entraîner spécifiquement la mémoire de travail. Il a été discuté une évaluation complémentaire de la patiente adulte afin de déterminer si elle bénéficierait d'un traitement par modulateur glutamatergique utilisés dans les démences type Alzheimer (MEMANTINE®). Ce travail a fait l'objet d'une collaboration fructueuse entre notre équipe de génétique humaine et le laboratoire de recherche fondamentale CNRS du LEAD (Laboratoire d'Etude des troubles des Apprentissages et du Développement). Cette approche transversale a permis une étude précise des cas rapportés et améliorer notablement le contenu de notre étude.

3. Article

A. Description d'anomalies génétiques impliquant des facteurs synaptiques

Hemizygous deletion of the *SALM1* gene is responsible for a selective working memory deficit.

1. *Résumé de l'article*

L'usage de la CGH-array dans les troubles des apprentissages (LD) a notablement accru le nombre de gènes impliqués dans ce groupe hétérogène de pathologies. Les gènes candidats ont renforcé l'implication de facteurs de transcription post-mitotiques et de facteurs synaptiques dans les troubles des fonctions cognitives.

Nous rapportons une famille unique diagnostiquée avec une LD co-ségrégeant de manière dominante avec une microdélétion de 870kb au locus 6p21. Ce réarrangement chromosomique comprend le gène *LRFN2/SALM1*, codant pour une protéine d'expression cérébrale de localisation post-synaptique.

L'évaluation neuropsychologique de cette famille a identifié un déficit sélectif de la mémoire de travail, sans DI associée. Des évaluations plus poussées ont caractérisé un déficit des fonctions exécutives et des processus auditoro-verbaux. Ces données étaient cohérentes avec l'imagerie cérébrale fonctionnelle montrant une atrophie modérée et un hypométabolisme de régions impliquées dans le réseau neuronal de la mémoire de travail. Une immuno-colocalisation a démontré l'étroite co-localisation de LRFN2/SALM1 avec la sous unité NR1 du récepteur NMDA (N-Methyl-D-Aspartate) dans les parties latérales de la densité post-synaptique de neurones hippocampiques et cérébelleux de rats.

Ces données issues d'approches combinées soulignent l'implication de LRFN2/SALM1 dans les troubles des apprentissages, et plus spécialement dans les processus de mémoire de travail et des fonctions exécutives. En conclusion, l'identification de cas familiaux porteurs d'endophénotypes homogènes de LD participera à l'amélioration de la prise en charge des patients.

2. *Discussion et Perspectives*

La mémoire de travail (WM) est un concept de neuroscience représentant la part de mémoire « active » ou « à court terme ». Ce type de mémoire permet De

traitement des informations visuelles ou auditives, mais conditionne également le stockage de données vers la mémoire à long terme. Le modèle de Baddeley, faisant toujours référence bien que remanié, compose la mémoire de travail de 4 éléments clés : le calepin visuo-spatial et le registre auditoro-verbal (ou boucle phonologique), gérant les informations affluentes, le ‘’buffer’’ épisodique permettant un stockage transitoire de l'information et l'administrateur central qui traite les données, limite l'impact des ‘’interférences’’ sur le traitement de la tâche débutée et relie le système de la WM aux autres types de mémoire.

Les données d'imageries fonctionnelles suggèrent l'existence d'un réseau cérébello-cérébral de la WM. Les données afférentes seraient traitées via les cortex sensoriels par le système limbique. Les données efférentes seraient initiée par le vermis supérieur et relayé par le cortex préfrontal vers les zones cérébrales impliquées dans la réponse demandées. Ces données proviennent de modèles lésionnels issus de pathologies acquises de l'adulte ou d'études IRM fonctionnelles avec des tâches de sollicitation de la mémoire de travail. Bien que l'IRM fonctionnelle avec tâches dédiées soit l'examen de choix pour étudier l'activation de la WM chez nos patients, cet examen nécessite un groupe de patients afin de normaliser les résultats obtenus.

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3. Article

TITLE

Hemizygous deletion of the SALM1 gene is responsible for a selective working memory deficit.

AUTHORS

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Keywords
Array-CGH, *SALM1/LRFN2*, learning disability, working memory deficit

ABSTRACT

Learning disabilities (LD) are a clinically and genetically heterogeneous group of disease. Array-CGH and high-throughput sequencing dramatically expand the number of genes implicated in isolated Intellectual disabilities (ID) and LD, highlighting the implication of neuron specific post-mitotic transcription factors and synaptic proteins as candidate genes. We report on a unique family diagnosed with autosomal dominant learning disability and a 6p21 microdeletion segregating in 3 patients. The 870kb microdeletion encompassed the brain-expressed gene *LRFN2/SALM1* encoding for a postsynaptic protein. Neuropsychological assessment identified a selective deficit of working memory, without ID. Further investigations identified a defect in the executive functions, and auditoro-verbal processes. These data were consistent with brain MRI and FDG-PET functional brain imaging that revealed atrophy and hypometabolism of grey matter structures strongly implicated in working memory processes when compared to controls. We performed an electronic microscopy immuno-colocalization demonstrating the tight co-localization of LRFN2/SALM1 with the NR1 subunit of the NMDA (N-Methyl-D-Aspartate) receptors in the lateral part of the postsynaptic density of cerebellar and hippocampal rat neurons. Altogether, the combined approaches highlighted the implication of *LRFN2/SALM1* in LD, specifically by its implication in working memory processes and executive functions. A medication by NMDA modulator could be discussed because of the interaction of LRFN2/SALM1 with the NMDA receptors. In conclusion, the identification of familial cases of clinically homogeneous endophenotypes of LD might help the management of the patients, as well as the genetic counseling of families.

INTRODUCTION

Genetic basis of learning disability are mostly unknown and fully penetrant causal genes responsible for isolated learning disabilities in patients remain limited. Indeed, difficulties in fine phenotyping requiring non-routine investigations, possible incomplete penetrance or variable expressivity, socio-environmental contribution and lack of large familial cases are suggested to limit genetic investigation in such clinical entities. However, as previously suggested, chromosomal imbalances with a purely cognitive phenotype may provide clues towards candidate genes that can have a direct impact on cognition (Flint, 1999). In this context, the identification of precise clinical phenotyping and description of unique families with inherited learning disabilities is crucial for the patho-physiological understanding of specific learning disabilities (Lai et al., 2001). However, most of the genetic determinants of higher cognitive functions including learning, attention, memory and executive functions remain unknown (Graham and Fisher, 2013; Newbury et al., 2010) . Here, we report on a family presenting with a non-syndromic specific cognitive phenotype mainly characterized by short-term memory and/or working memory deficits, without general intellectual disability (ID).

Working memory (WM) is a neural system that provides temporary active maintenance of necessary information while performing complex cognitive tasks such as reasoning, learning, understanding, thinking, decision making and planning (Baddeley and Hitch, 2000; Baddeley, 1981; Khan and Muly, 2011). Selective WM deficit is a rare condition, but might have previously been underestimated (Holmes et al., 2010). However, recent technological advances in genetics and WM sub-tests might help to identify and diagnose such disorders (Kaufman and Lichtenberger, 2005). This is of particular importance as many studies have shown that poor WM performance can impact on academic performance (Gathercole and Alloway, 2006; Gathercole et al., 2006) and is sometimes associated with a range of developmental disorders, such as Down syndrome. Early identification of patients with selective WM deficit is thus critical for better target intervention and to improve long-term outcomes. In this context, experimental paradigms have been developed to improve working memory performance. For example a recent study by Bennett, Holmes and Buckley (2013) demonstrated successfully that working memory could be enhanced following training in children with Down syndrome (Bennett et al., 2013). These findings thus support the importance of early recognition of working memory difficulties.

Most WM models distinguish between the storage capacity of short-term memory (STM) and the storage and processing functions of WM. Storage capacity can be measured using simple span tasks requiring the storage of either verbal or visual information. Working memory can be assessed by complex span tasks requiring participants to process the information (e.g., reading or listening span) (Daneman and Carpenter, 1980). One of the main components of the working model developed by Baddeley and Hitch (Baddeley and Hitch, 1974, 2000) is the central executive. Several functions have been proposed for the central executive including the coordination of multiple tasks (Baddeley et al., 1997), shifting between tasks (Baddeley and Della Sala, 1996) and the capacity to attend and inhibit information (Baddeley et al., 1998). More recent models identified three partially overlapping key executive functions: shifting (moving from one task to the other), updating (replacing non-relevant information) and inhibition (inhibiting prepotent responses) (Miyake et al., 2000, 2010). Patient studies first placed the emphasis on the frontal lobes as the main neural substrates of executive functioning (Collette et al., 2006). However, functional neuroimaging studies revealed a more complex picture (Collette et al., 2006). In fact, despite the fact that this field of research is very well-developed, the studies revealed a considerable heterogeneity of findings. The main outcome of these studies was to demonstrate that the different executive functions are associated with both prefrontal and posterior (mainly parietal) regions (Collette et al., 2006). The posterior parietal cortex (PCC) has also been shown to be involved in visuo-spatial short-term memory (Todd and Marois, 2005). Finally, recent studies have emphasized the role played by the hippocampus in short-term memory functions (Finke et al., 2008. Henke et al., 2010) and in binding of object-location associations in working memory (Piekema et al., 2006).

Executive or working memory deficits have been reported in neurodevelopmental genetic disorders associated with mental retardation and physical abnormalities such as Down syndrome or Williams syndrome (Baddeley and Jarrold, 2007; Carney et al., 2013). Despite the fact that genetic influences on cognitive processes have often been considered to be non-specific, the existing literature suggests evidence that single genes might be involved in selective WM deficit (Karlsgodt, et al., 2011). The dopaminergic excitatory pathway was first studied but recent studies point the critical role of the glutamatergic pathway and post-synpatic regions in cognitive processes (Karlsgodt, et al., 2011; Nithianantharajah et al., 2013). Various approaches including linkage analysis and association studies pointed some candidate genes, such as *ROBO1 or CHD13* associated with lowered WM

performances as a specific endophenotype of a more complex trait (Arias-Vásquez et al., 2011; Bates et al., 2011). More precisely, the genetic variations of *ROBO1* were associated with impaired phonological buffer performance (assessed with word, pseudo-word and non word repetition, as well as forward digit span) and CDH13 variations were associated with lowered WM performance (inhibition, verbal and visuo-spatial working memory were assessed). These two genes were implicated in neuronal type specification, axonal growth and synapse formation (Gonda et al., 2013; Redies et al., 2012). The postsynaptic region seemed relevantly involved in cognitive performance and learning processes (Kaufman et al., 2010).

Proteins belonging to the transmembrane leucine-rich super-family proteins (LRR proteins) were suggested to be crucial for the neuronal postsynaptic organization (Ko, 2012; de Wit et al., 2011). The SALM/LRFN protein family is a brain specific group of proteins characterized by leucine-rich repeat motifs (LRR), as well as Ig-like and fibronectin-III interacting domains (Ko et al., 2006; Morimura et al., 2006; Nagase et al., 1998; Wang et al., 2008). The SALM/LRFNs are transmembrane cell adhesion molecules, that interact with both glutamatergic receptors and membrane associated guanylate kinase (MAGUK) family members like postsynaptic density 95 (PSD-95) (Morimura et al., 2006; Seabold et al., 2012; Wang et al., 2008). SALM/LRFN proteins were able to recruit postsynaptic factors to functional excitatory synapses, as well as modify synaptic plasticity (Ko et al., 2006; Mah et al., 2010). Among them, *SALM5/LRFN5* has already been associated with human diseases with a variable expressivity from autism spectrum disorder (ASD) to schizophrenia, with or without epilepsy (de Bruijn et al., 2010; Mikhail et al., 2011).

Here, we report on a family (a mother and her two daughters) presenting for the first time a *SALM1/LRFN2* gene haploinsufficiency, associated to a non syndromic specific cognitive phenotype mainly characterized by short-term memory and/or WM deficits, without intellectual disability (ID). This paper presents the outcome of the standardized neuropsychological assessment given to all three patients and findings from further working memory tests given to the mother. Functional brain imaging completed the neuropsychological study. The implication of the *SALM1/LRFN2* protein in the post-synaptic density and its co-localization with the NR1 subunit of the NMDA receptor was demonstrated by cellular experiments.

PATIENTS AND METHODS

Patients (Figure 1a)

Patient 1 was the second child of non-consanguineous young parents. She was right handed and 7 years of age at examination. Pregnancy was normal with natural delivery at 38 weeks of gestation. Birth measurements were 2.680 kg for weight (25th centile), 48.5 cm for length (25-50th centile) and 31 cm for occipito-frontal circumference (OFC) (<3rd centile). Psychomotor acquisitions were within the normal range with sitting at the age of 9 months, and walking at the age of 12 months. She was referred because of a speech delay interfering with scholar skills. Speech was absent at 36 months, and secondarily improved with scholarship. No behavioural trouble was associated to the learning disabilities. A speech evaluation was performed in the fifth year because of non-understandable speech. No specific diagnosis could be made. Neuro-pediatric evaluation identified an asymmetric spasticity with brusk patellar reflexes and positive babinsky sign. To explore this symptom, a brain MRI was therefore performed and a mild atrophy of the superior vermis was diagnosed. Other investigations included normal standard chromosomal analysis, absence of fragile-X, as well as normal creatine and guanidoacetate urinary assays, thyroid hormones and creatine kinase dosage, urinary organic acids and plasmatic amino acids chromatographies.

Neuropsychological evaluation (WISC-IV) (Wechsler, 2004) showed a total IQ of 72 and identified a comprehension score in the low range with a vocabulary level clearly not appropriate for her age (*Verbal comprehension*=79). Her perceptual reasoning was also impaired, with difficulties in planning complex spatial actions (*Visual Puzzles=4*), difficulties in spatial reasoning (*Matrix=6*) and, finally, problems when asked to quickly perceive visual details (*Picture completion=6*). On the other hand, her performance on processing speed tasks (Digit symbol-coding=8, Symbol Search=9) was normal. Finally, the results on the working memory tasks indicated clear deficits with performance in the very low range on both tasks (*Digit Span*= 2, *Arithmetic=1*). Speech evaluation pointed very low short term memory skills including low repetitive skill in both logatomes (-3.09 SD) and sentences (-3.64 SD) as well as low recognition of her own errors (-4.73 SD). These results contrasted with appropriate lexical, comprehensive and syntaxic skills for age and good attention (Details in Suppl Table 1). The clinical evaluation was suggestive of an atypical learning disability with non-specific speech delay.

Patient 2 was 5 years of age and right handed at the examination. She was born premature at 33 weeks of gestation because of a retro-placental haematoma. Immediate APGAR was null, and 10 at 5 and 10, minutes after initial reanimation. Birth weight was 2.080 kg. She was hospitalised during 15

days without further complication. Psychomotor acquisitions were at the limit of normal range with sitting acquired at 9 months, crawling acquired at 10 months, and walking at 18 months. Speech was delayed and was absent at the beginning of school enrollment. Mild behavioral troubles were noticed, including aggressivity with frustration. Speech evaluation was perfomed and dysphasia was diagnosed. Speech therapy was started, and standard scholarship with personal assistant was possible. Clinical examination was normal and no facial dysmorphism was noticed. No etiological exams were performed.

Neuropsychological evaluation was done at 5 years old using the WPPSI-III (Wechsler, 2002). On the verbal tests, performance was very poor (*Information=1*, *Vocabulary=1*) contrasting with a better achievement of non-verbal tests (*Block Design=12*, *Matrix=12*, *Picture Completion=9)*. She could, however, name pictures suggesting some preserved abilities.

Patient 3, the mother of the children, was interviewed after the diagnosis for complementary examinations. She was 29 years pf age and right handed. She was born at term. She did not mention delayed psychomotor acquisitions, although a speech delay was a major concern of her childhood. She was oriented towards a school for special needs before the age of 10 years old. She had a vocational course to become a horticulturist but has never worked. The clinical examination was normal. A mild non-specific facial dysmorphism was noticed with hypotelorism and deep-set eyes. A brain MRI was performed and evidenced superior vermis atrophy, associated with mild bilateral atrophy of cerebellar lobes 6 and 7, and bilateral parietal atrophy (Figure 1). No hippocampal atrophy was diagnosed. Neuropsychological evaluation (WAIS-IV) revealed a Total IQ of 78 with heterogenous results in the sub-tests (Wechsler, 2003). While perceptual organization was found to be normal (98) verbal comprehension was within the inferior normal range (84), mainly due to low performance on the *Similarities test*. There was a deficient processing speed (72) and deficient working memory (68) with significant impairments on all tests in the extremely low range (Table 1). Long-term memory was explored using the RLRI test, a task similar in its construction to the California verbal learning test (Delis et al., 1987). Recall (as measured by the number of words correctly recalled) and recognition memory (as measured by the number of items correctly recognized) were overall above average (see Table 1), showing thus overall good episodic memory performance.

Array CGH

The platform used the Human Genome CGH Microarray 180K from Agilent® according to the manufacturer's protocol (Agilent Technologies, Santa Clara, CA). Data were processed with feature extraction (v. 9.1) software and the results were analysed with CGH analytics (v. 4.0) software (Agilent®) in the Hg19 genome assembly. When a chromosomal rearrangement was identified, the anomaly was confirmed by an independent method (diagnosis was made using quantitative PCR) (Thevenon et al., 2012).

Working memory assessment

Further testing of patient 3's WM combined standard neuropsychological tests and experimental tasks. WM was first assessed with neuropsychological tests including the Spatial and Digit Span of the WMS-III (Wechsler, 1997) and the WM Test of Attentional Performance (TAP, (Zimmermann and Fimm, 1995). To explore the awareness of her own memory deficits, we asked patient 3 to predict her memory performance using the classical digit span task of the WAIS-IV. For this purpose, after completing each trial at each sequence length, patient III was asked if she could recall in order the number of items presented (Murphy et al., 1987).

Classic span experimental tasks such as the phonological span (Corbin & Marquer, in press) and the counting span task (Case et al., 1982) were also administered and performance compared to the literature norms. The phonological task consisted of memorizing pseudo-words in sequences of increasing size (1 to 6). Two trials were presented to patient 3 for each length and the task was to recall each sequence immediately after its presentation, without order constraint. The test was stopped when patient 3 failed to recall the two consecutive trials at the same length [Corbin & Marquer, in press]. In the counting span task, patient 3 was asked to count aloud the number of red dots in a series of arrays, and then recall in order the successive tallies of each array. The array cards were presented one at a time in series of ascending length, from 2 to 6 consecutively. When failing to recall the count array on each set of a given length series correctly, the series of higher lengths was not presented and the task was interrupted (Case et al., 1982). The mean span of this task was calculated 25.7, -1.5 SD, (Conway et al., 2002).

Finally, in order to determine whether or not patient 3 also presented attention impairments, subcomponents of the TAP were administered (Alterness and Incompatibility) as well as the D2 test of attention (Brickenkamp, 1981). Executive functioning was assessed using the Trail-Making Test (Reitan, 1986) and the Wisconsin Card Sorting Test (Heaton, 1981).

Functional Brain imaging

Functional imaging with (18F) fluoro-2-deoxy-D-glucose positron emission tomography (18FDG-PET/CT) was performed in patient 3 only. Prior to undergoing the 18FDG-PET/CT, the patient had fasted during 6 hours. After 10 minutes of resting time and wearing an eye mask, she received the injection of 189,5 MBq of F^{18}-FDG. Thirty minutes later, the scan was performed using a dedicated PET/CT system (GEMINI TOF, Philips Medical Systems, Eindhoven, The Netherlands) in 3D mode. Emission data were corrected for dead time, random and scatter coincidences and attenuation correction was performed using a low dose CT scan. Visual inspection of images was performed first. After intensity normalization these images were compared with those of 20 controls by voxel-based method using Statistical Parametric Mapping (SPM8, http://www.fil.ion.ucl.ac.uk/spm).

Cellular experiments

For electron microscope (EM) studies, DAB (3',3-diaminobenzidine tetrahydrochloride)/immunoperoxidase-labeled parasagittal brain sections were prepared in several experiments from three animals, as described previously (Petralia and Wenthold, 1992, 1999; Petralia, 2012; Petralia et al., 2009, 2010). Adult male Sprague-Dawley rats were anesthetized with ketamine/xylazine and perfused transcardially with 0.12 M phosphate buffer (PB) followed by 4% paraformaldehyde in PB and postfixation in the fixative. All animal experiments described in this paper were done according to the National Institutes of Health (NIH) Guidelines for Animal Use (NIH Animal Protocol #1167-07). Fifty-micrometer sections were cryoprotected in 30% sucrose, frozen, and stored at -80ºC. For each experiment, a set of sections were thawed, washed 3X in phosphate-buffered saline (PBS), and then were incubated in 10% normal goat serum (NGS) in PBS for 1 h, then in primary antibody in PBS overnight, then processed with the Vectastain kit and 3',3-diaminobenzidine tetrachloride (DAB; Vector Laboratories, Burlingame, CA, USA). Sections were washed 2x20 min in Cac, fixed in 2% glutaraldehyde/Cac for 15 min, washed 3x10 min in Cac, fixed in 1% osmium

tetroxide/Cac for 1 h, and washed 3x20 min. Further processing was done as described previously, including alcohol dehydration and embedding in epon. As in previous studies, ultrathin sections for electron microscopy were cut from the epon-embedded sections by cutting parallel to the edge of the section. This allowed us to examine both sides of the section since significant labeling is found only to about 5 μm deep on each side. Note that detergents, which can improve penetration of antibodies, were never used in these studies because they will macerate the tissue and may introduce artifacts in the distribution (Petralia et al., 1998). Studies of the ultrastructural distribution of DAB reaction product are limited by differential penetration of antibodies and DAB from the surface (i.e., more penetrates through surface-cut processes) and by DAB "bleeding," typically from a membrane to the adjacent cytoplasm or v.v. (Petralia et al., 1998, 1998). PBS control sections that lacked the affinity-purified primary antibodies were run in all experiments and always were unlabeled.

Electron microscopy of immunogold-labeled brain sections of hippocampus and cerebellum from two animals followed previous protocols (Petralia and Wenthold, 1992, 1999; Petralia, 2012; Petralia et al., 1998, 2009, 2010; Zhao et al., 1998). Adult rats were anesthetized as above and perfused and postfixed with 4% paraformaldehyde plus 0.5% glutaraldehyde in PB. Three hundred micrometer parasagittal sections were cryoprotected in 30% glycerol and frozen in liquid propane in a Leica EM CPC (cryopreparation chamber; Vienna, Austria). Then, frozen sections were immersed in 1.5% uranyl acetate in methanol at -90ºC in a Leica AFS (automatic freeze-substitution instrument), infiltrated with Lowicryl HM-20 resin at -45ºC, and polymerized with ultraviolet light. Ultrathin sections were incubated in 0.1% sodium borohydride plus 50 mM glycine in Tris-buffered saline plus 0.1% Triton X-100 (TBST), followed by 10% NGS in TBST, primary antibody in1% NGS in TBST overnight at 4ºC, and immunogold (10 nm F(ab')2 gold; Ted Pella, Redding, CA) in 1% NGS in TBST plus 0.5% polyethylene glycol (20,000 MW). Sections finally were stained with 0.1% uranyl acetate and 0.03% lead citrate (with 0.1% sodium hydroxide). All labeling used SALM1 antibodies (see above) or NR1 antibodies (Petralia et al., 2002, 2010). Images were processed in Adobe Photoshop with minimal use of levels, brightness and contrast applied evenly throughout the section.

RESULTS

Array CGH analysis and interpretation (Figure 1)

Array CGH was performed on patient 1, diagnosing a 876 kb chromosomal microdeletion at the 6p21 locus, mapped to chr6:39893669-40766735, Hg19. No similar chromosomal rearrangement was identified in public databases such as DGV or Decipher (See URLs). Only 2 truncating events were reported over 6500 healthy patients (See URLs). Familial segregation showed that both sisters carried the 6p21 microdeletion, inherited from the mother. Familial segregation could not be further studied since the mother had no further contact with her parents and other family members. Three genes were included in this deletion, namely *TRDG1*, *SALM1* and *MOCS1* (Figure 1b). The *TRDG1* gene is a testicular factor, with no identified brain expression (see URLs). The *MOCS1* gene (OMIM *603707) is a gene implicated in severe autosomal recessive neonatal epileptic encephalopathy. However, no symptoms have been reported in heterozygous carriers. Accordingly, genic deletions and truncating mutations have been reported frequent in healthy individuals.

Working memory assessment (Table 1)

Tasks assessing short-term and working memory were mostly deficient (see Table 1). On the WAIS-IV, Patient 3 achieved a digit span forward of 3 and backward of 4, thus suggesting difficulties in storing and manipulating verbal information. On the WMS-III, patient 3 achieved a spatial span of 4 forward and a spatial span of 6 backward. In other words, these results suggest that spatial working memory is less impaired than verbal working memory. To further explore patient 3's short-term memory and working memory, several experimental tasks, as presented in the method section, were administered and results compared to findings in the scientific literature on healthy populations. On the phonological span task, patient 3 successfully recalled, at best, a sequence of two consecutive pseudo-words (her score was less than 1.5 SD compared to mean performance of 50 students, Corbin & Marquer, in press). Patient 3's working memory was also measured using a counting span task. During the complex span task, patient 3 recalled two series of length two and one series of length three giving her a span score of 7 (lower than the mean span of 25.7,SD=12.8, obtained by Conway et al., 2002 on 120 students). These results thus confirmed Patient's 3 low span performance. Testing the awareness of memory deficits showed total accordance between predictions and span

performance (predicted 4 and recalled 4), thus showing that patient 3 has a good awareness of her low span.

Performance on the D2 attention test revealed mainly low concentration performance (see Table 1). Subtests of the TAP showed a relatively preserved attentional focus for simple tasks. On the other hand, results at the TAP incompatibility and the WCST do not suggest severe inhibition deficits. The performance at the Trail-Making test shows slow speed of processing probably associated with shifting difficulties.

Brain functional imaging (Figure 2)

The visual analysis of 18FDG-PET/CT images revealed hypometabolism of left temporal region (Figure 2). Images from patient 3 were compared to controls using the SPM statistical model with a threshold masking value of p= 0.001, intensity peak=3,57, cluster size=19 and using the *t-test* in the SPM package. As a result, we confirmed the existence of hypometabolism in i) left temporal cortex (Peak intensity=6.38 and number of voxels=124 for middle temporal gyrus and Peak intensity=4.11 and number of voxels=20 for inferior temporal gyrus), ii) left orbito-frontal cortex (Peak intensity=7,78; number of voxels=31), iii) left lingual gyrus (Peak intensity=3.93 and number of voxels=61), iv) right thalamus (Peak intensity=3.93 and number of voxels=45), v) right superior and medial frontal gyrus (Peak intensity=4,95 and number of voxels=19) and vi) left and right posterior cingulate (Peak intensity=4.04 and number of voxels=35).

Neuronal localisation of SALM1 (Figure 3)

Labeling of synapses in the hippocampus CA1 stratum radiatum with EM/DAB immunoperoxidase and immunogold was localized to both the postsynaptic and presynaptic sides (Figure 3A). Immunogold distribution was prominent in the postsynaptic membrane/density and the presynaptic membrane, as well as in perisynaptic and extrasynaptic locations on the postsynaptic and presynaptic sides. Labeling for SALM1 colocalized at the synapse with labeling for NR1. Distinct labeling of the large mossy terminal synapses in the cerebellar granular layer was not common with EM/DAB immunoperoxidase but was prominent with immunogold, and distribution (Figure 3B) was similar to that seen in the hippocampus. In addition, the granule cell dendrites at mossy terminal

synapses have attachment plaques (puncta adherentia) that contain NMDARs and MAGUKs (Petralia et al., 2002), and SALM1 was localized to these also.

DISCUSSION

We report on the first family with chromosomal micro rearrangement encompassing *SALM1*. We can argue for the pathogenicity of hemizygosity for *LRFN2/SALM1* because: i) no gene deletion has ever been reported in public database of healthy patient (See URLs); ii) Exceptional truncating mutations were reported in the exome sequencing of more than 6500 individuals from the NHLBI cohort (See URLs); iii) SALM1/LRFN2 co-localizes with NR1 (N-Methyl-D-Aspartate Receptor 1) in the post synaptic region; iv) reduced expression of an NR1 partner has been demonstrated to alter excitatory synapse functioning and WM processes (Karlsgodt, Robleto, et al., 2011). The reported individuals are presenting with an endophenotype of learning disability, with selective working memory deficit, demonstrated by standard neuropsychological assessment as well as experimental tasks. Brain imaging reinforced neuropsychological findings. In our patients, *SALM1* haploinsufficiency was associated with mild progressive atrophy of cerebrum and cerebellum cortices. Functional imaging in patient 3 revealed a hypometabolism of the left prefrontal and temporal regions, already implicated in WM processes (Collette et al., 2006). Together with the co-localisation of SALM1 with the NMDAR, this result supports the implication of *SALM1* as a new gene for learning disability with selective WM deficit.

In the literature, WM performance is described as a highly heritable trait although the underlying molecular factors remain to be determined (Ando et al., 2001; Chen et al., 2009). More generally, the molecular basis of non-syndromic learning disability remains largely unidentified. The clinical heterogeneity of such conditions has limited the application of classical genetic studies. The identification of de novo or familial CNV segregating with the phenotype, combined with fine phenotyping enabling the emergence of clinical learning disorders endophenotypes will be a clue. This strategy allowed the identification of genes or chromosomal loci implicated in learning disorders such as developmental verbal dyspraxia (Kang and Drayna, 2011; Thevenon et al., 2013). Remediation of these types of learning disabilities can often be more effectively achieved when the precise cognitive dysfunction has been identified (Murray et al., 2012).

The delineation of cognitive disorders has been improved through the generalisation of neuropsychological assessment of the patients. Specific subtests investigating WM processes were introduced in 2006 (Kaufman and Lichtenberger, 2005). WM deficits are often associated with

complex traits observed in neuropsychiatric disorders such as autism (Holdnack et al., 2011), schizophrenia (Michel et al., 2013) or attention-deficit-hyperactivity disorder (ADHD) (MacAllister et al., 2012); or chromosomal anomalies such as Down syndrome or Williams syndrome (Baddeley and Jarrold, 2007); Carney et al., 2013). In those examples, WM deficits are variously associated with ID, behavioural anomalies and learning disabilities (Costanzo et al., 2013; Farran, 2008). To the best of our knowledge, congenital and isolated deficit of WM without more severe ID or psychiatric traits were not reported. Of particular interest, Patient 3 whilst presenting clear working memory deficits does not seem to have episodic memory problems (as measured by the RLRI). In other words, Patient 3 is still able to learn new information and retrieve this information from long-term memory despite presenting a very low span and measurable working memory deficits. This dissociation between short-term and long-term memory has been reported many times in neuropsychological studies (Scoville and Milner, 2000; Shallice and Warrington, 1977) and suggests in the case of Patient 3 that episodic memory has developed relatively normally despite working memory impairment. This type of dissociation challenges Tulving's hierarchical model of memory (1995) in which working memory would play a role in episodic memory. Another similar challenge comes from developmental amnesia, characterized by dissociation between episodic and semantic memory (Vargha-Khadem et al., 2001). Advances in the understanding of genetic contributions to developmental disorders of cognition represent an opportunity to better understand the contributions of separate sub-components of memory and challenge exisiting neuroanatomical models of memory (Ranganath and Blumenfeld, 2005).

Previous volumetric correlation of brain MRI suggested an anatomical substrate to WM overlapping with the provided data from patient 3 (Collette et al., 2006; Kang et al., 2011; Karlsgodt, Bachman, et al., 2011). In the reported patient, the atrophic brain regions identified namely the superior vermis of the cerebellum and the parietal cortexes were different from the hypometabolic regions. Hypometabolism in pre-frontal cortex was noticed. This region has roles in cognitive processes, including the selecting, organization, manipulation and monitoring of information held in memory, all of which are crucial for successful WM. These roles, in combination, bear close resemblance with the central executive component in Baddeley and Della Sala's WM models (Khan and Muly, 2011). The limbic and cingulate lobes were not atrophic, but their hypometabolism seems consistent with the expression pattern of the candidate gene *SALM1*/*LRFN2* (Morimura et al., 2006). Conversely, mice with Lrfn2 gene deletion exhibit social withdrawal, hyperactivity in homecage,

impaired prepulse inhibition and scarce ultrasonic vocalizations but showed better performance in learning and memory tasks. In hippocampus, the level of PSD-95 was decreased, and synapses were abnormal [Morimura Abstract Neuroscience 2012].

Currently, the functional roles of genes that have been identified in cognitive disorders can be separated into 2 main functional groups: i) promoting neuronal differentiation and specification; and ii) synaptic scaffolding and functioning (Kaufman et al., 2010). The latter group is involved in a broad spectrum of neurodevelopmental disorders. Hypotheses favour the implication of the scaffolding proteins of the postsynaptic region in higher cognitive processes (Nithianantharajah et al., 2013). The Leucine rich repeat (LRR) superfamily of proteins is an example of accelerated evolution involving genomic duplication, with 139 LRR proteins in humans compared to 66 in flies. This observation suggests a critical role for those proteins in the human specific higher cognitive processes (Dolan et al., 2007). More precisely, several *LRR* genes were diagnosed as deleted or mutated in patients with WM and executive function disorders associated with neurological disorders such as Parkinson's disease (*LRRK2*)(Thaler et al., 2012), and with neuropsychiatric phenotypes (*LRFN5, LRRMC*…)(de Bruijn et al., 2010; Kleffmann et al., 2012; Mikhail et al., 2011; Sousa et al., 2010). Null mice for different LRR proteins interacting with the PSD complex showed learning disabilities associated with behavioural abnormalities (Carlisle et al., 2011; Katayama et al., 2010; Takahashi et al., 2012; Takashima et al., 2011). Among the LRR proteins, the SALM/LRFNs are a specific transmembrane protein group, promoting changes in neuronal morphology, dendritic outgrowth and synapse formation (Mah et al., 2010; Seabold et al., 2012; Wang et al., 2008). It was further demonstrated that the SALMs form complexes with NMDA receptors, promoting the specialization of excitatory synapses (Ko et al., 2006; Wang et al., 2008). In the pre-frontal cortex, which is highly implicated in WM processes, the NMDA glutamate receptors are key actors for cellular memory mechanisms such as long-term potentiation (Karlsgodt et al., 2011). Dosage of NR1 subunit partners was shown to be critical for glutamate signaling. This was demonstrated with the dystrobrevin binding protein-1 (dysbindin, or DTNBP1) null mice displaying deficits in WM performance, secondary to NR1 dysfunction (Karlsgodt, Robleto, et al., 2011).

To conclude, we report on a family harbouring a unique microdeletion, with selective WM deficit and notably encompassing *SALM1/LRFN2*, giving further evidence that selective LD can be of monogenic inheritance. Neuropsychological evaluation with experimental tasks, brain anatomical and

functional imaging together with cellular experiments emphasized the role of SALM1/LRFN2 in learning disability with selective WM deficit. This study highlights the power of multidisciplinary fine phenotyping in describing new genetics disorders.

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LEGENDS TO THE FIGURES

Figure 1: Family tree, genetic anomaly, Brain anatomical imaging, predicted LRFN2 expression

Figure 2: Functional brain imaging showing hypometabolism in 6 clusters: i) left temporal cortex, ii) left orbito-frontal cortex, iii) left lingual gyrus, iv) right thalamus, v) right superior and medial frontal gyrus and vi) left and right posterior cingulate. Hypometabolism is represented by the yellow – red gradient (red for strongly hypometabolic).

Figure 3: Panel A. Localization of SALM1 at synapses in the hippocampus CA1 stratum radiatum with EM/DAB immunoperoxidase (a,b) or immunogold (c-i). DAB labeling is found in patches throughout the postsynaptic spine (a) and in the presynaptic terminals (p) also (b; arrowheads). Immunogold labeling for SALM1 (5 nm gold; arrowheads) colocalizes with labeling for the NMDA receptor subunit, NR1 (15 nm gold) in the postsynaptic membrane/density (d,f,g; f is very oblique). Labeling for SALM1 also is found extrasynaptic (c,e,h,i; colocalized with NR1 in e). p, presynaptic terminal. Scale bar is 100 nm for immunogold. Panel B. Localization of SALM1 at synapses in the cerebellar granular region with EM/DAB immunoperoxidase (a) or immunogold (b-n). DAB labeling is generally weak, but can be found in the postsynaptic density (arrowhead; compare to an unlabeled synapse in the lower right corner). Immunogold labeling for SALM1 (5 nm gold; arrowheads) is found in the postsynaptic membrane/density (b,c) where it colocalizes with labeling for the NMDA receptor subunit, NR1 (15 nm gold; d,e). Labeling for SALM1 also is seen on the presynaptic membrane or in extrasynaptic

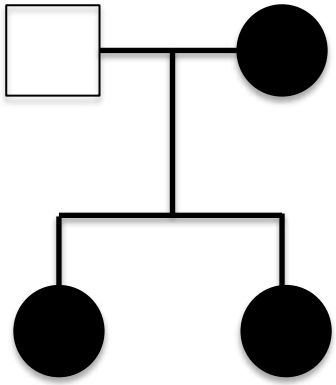
membrane locations (f-i). In addition, labeling is seen in attachment plaques (j-n) where it colocalizes with NR1 (in j, a synapse is labeled with a large gold particle for NR1 and the adjacent attachment plaque is labeled for SALM1). m, mossy fiber terminal. Scale bar is 500 nm for the EM/DAB and 100 nm for immunogold.

Table 1. Neuropsychological Assessment of N.G

	Score	Rank
WAIS-IV		
Verbal Comprehension	84	14
Information	8	Average
Similarities	6	Low average
Vocabulary	8	Average
Working memory	68	2
Arithmetic	5	Low average
Digit span	4	Low average
Perceptual organization	98	Average
Block Design	10	Average
Matrix	11	Average
Picture Completion	8	Average
Processing speed	72	3
Digit Symbol-Coding	4	Low average
Symbol search	6	Low average
Long-term memory		
Free Recall Trial 1	8	Above average
Free Recall Trial 2	9	Low average
Free Recall Trial 3	12	Above average
Delayed Recall	12	Above average
Cued-Recall Trial 1	16	Above average
Cued-Recall Trial 2	16	Above average
Cued-Recall Trial 3	16	Above average
Recognition task	16	Above average
Short-term – Working memory		
WMS III- Spatial Span	8/19	Percentile 25
WMS III-Spatial Span Forward	6/19	Percentile 9
WMS III-Spatial Span Backward	10/19	Percentile 50
TAP-Working Memory	716 (324)	Percentile 54
Attention		
D2 Test		
Total Letters identified	263	<Percentile 5
Omissions + Errors	14 (5,32%)	Percentile 25-50
Total identified – Errors	103	Percentile 5-10
TAP-Alertness	0.011	Percentile 34
Intrinsic alertness	266 (45)	Percentile 24
Attentional focus	271 (46)	Percentile 18
TAP-Incompatibility	0.475	Percentile 54
Executive function		
Trail-Making test		
Trail Making-Part A	51s	<10th Centile
Trail Making-Part B	167s	<10th Centile
WCST		
Categories	5	> Percentile 16
Total errors	42 (33%)	Percentile 42
Perseverative errors	7 (5%)	Percentile 82
Non perseverative errors	29 (23%)	Percentile 14
Failure to maintain set	5	< Percentile 1

D2 test=Concentration Endurance Test (BrickenKammp, 1981). TAP= Test of attentionalperformance (Zimmermann &Fimm, 2002). WMS III=Wechsler Memory Scale-II, REFS :MEM-III Echelle Clinique de Mémoire - TROISIEME EDITION, 2001, WECHSLER D. ECPA

A



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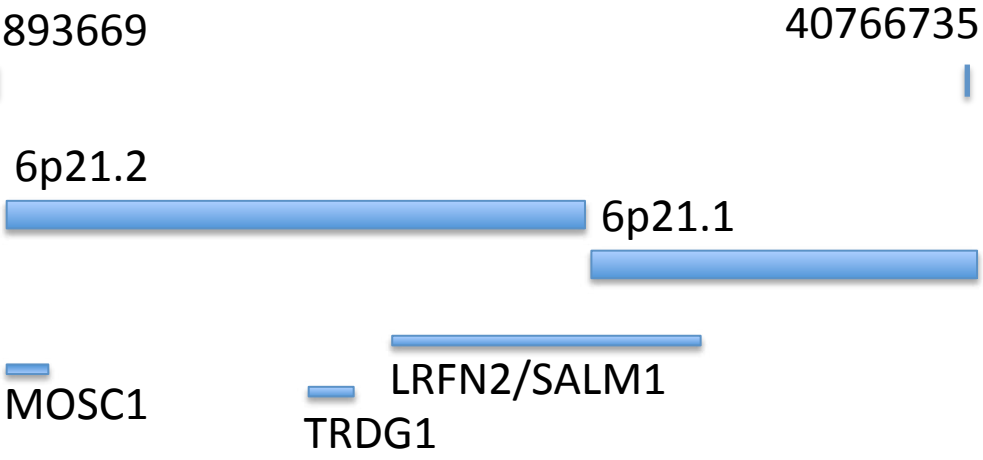
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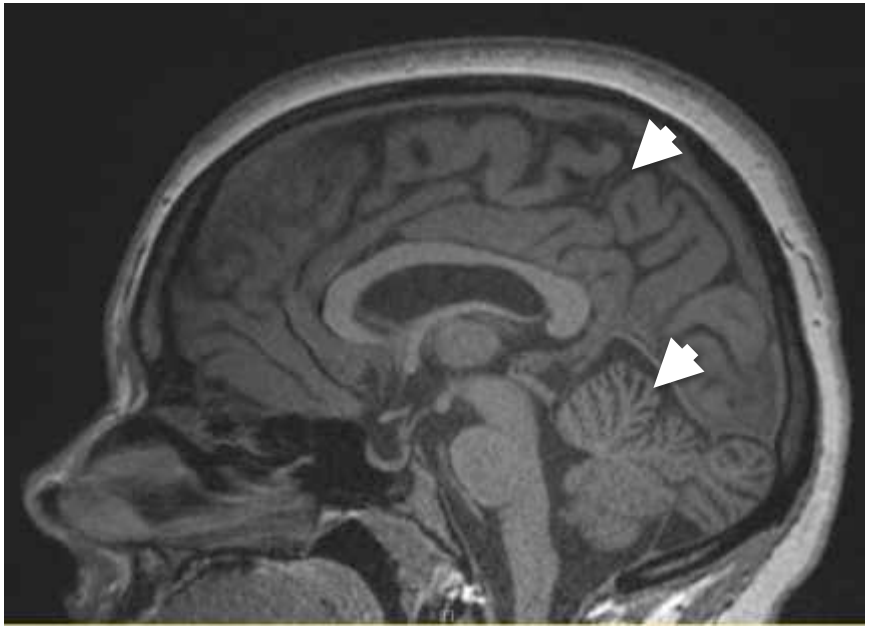

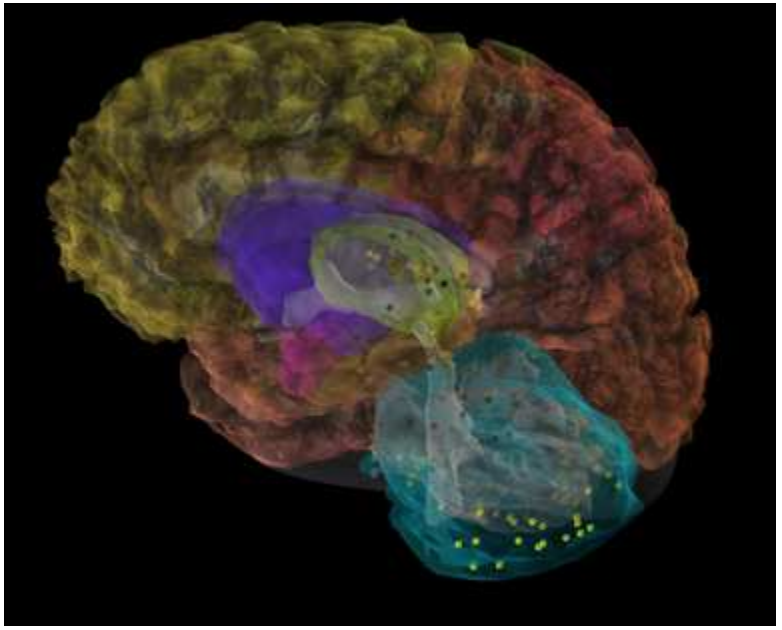

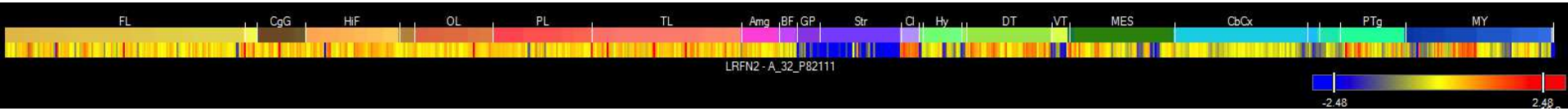
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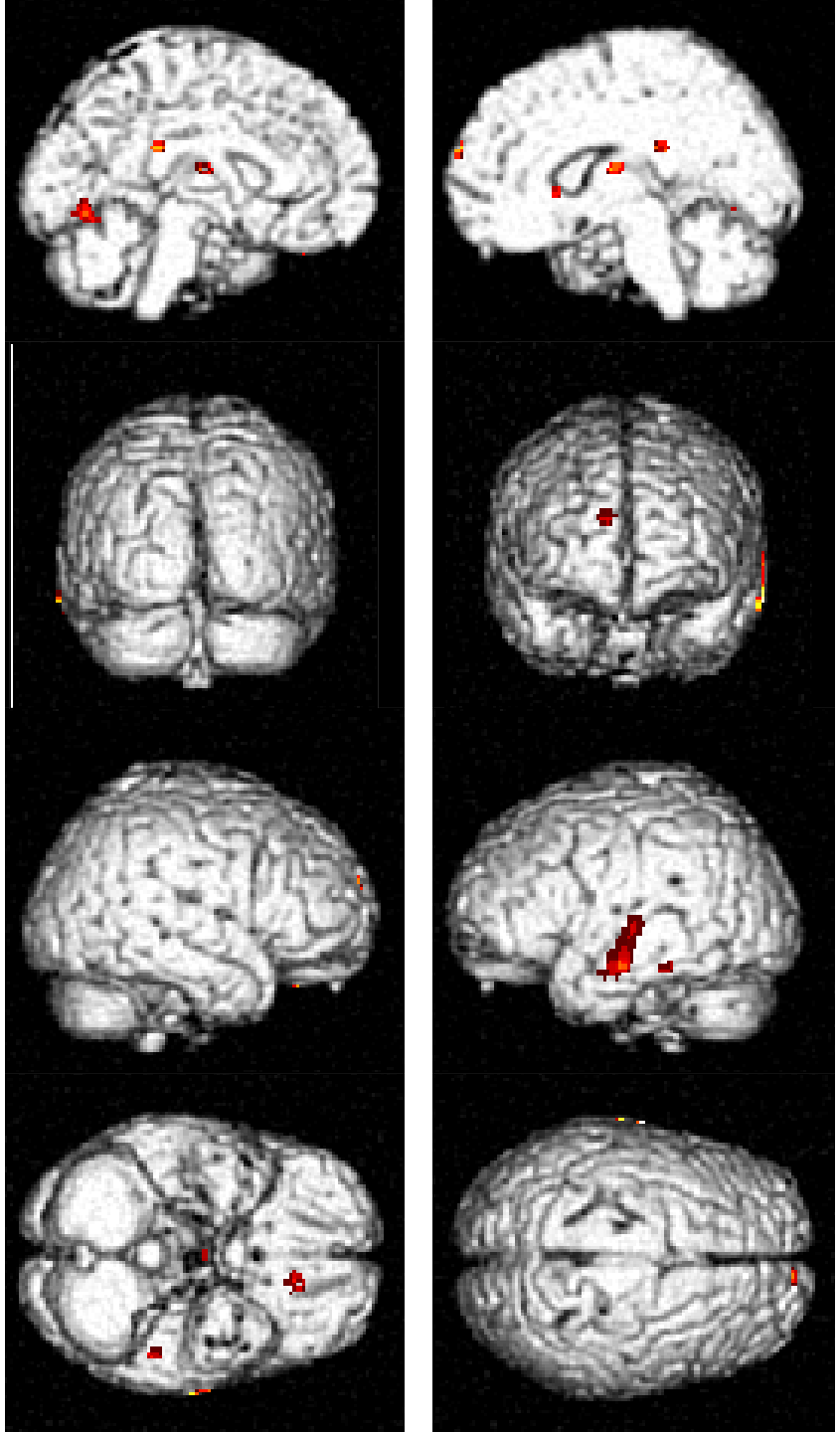

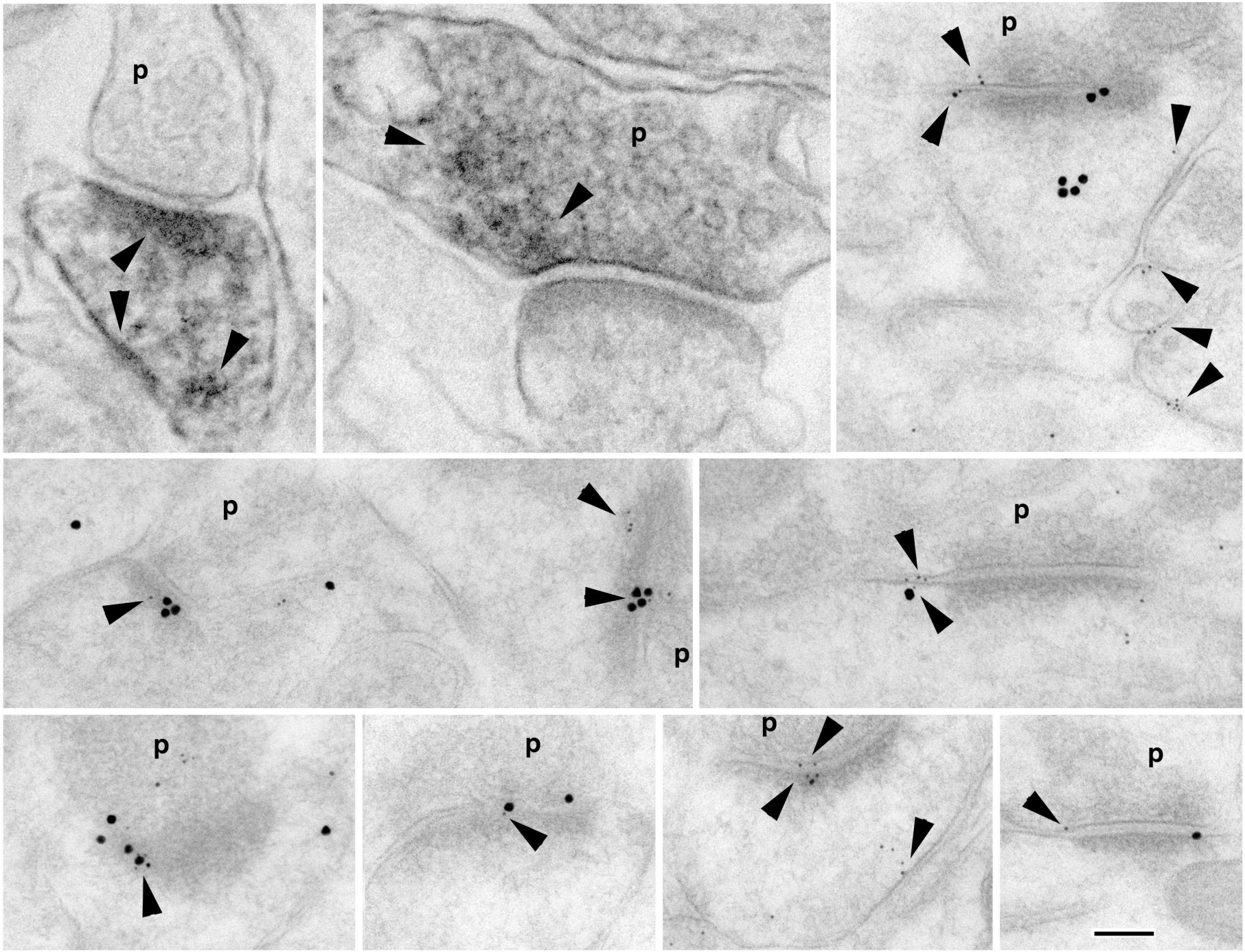
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12p13.33 microdeletion including *ELKS/ERC1*, a new locus associated with childhood apraxia of speech

1. *Résumé de l'article*

Les troubles de production du langage sont une condition hétérogène où des cas sporadiques comme familiaux sont décrits. Cependant, un mode d'hérédité monogénique n'explique qu'une minorité des cas en particulier dans la dyspraxie verbale (Childhood Apraxia of Speech, CAS). Les délétions impliquant le télomère du bras court du chromosome 12 sont parmi les plus rares délétions sub-télomériques et seuls 4 patients ont été rapportés avec des anomalies de moins de 5Mb.

Afin de caractériser ce syndrome microdélétionnel rare, une collaboration internationale a été entreprise via la base de données Decipher et 9 cas ont pu être recrutés. Un retard de langage était rapporté chez tous les patients, diagnostiqué dyspraxie verbale lorsque le patient avait pu être évalué par un orthophoniste (5/9 patients). Une DI était associée chez 5/9 patients, associée à des manifestations psychiatriques de sévérité variable. Deux des délétions étaient héritées de parents qualifiés initialement sains. Cependant, une réévaluation clinique et orthophonique a identifié des troubles de production du langage, suggérant une expressivité variable.

La plus petite région chevauchante chez ces patients contenait le gène *ELKS/ERC1* codant pour une protéine de la jonction synaptique. Ces résultats suggéraient que la délétion 12p13.33 est responsable de phénotypes variables incluant la dyspraxie verbale, associée à des troubles neuro-comportementaux. Le diagnostic clinique de dyspraxie verbale justifie une évaluation génétique.

2. *Discussion et perspectives*

Les troubles du langage sont un domaine hétérogène cliniquement. Cependant, il existe de nombreux regroupements phénotypiques et les déficits spécifiques du langage restent une entité mal déterminée. A l'inverse, les troubles productifs du langage sont segmentés en une nosologie plus homogène et c'est dans ce groupe de pathologie que les rares causes monogéniques de troubles du langage ont été identifiées : *FOXP2, CNTNAP2, FOXP1…*

La dyspraxie verbale est un diagnostic clinique qui associe des troubles phasiques et praxiques de production du langage. Selon certains auteurs, la dyspraxie verbale est sous diagnostiquée. Cela peut avoir des conséquences en

termes de prise en charge puisque des remédiations spécifiques ont fait preuve d'efficacité sur ce trouble du langage.

La collaboration étroite avec le Centre de Référence des troubles du Langage du CHU de Dijon a permis de décrire au mieux les manifestations cliniques du cas index, pour porter un diagnostic précis. Le diagnostic de dyspraxie verbale chez le cas index a imposé une réévaluation orthophonique spécialisée des autres cas porteurs de microdélétions 12p13.33. Cela a permis la mise en évidence de troubles de production langagière suggérant une dyspraxie verbale chez les patients évalués et même d'affirmer un diagnostic de dyspraxie verbale chez certains.

Depuis la publication de l'article, nous avons été directement contacté par deux familles nord américaines dont les enfants étaient porteurs de délétions 12p13.33 et présentaient des troubles du langage. En parallèle, au congrès de l'ESHG 2013, à Paris, un poster australien détaillait la présentation clinique de 2 nouvelles familles avec délétion 12p13.33 (P05-002). Les patients semblaient présenter des troubles de production du langage, associés de manière variable à une DI et des troubles du comportement. Les délétions de ces patients, bien que chevauchant avec nos 9 cas semblent pointer le gène *CACNA1C* comme principalement impliqué dans le phénotype. Ces données externes semblent valider la présentation clinique de nos patients. Cependant, *CACNA1C* n'était pas délété chez tous nos patients.

Le groupe de Simon E. Fisher (Max Planck Institute for Psycholinguistics and Professor of Language and Genetics at the Donders Institute for Brain, Cognition and Behaviour in Nijmegen, the Netherlands) avait été contacté afin d'intégrer notre gène candidat à la capture de SHD prévue sur des groupes de patients avec troubles productifs du langage.

3. Article

European Journal of Human Genetics (2013) 21, 82–88

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ARTICLE

12p13.33 microdeletion including *ELKS/ERC1*, a new locus associated with childhood apraxia of speech

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Speech sound disorders are heterogeneous conditions, and sporadic and familial cases have been described. However, monogenic inheritance explains only a small proportion of such disorders, in particular in cases with childhood apraxia of speech (CAS). Deletions of <5 Mb involving the 12p13.33 locus is one of the least commonly deleted subtelomeric regions. Only four patients have been reported with such a deletion diagnosed with fluorescence *in situ* hybridisation telomere analysis or array CGH. To further delineate this rare microdeletional syndrome, a French collaboration together with a search in the Decipher database allowed us to gather nine new patients with a 12p13.33 subtelomeric or interstitial rearrangement identified by array CGH. Speech delay was found in all patients, which could be defined as CAS when patients had been evaluated by a speech therapist (5/9 patients). Intellectual deficiency was found in 5/9 patients only, and often associated with psychiatric manifestations of various severity. Two such deletions were inherited from an apparently healthy parent, but reevaluation revealed abnormal speech production at least in childhood, suggesting variable expressivity. The *ELKS/ERC1* gene, which encodes for a synaptic factor, is found in the smallest region of overlap. These results reinforce the hypothesis that deletions of the 12p13.33 locus may be responsible for variable phenotypes including CAS associated with neurobehavioural troubles and that the presence of CAS justifies a genetic work-up.

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Keywords: array CGH; 12p13.33; inherited deletion; *ELKS/ERC1* gene; speech delay; childhood apraxia of speech (CAS)

INTRODUCTION

Intellectual disability (ID) is a major social, educational and health problem affecting 3% of the population. Speech delay is frequently associated with intellectual deficiency, but is rarely the predominant symptom. Specific language impairment is the most frequently diagnosed form of developmental language disorder, affecting up to 7% of 5- and 6-year-old children.[1] There is a considerable variation in the profile of the linguistic deficits observed and the functions affected, which may be expressive, receptive or both.[2] Among speech sound disorders, childhood apraxia of speech (CAS) also labelled developmental verbal dyspraxia (DVD) generally refers to the productive aspects of verbal communication.[3] CAS/DVD is defined by the association of: (1) inconsistent error production on both consonants and vowels across repeated productions of syllables or words; (2) lengthened and impaired coarticulatory transitions between sounds and syllables; and (3) inappropriate prosody.[4] Point mutations and chromosomal abnormalities that affect *FOXP2* were the first known genetic bases in such phenotypes.[5–10] The affected persons also had variable levels of impairment in expressive and receptive language, extending to abnormal production and comprehension of grammar. *FOXP2* disruptions were found in approximately 2% of a recruitment sample with 'DVD' (1/49).[6,10,11] Other causative genes were secondarily described, associating speech disorders to neurobehavioural abnormalities, including the *CNTNAP2*, *FOXP1* and *SRPX2* genes.[12–17]

Herein, we report on a series of nine patients with 12p subtelomeric deletions, including two familial cases with severe speech sound disorders, defined as CAS/DVD when evaluated. We therefore aimed to further clinically delineate the '12p13.33 microdeletion syndrome' and determined the smallest region of overlap and a candidate gene for speech sound disorders strongly suggesting CAS/DVD.

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METHODS

French array-CGH network

A collaborative study was set up to colligate all of the French cases carrying a 12p13.33 microdeletion. Informed consent was obtained for all tested patients. These platforms used either the Human Genome CGH Microarray 44, 105, 180 or 244K from Agilent according to the manufacturer's protocol (Agilent Technologies, Santa Clara, CA, USA). Data were processed with feature extraction (v. 9.1) software and the results were analysed with CGH analytics (v. 4.0) software (Agilent Technologies) in the Hg18 genome assembly. When a 12p13.33 deletion was identified through array CGH, the anomaly was confirmed either by fluorescence *in situ* hybridisation (FISH) or quantitative PCR (qPCR).[18] In cases of inherited deletion, the size of the parental deletion was determined by array CGH.

European decipher database

The decipher database (http://decipher.sanger.ac.uk) was interrogated for 12p13.33 deletions and allowed us to find new cases in England (patient 6) and Canada (patient 8). An SNP array study using the Affymetrix SNP6.0 array was used and the deletion was confirmed on FISH studies using the BAC probe RP11-359B12. Parental analyses were performed using FISH analyses.

Specialised evaluation of speech

The patients from family 1 and 2 were evaluated by a specialised speech therapist from a reference centre for speech disorders. Patient 6 and 9 were evaluated by their speech therapist, using the standardised scales discussed below. In order to determine the cause of speech production troubles, specific tests were performed including comparison between receptive and expressive abilities, a search for errors on consonants and vowels in repeated production of syllables or words, and its correlation with the length of the words (according to the *BALE* scale, ELO battery, ERTL-4 scale),[19–21] a study of the ability to repeat nonsense words, a study of fine coordinated movement sequences of the mouth, tongue, lips and eyes in order to investigate the hypothesis of CAS/DVD. As an example, the results from the evaluation of patient 1 are available in Supplementary Data (Supplementary Table 1). Oro-facial praxis was assessed using the Henin–Dulac scale.[22] Neuropsychological evaluations were conducted using the standardised Weschler's scales. A diagnosis of CAS/DVD was made when a patient met the following criteria: (1) presence of significantly abnormal oro-facial praxis and (2) presence of five or more key features of CAS published by Forrest.[23]

RESULTS

Patients 1 and 2, family 1

The proband (patient 1) was a 3-year-old boy, the second child born to healthy parents. His older brother had normal development and schooling. During the third trimester of pregnancy, asymmetrical cerebral ventricular dilatation was noticed (left ventricle: 11 mm). Fetal encephalic MRI, maternal CMV serology and foetal karyotype were performed and showed no additional abnormalities. At birth at 39 weeks of gestation, weight of the patient was 3270 g, length 51 cm and OFC 35 cm. Post-natal transfontanellar ultrasonography was interpreted as normal. He was described as a calm baby, with no interest in toys. Acquisition of developmental milestones was slow: walking was acquired at 21 months and speech was delayed until around the age of 3 years. He communicated mostly by shouting. He had disturbed sleep. At the first examination at 3 years of age, height and weight were on the mean curve, but he had macrocephaly at + 2.5 SD. Mildly dysmorphic features were noted, including a square, coarse face, mild frontal bossing, enophtalmia, low-set ears, with anteverted and thick ear lobes, a marked philtrum, large nares, a thin upper lip and irregular and narrowly spaced teeth (Figure 1). He had hypertrichosis located on the lower region of his back. There were no other malformations at the clinical examination. Cerebral MRI, renal and cardiac ultrasounds, skeletal X-rays and fragile-X testing were unremarkable. Urinary oligosaccharides and mucopolysaccharides, assessed because of the hypertrichosis and coarse face, were normal. Audition tests were normal. At the age of 3 years, the neuropsychological evaluation identified a dysharmonic neuropsychological profile (WPPSI-III, comprehension SN 4/19, reading ability SN 8/19, cubes SN 1/19, object assembly and block design SN 11/19). The IQ could not be calculated because of the dissociation between performances and verbal abilities. Reading abilities were different from word comprehension, which was very low, and aggravated by a visual perception deficiency evidenced by difficulties in picture naming. The performances were comparable to those of children of his age. Attention deficit was noticed, but the patient was too young to confirm a diagnosis of ADHD. A specific rehabilitation programme focusing on abstraction and perception abilities was prescribed to improve adaptation. At 46 months, a speech evaluation diagnosed speech impairment with no communication troubles. Comprehension skills were low. Spontaneous speech associated with unarticulated words was understood and translated by the family. A neuropediatric examination ruled out a neurological defect. Array CGH diagnosed a 3.2-Mb telomeric deletion of the short arm of chromosome 12 (arr 12p13.33p13.32 (179 123–3 264 542) x1). qPCR analysis in the parents revealed that the deletion was inherited from the mother (patient 2).

Figure 1 Photos of patients 1, 3 and 9. Note the mild non-specific dysmorphic features. Patient 1: macrocephaly, square and coarse face with large forehead, enophtalmia, down-slanting palpebral fissures, low-set ears, anteverted and thick ear lobes, a marked philtrum, large nares, a thin upper lip and irregular and narrowly spaced teeth; patient 3: no dysmorphic features; and patient 9: hypotelorism, horizontal eyebrows, up-slanting palpebral fissures, large nares and downturned corner of the mouth. Photos published with parental consent.

The mother (patient 2) had a past history of severe speech delay with speech production troubles. She said her first words at 4 years of age and had intensive speech therapy. She had difficulties during her schooling, but did not attend a school for special needs. She was not able to obtain a high school diploma, but followed a vocational course. She is now working as a home-care provider and has good social integration. Her IQ is 89 (WAIS-III. IVQ: 86 with VCI 92, WMI: 94, IPQ: 96 with OPI: 95 and PSI: 102). The size of the deletion was determined by array CGH and found to be identical in patient 1 and his mother. Further investigations of the family showed that the deletion in the mother was *de novo*.

Specialised speech evaluation revealed several characteristic signs of CAS/DVD, including better receptive than expressive abilities, inconsistent errors on consonants and vowels in repeated production of syllables or words with an increase in errors with longer words, especially over two syllables, impaired ability to repeat nonsense words and oral apraxia in fine coordinated movement sequences of the mouth, tongue, lips and eyes. As an example, we phonetically translated an evaluation in the Supplementary Data (Supplementary File 1). A similar pattern was found in her child when testing was possible (Table 2).

Patients 3, 4 and 5, family 2

Patient 3 was the only child of young healthy non-consanguineous parents. The pregnancy was uneventful. He was born at 40 weeks of gestation. His birth weight was 2800 g, length 47 cm and OFC 34 cm. He was able to walk at 13 months. Speech was delayed with the first sentences pronounced at 3½ years. Hearing tests were normal. Behavioural abnormalities were noted at 3 years of age with hyperactivity, anxiety, solitariness and low social interaction. Several stereotypies were noticed in response to stress. There was no sleep disturbance. Standard schooling was interrupted before the child started primary school, and it was recommended that he attend a school for special-needs children. When he was referred for a neuropediatric consultation at 5 years of age, measurements were above normal with a height of 118 cm (+ 2.1 DS), a weight of 21 kg (+ 2.2 DS) and OFC 53 cm (+ 1.2 DS). No dysmorphic features were noted (Figure 1). Expressive speech remained insufficient with speech production trouble. The Henin–Dulac test revealed praxis troubles (*Z*-scores between −4 and −7 SD) associated with dysphasic symptoms (Table 2). Standard karyotyping was normal, fragile X was ruled out. Array-CGH 150K identified a 1.3-Mb deletion on the short arm of chromosome 12 (arr 12p13.33 (192 403–1 346 471) x1). This result was checked by FISH analysis using BAC probes (BAC RP11-359B12). Familial studies revealed that the deletion was inherited from the father and the paternal grandfather. Incomplete penetrance was first suggested, but targeted medical interviews suggested variable expressivity. Indeed, the father (patient 4) had a past history of speech delay with severe speech production troubles, stammering and unarticulated words. He had difficulty learning how to read and write. Hyperactivity was noticed in infancy and he had to stop school before the 7th grade at 15 years of age. He is now working as a technical operator. The paternal grandfather (patient 5) had a similar past history. The age at first words could not be defined, but he still displays speech production troubles with many unarticulated words. He was also hyperactive and stopped school before high school at 17 years of age. He has borderline intelligence and used to work as a factory worker. Clinical examinations of the three patients were normal. No dysmorphism was noticed in patients 4 and 5.

Patient 6, family 3

The proband was one of non-identical twins born to a healthy Caucasian couple following assisted conception by intracytoplasmic sperm injection. He was delivered by Caesarean section at 28 weeks of gestation due to premature rupture of the membranes and cord prolapse. His birth weight was 1320 g. He was ventilated for 2 days and then on continuous positive airway pressure for several weeks. He remained in a neonatal intensive care unit for 8 weeks where he had problems with jaundice and anaemia but no major collapses. He passed his neonatal hearing test and started feeding well. He smiled at 18 weeks, sat between 6 and 9 months and walked at 14 months but was uncoordinated. Developmental delay with late speech was noticed and there was concern that he had some autistic features. His general health was good. When he was seen at 3 years, he was able to say a dozen words with first associated words. There was no neurological defect identified during clinical examination. Most of his problems were in the area of speech and language. Speech evaluation was limited by ID and concentration troubles. Although delayed, comprehensive skills were satisfactory and expressive skills were lower, the therapist noticed very slow progress in therapy and inability in repeating words before the age of 10 years. Oro-facial praxis was not acquired and was associated with fine psychomotor difficulties (Table 2). His height was 98.7 cm (75th centile), weight 16.14 kg (75th centile) and OFC 52 cm (50th centile). He had rather myopathic facies with a tented upper lip and a tendency to drool. He snored excessively at night. His palate was highly arched. He had mild hypotonia with generalised joint laxity and an umbilical hernia. His ear lobes were prominent. The routine karyotype, 22q11 FISH, fragile X and 11p methylation were normal, as was a brain MRI scan. By 5 years of age, his speech was improving. He was declared to have special educational needs and receives extra help at school. He was in good health, and the main concern was his behaviour. He did not mix well with other children, had poor communication skills, was not motivated to learn and was late with toilet training. A diagnosis of autism was suggested but not formally confirmed. Unfortunately, he could not be evaluated by a specialised speech therapist. An SNP array study using the Affymetrix SNP6.0 array showed a 3.1-Mb deletion of 12p13.33, which was confirmed on FISH studies using the BAC probe RP11-359B12. The deletion was not present in either parent. His twin brother was developing normally.

Patient 7, family 4

The proband was the first child of young healthy parents. A younger sister aged 11 years had normal psychomotor development and schooling. The pregnancy was unremarkable and the patient was born at 40 weeks of gestation with a birth weight of 2820 g, birth length 47 cm and OFC 34 cm. There was no problem during the neonatal period. Crawling was acquired at 18 months. The first symptom was a delay with walking, still not acquired at 22 months. Later, speech delay was also noticed. The first words were pronounced at 3½ years. At 5 years of age , height was 113 cm (+ 1 DS), weight 7.3 kg (−1 DS) and OFC 47.5 cm (−3.5 DS). Clinical examination revealed a long face with large ears and prominent lobes, epicanthus and large incisors with dental malocclusion. Anxiety and attention-deficit hyperactivity disorder was diagnosed. Unfortunately, he could not be evaluated by a specialised speech therapist. Complementary investigations included a normal brain MRI, normal karyotype, fragile X, plasma amino-acid and urinary organic-acid chromatography and transferrin isoelectrical focusing. The array CGH identified a *de novo* 2.76-Mb deletion in the 12p13.33 band (arr 12p13.3 (1 080 000–3 850 000) x1). This deletion was confirmed by FISH

analysis and not present on the parental chromosomes using BAC probes (RP5-927J10 and RP11-476M19).

Patient 8, family 5

The proband was a 10-year-old child of young non-consanguineous parents of Indian origin. His younger brother was healthy. A maternal uncle had low academic skills. The pregnancy was normal but complicated with a premature rupture of membranes at 31 weeks of gestation. The patient was born at 32 weeks with a birth weight of 1.9 kg. The neonatal period was complicated by hospitalisation in an intensive care unit for hypothermia and feeding difficulties. Psychomotor development was delayed with independent walking acquired at around 30 months and poor fine motor skills. The first words were pronounced at 36 months. Abnormal speech production was noticed and speech therapy was quickly started. At 9 years of age, measurements were 125 cm for height (25e percentile) and 20 kg for weight (3e percentile). There was relatively mild microcephaly with an OFC of 49.5 cm (3e percentile). Mild non-specific dysmorphism was noticed with micrognathia and prominent ears. He had surgery for inguinal hernia at 9 years of age. The patient went to a normal school with occupational therapy until the age of 10 years and was then after-orientated to a school for special-needs children. He was described as a fearful child with poor concentration skills. A diagnosis of ADHD was raised after a neuropsychological evaluation. Ritalin was tried then stopped because of a lack of effectiveness. This evaluation also noted a marked delay in arithmetic problem solving, sub-normal skills in reading and normal skills in spelling. Unfortunately, he could not be evaluated by a specialised speech therapist. Additional investigations included normal haemoglobin electrophoresis, 22q11 FISH, fragile X, plasma and urine amino acids, urine organic acids, brain MRI and EEG. Blood TSH analysis revealed moderate hypothyroidism, treated with L-thyroxine. Array CGH identified a 2.5-Mb deletion on the short arm of chromosome 12 (arr 12p13.33 (33 879–2 537 524) x1). The deletion was not found in either parent.

Patient 9, family 6

The patient was a 4-year-old child presenting initially with speech delay, contrasting with normal motor skills. Pregnancy was marked by intra-uterine growth retardation. The mother had a personal history of speech delay with no consequences on her education or professional career. For the patient, sitting was acquired at 7 months and walking at 16 months. Speech was delayed and the first words were uttered between 36 and 40 months. Clinical examination was normal. The morphological examination revealed hypotelorism, microcephaly with a prominent metopic suture, moderate joint laxity and brittle first toe nails. Chronic otitis was diagnosed and grommets were implanted. The patient was orientated to a school for special-needs children at the age of 6 years because of developmental delay associated with behavioural abnormalities. Speech evaluation identified speech production troubles contrasting with better lexical acquisition. The standardised speech evaluation identified oro-facial apraxia, associated with poor intelligibility of speech. He presented with frequent sound omissions and vowel errors (Table 2). Speech was only understood by the parents. The first sentences were noticed at the age of 8 years. Poor fine motor skills were also noticed. At the age of 11 years, growth was normal (height +1 SD and weight +1 SD). Microcephaly was persistent (OFC −2.2 SD). Cerebral MRI was normal. Standard chromosomal analysis was normal. Subtelomeric FISH analysis diagnosed a terminal deletion of the short arm of chromosome 12. Parental analysis revealed that the patient had a *de* novo deletion. Mapping was refined by array CGH 4 × 180k (Agilent Technologies) and identified a *de novo* 4.76-Mb deletion (arr 12p13.32p13.33 (163 393–4 790 279) x1).

DISCUSSION

Subtelomeric deletions associated with developmental disabilities account for 2.5% of the etiologies of learning disability. Approximately half of the clinically significant abnormalities identified are isolated terminal deletions, the majority of which are *de novo*. In the largest study, which investigated the telomeres of 11 688 individuals with developmental disabilities, the 12p subtelomeric deletion was the least frequently encountered variation.[24]

12p13.33 microdeletion is a rare condition that has only been described in four case reports (three subtelomeric and one interstitial).[25–28] It is associated with ID and various psychiatric manifestations (Table 1). The age at which the first words are uttered was delayed in 2/4 patients, but no specific data regarding speech production troubles have been reported. Larger 12p terminal deletions identified by conventional cytogenetic techniques have been described in 12 patients since 1975.[29–32] A recognisable '12p deletion syndrome' was once suggested but ruled out because of a lack of specificity.[31] From our patients and the review of the literature, we have further delineated the clinical phenotype of 12p13.3 microdeletions (Table 1). Pregnancies were generally unremarkable, except for one case with unilateral ventricular dilatation and one with intra-uterine growth retardation. No malformations were found except for one case associated with oculo-auriculo-vertebral spectrum, which may have been coincidental.[26] Absent to non-specific dysmorphic features were noted, but prominent ear lobes seem to be a frequent feature. The neonatal period was in general unremarkable, and in most of the cases, the first or unique symptom was the speech delay, with the first words uttered at around 36–40 months, associated or not with a delay in walking acquisition. Interestingly, speech production troubles were identified in 8/9 patients of our series (Table 1), and this feature could have oriented clinicians towards this diagnosis. The cause of the speech production troubles was CAS/DVD in investigated cases (5/9). Patients presented an association of oro-facial praxis defects and dysphasic symptoms (Table 2). They were mostly unable to imitate sounds and had better skills on automatic than imitated speech, had difficulty with articulatory movements for speech, gross and fine motor difficulties, with general oral-motor difficulties, effortful productions, groping and increased errors with increased utterance length, frequent sound omissions, prosodic irregularities and slow progress in correcting deficits in therapy (Table 2). The diagnosis of CAS/DVD was made when patients presented five or more characteristic symptoms reported in the ASHA technical report or by Forrest.[4,23] A neuropediatric examination could also rule out a neurological deficit. The association with behavioural abnormalities and developmental delay could lead to the classification of 'apraxia of speech with complex neurobehavioral disorder'.[4]

Although intellectual deficiency was first suspected in most of the reported patients (Table 1), it was ruled out in some of them following a neuropsychological evaluation. As an example, the neuropsychological evaluation of patient 1 in this study revealed dissociation between low verbal and normal non-verbal performances; it was therefore not possible to calculate the total IQ. We went back to some of the authors of previously reported cases for additional information (Table 1). When our data were pooled with other reported cases, it was interesting to note that the deletion was inherited from one parent in four of the nine probands. In each case,

Table 1 Summary of the main clinical features presented by the patients from each family from this report and from the literature and brain-expressed genes deleted in patients

	Family 1		*Family 2*			*Family 3*	*Family 4*	*Family 5*	*Family 6*	*Literature data*			
	Patient 1	*Patient 2*	*Patient 3*	*Patient 4*	*Patient 5*	*Patient 6*	*Patient 7*	*Patient 8*	*Patient 9*	*Baker et al*[25]	*MacDonald et al*[27]	*Rooryck et al*[26]	*Abdelmoity et al*[28]
Sex	M	F	M	M	M	M	M	M	M	M	M	F	F
Age at diagnosis	3 y	35 y	5 y	37 y	67 y	3 y	5 y	10 y	6 y	16 y	6 y	ND	8 y
Walking acquisition (months)	21	ND	13	ND	ND	14	22	30	16	16–18	ND	36	18
First associated words (months)	36	42	42	36	ND	36	40	36	40	36	ND	30	30
Orthophonic findings	CAS/DVD	CAS/DVD	CAS/DVD	Speech production trouble	Speech production trouble	CAS/DVD	ND	Speech production trouble	CAS/DVD	ND	ND	ND	ND
Psychiatric evaluation	Solitariness and low interactions	Normal	ASD, ADHD and low interactions, stereotypies	ADHD	ADHD	ASD and ADHD	Anxiety and ADHD	Anxiety and ADHD	Abnormal behaviour	Abnormal behaviour aggressivity	Anxiety and ADHD	Normal	ADHD and staring episodes
Intellectual disability	No	No	Yes	No	Borderline	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Schooling	Normal with personal assistance	Normal but no high school diploma	Special needs	Stopped before high school	Stopped before high school	Special needs	Special needs	Normal school until grade 4	Special needs	Special needs	Normal with personal assistance	Normal with personal assistance	Stopped in 8th grade
Others	—	—	—	—	—	—	—	Hypothyroidism		Right-sided aortic arch, VSD	—	OAVS	—
Deletion size (Mb)	3.2	3.2	1.3	1.3	1.3	3.1	2.76	2.5	4.79	1.65	2.95	2.3	1.39
Inheritance	Maternal	*De novo*	Paternal	Paternal	ND	*De novo*	*De novo*	*De novo*	*De novo*	Maternal	*De novo*	*De novo*	Paternal

Abbreviations: ADHD, attention-deficit hyperactivity disorder; ASD, autistic spectrum disorder; F, female; M, male; ND, not defined; OAVS, occulo-auriculo-vertebral syndrome; VSD, ventricular septal defect; y, years.

the inheritance of the deletion was first an unexpected finding because the parent had a normal life with a job and family. Retrospective targeted interviews revealed that they all had speech delay and learning difficulties during childhood. None of them graduated from high school and they all had jobs that did not require qualifications, which favours variable expressivity rather than incomplete penetrance. When tested, they displayed a normal IQ (family 1).

Table 2 Speech evaluation with the BALE scale and association with the diagnosis criteria for CAS/DVD published by Forrest[23]

	Patient 1	*Patient 2*	*Patient 3*	*Patient 6*	*Patient 9*
Inconsistent production	+	+	ND	ND	ND
Groping	+	+	+	ND	ND
Presence of vowel errors	+	−	−	+	+
Increased errors with increased utterance length	+	+	+	ND	ND
General oral-motor difficulties	+	+	+	−	+
Poor intelligibility	+	−	−	−	+
Slow progress in therapy	+	+	+	+	+
Difficulty with articulatory coordination for speech	+	+	ND	ND	ND
Multiple errors	+	+	+	ND	ND
Prosodic irregularities	ND	+	+	+	ND
Unable to imitate sounds	+	−	ND	ND	+
Expressive language worse than receptive	+	−	+	+	−
Limited early vocalisations/sound play	+	+	ND	+	ND
Effortful productions	+	+	ND	−	ND
Gross and/or fine motor difficulties	+	+	+	+	+

Abbreviations: +, present; −, not present; ND, not defined.
The 15 most frequent features that could be identified in patients with CAS are listed. Speech evaluation of patients 1, 2, 3, 6 and 9 were summarised in the table.

These data give further examples that go against the general view that subtelomeric imbalances will lead to MCA/MR, because apparently phenotypically normal individuals can also carry subtelomeric aberrations.[24] Ravnan *et al*[24] showed that the majority of terminal deletions were found to be *de novo* (48/60 familial studies). The remaining cases were inherited from a single parent carrying the same deletion, the majority of whom had been reported to be phenotypically normal by the referring physician (10/12). Therefore, parental FISH studies were recommended for all patients in whom a subtelomeric rearrangement was found. Similarly, Balikova *et al*.[33] reported subtelomeric copy-number changes in 12 families, in which the imbalance was inherited from a phenotypically normal parent (subtelomeric 2q, 3p, 4p, 4q, 6q, 10q, 17p, 17q, Xp and Yq deletions, 1q, 4q, 10q and 11q duplications). A careful clinical history and neuropsychological investigations are therefore needed in so-called asymptomatic patients. At the present time, there are a number of hypotheses to explain the variability of clinical expression encountered in these microdeletional syndromes. These include variations in genetic background, epigenetic phenomena like imprinting, expression or regulatory variation among genes in the rearrangement region and the unmasking of recessive variants residing in the single remaining allele.

From these nine new patients with a 12p subtelomeric microdeletion, we tried to define the smallest region of overlap for the speech abnormalities. The 12p subtelomeric region is not a gene-rich region with approximately 35 genes spanning the four telomeric megabases (Figure 2). Four of these genes are known to be implicated in either neuronal exchange (*IQSEC3* and *ELKS/ERC1*),[34,35] or psychiatric disease and intellectual disabilities (*SLC6A13* and *CACNA1C*).[36,37] The molecular data from our group of patients added to other observations in the literature allowed us to identify a small region of overlap of 260 kb containing the *ELKS/ERC1* gene. This gene seems to

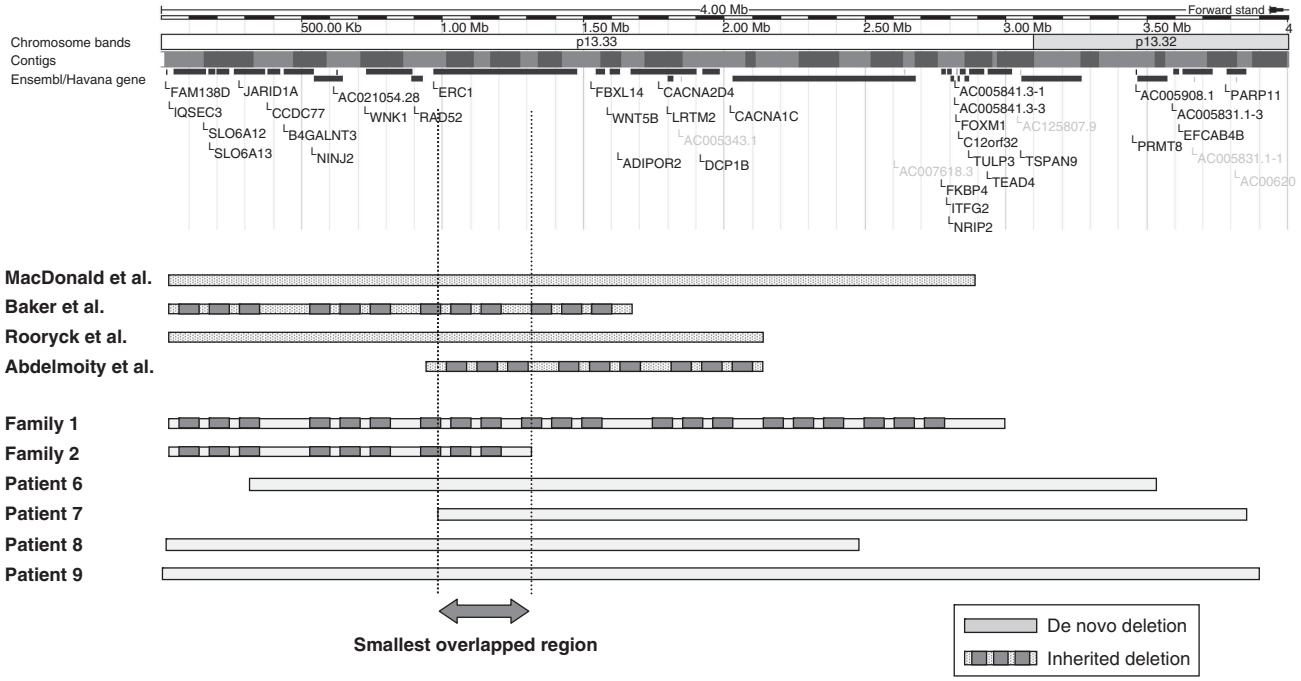

Figure 2 Alignment of the deletions identified with array CGH. The first four megabases from the telomere comprise 35 genes. Four of them are known genes expressed in the brain and implicated in neurotransmission (*IQSEC3* and *ELKS/ERC1*) or have been assigned to psychotic phenotypes (*SLC6A13* and *CACNA1C*). The smallest region of overlap in all the patients with speech delay (260 kb) only contains the *ELKS/ERC1* gene.

be the best candidate for the speech sound disorder in the 12p13.33 region. The ELKS protein is not brain specific.[38] In neuron cells, the ELKS protein is concentrated in the pre-synaptic active zone.[35,38] It was shown to be necessary for vesicular exocytosis in various cell types.[35,38,39] This protein was shown to be expressed at neuromuscular junctions,[39] raising the hypothesis of altered fine cortical control of vocalisation muscles.

Monogenic causes of CAS are rare.[3] CAS have been described occasionally with genetic disorders such as galactosemia, type 1 neurofibromatosis or in chromosomal rearrangements, in particular encompassing the *FOXP1* or *FOXP2* genes.[40–43] Four genes have been reported in association with speech disorders, often associated with other neurobehavioural abnormalities. These include mutations in the *CNTNAP2*, *FOXP1 FOXP2* and *SRPX2* genes.[13–17,42,43] In this series, 7/9 patients presented with behavioural troubles and 6 of them with ADHD, which could be explained by hemizygosity of *ELKS/ECR1*, located in the smallest region of overlap, given the similarity in the patients' phenotype. However, other genes expressed in the brain comprised in the microdeletion, such as *CACNA1C*, could also in part explain the phenotype.[44,45]

To conclude, 12p13.33 subtelomeric microdeletion is a rare genetic rearrangement that predisposes patients to speech sound disorders that could be defined as CAS/DVD when evaluated by a speech therapist. The *ERC1/ELKS* gene found in the smallest region of overlap could be a good candidate gene for CAS/DVD.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on European Journal of Human Genetics website (http://www.nature.com/ejhg)

Patient 1

1. Oro-facial praxis (Henin-Dulac Scale)

	Result	*Expected success rate at this age*
Mouth		
Tight the teeth	Unable to coordinate	95 %
Send a kiss with a sound	Bites his lips	90 %
Tongue		
Protrude tongue up to his nose	Unable to orientate his tongue	95%
Getting the tongue	Difficult but possible,	95%
'In and Out' several times	with parasitic movements	
Cheeks		
Inflate cheeks	+	95%
Chewing laterally	+	95%
Breath		
Blow a candle	+	40%

2. Logatoms repetition (ERTL-4 Scale)

Target	*First try*	*Second try*
[jerwa]	[jegwa]	[ewa]
[gɔ̃tra]	[gora]	[gɔ̃ra]
[dimɑ̃ko]	[muko]	[omɑ̃ko]
[zylsø]	[zyzsø]	[yø]
[otrydire]	[tryie]	[re]
[favikɛr]	[iɛr]	[ikɛr]
[mønylivu]	[ivu]	[nivu]
Score	**0/7**	**0/7**

3. Word repetition (ELO Battery). *VP : Verbal paraphrase*

Target	*Production*	*Target*	*Production*
1 : [bato]	+	9 : [akwarjɔm]	[kwarjɔm]
2 : [ʃapo]	+	10 : [ɔrlɔʒ]	[gɔrlɔʒ]
3 : [robo]	+	11 : [tõmwar]	[tomwar]
4 : [sɔrti]	[sɔrtir] *PV*	12 : [karaf]	+
5 : [biskɥi]	[bukɥi]	13 : [anorak]	[ɛnorak]
6 : [albɔm]	[algɔm]	14 : [resɛrvwar]	[egovwar]
7 : [byfe]	[dyfe]	15 : [kɑ̃diʁe]	[kɥ̃ʒe]
8 : [wazo]	[mazo]	16 : [ʒ̃ud]	[ʒ̃dɔ̃ *PV*
	Score		***4/16 (-3.7 SD)***

4. Non-Word repetition (ERTL-4 Scale)

Target	*Production*
[atʃum]	+
[timid]	+
[prɔf]	[krɔf]
[ʒwajø]	+
[sɛ̃ple]	+
[grɛ̃ʃø]	[goʃø]
[dɔrmœr]	+
Score	***5/7***

Patient 2 (43 years old)

1. Oro-facial Praxis (Henin Dulac Scale). All the oro-facial praxis are usually acquired before the age of 12 years old.

Mouth	
Cover lower lip with upper lip	Unable
Tight the teeth	+
Send a kiss with a sound	Unable
Drink through a straw	+
Open and close mouth several times	+
Tongue	
Protude tongue up to her nose	+
Clap the tongue	Unable to make a sound
Getting the tongue 'In and Out' several times	+
Cheeks	
Infalte cheeks	Unable
Chewing mouth open	Possible with parasitic movements
Eyes	
Open and close eyes several times	Irregular with parasitic movements
Frown eyebrows	Unable
Breath	
Blow a candle	+

2. Logatoms repetition (BALE scale)

Target	***Production***	***Target***	***Production***
[pɑ̃bi]	+	[molyne]	+
[linu]	+	[favikɛr]	+
[ʃandy]	+	[□ifazø]	+
[gɔ̃tra]	+	[koguʃi]	+
[zylsø]	+	[todɔ̃kɛ̃]	+
[lyrir]	+	[bimɛ̃dal]	+
[bartɛ̃]	[martɛ̃]	[fɑ̃vereti]	+
[jerwa]	+	[mukorido]	+
[nyrɔ̃li]	[nyroli]	[farvikery]	[favikery]
[rikape]	+	[mɑ̃dyrlanoti]	[mɑ̃dylati]
		Score	***16/20 (-2,5 SD)***

3. Word repetition (BALE Scale)

Target	***Production***	***Target***	***Production***
[boks]	+	[goelɑ̃]	[guelɑ̃]
[spɛktakl]	+	[kjɔsk]	+
[ʒeografi]	+	[scrypyl]	+
[pœpl]	+	[povrøte]	+
[krokodil]	[trokodil]	[bibliotɛk]	[bibiotɛk]
[bruwɛt]	+	[filtr]	+
[elikoptɛr]	+	[ɛkstrɔrdinɛr]	+
[katastrɔf]	[takastatrɔf]	[brijoʃ]	+
		Score	***12/16 (-26 SD)***

4. Non-Word repetition (BALE Scale)

Target	***Production***	***Target***	***Production***
[krakabil]	[trakabil]	[faltr]	+
[bruwɛl]	+	[katastrɛf]	+
[djɔsk]	+	[biblionak]	[bibionak]
[alokaptɛr]	+	[spaktɛkl]	+
[povrito]	+	[trijaʒ]	+
[ʒeagrofe]	+	[pupl]	+
[scropal]	[stropal]	[goenɛ̃]	+
[biks]	+	[ikstradonɛr]	[itradonɛr]
		Score	***12/16 (-6 SD)***

A. Description d'anomalies génétiques altérant la physiopathologie des interneurones

3q27.3 microdeletional syndrome: a recognisable clinical entity associating dysmorphic features, marfanoid habitus, intellectual disability and psychosis with mood disorder.

1. *Résumé de l'article*

Depuis la généralisation de l'usage de la CGH-array, de nombreux syndromes microdélétionnels on pu être rapportés et de nombreux autres restent à décrire. Bien que la délétion sub-télomérique 3q29 soit un syndrome bien défini, il n'a jamais été défini de syndrome affectant les régions interstielles du 3q.

Nous avons rapporté les 7 premiers patients porteurs de microdélétions au locus 3q28q27.3 recrutés grâce à la base de donnée Decipher et suggérons ce locus comme un nouveau syndrome microdélétionnel. Les patients partageaient une dysmorphie faciale reconnaissable et un habitus marfanoïde, associés à des troubles psychotiques avec dysthymie et une DI modérée à sévère. La plupart n'avait pas de retard dans les grandes étapes d'acquisitions psychomotrices mais on développé des capacités de communication et d'adaptation très limitées.

Deux régions minimales chevauchantes ont pu être définies. La première était associée aux troubles psychotiques avec dysthymie ainsi que la dysmorphie faciale. Cette région était localisée au locus 3q27.3 et comprenait plusieurs gènes dont le gène SST. Ce dernier est considéré comme un bon candidat aux troubles psychotiques par son implication dans la migration et différenciation des interneurones. Un cas familial avec une présentation clinique différente nous a permis de définir une seconde région minimale chez les 4 patients restants. La seconde région minimale était associée à une DI sévère (4/4), une maigreur importante (BMI de 15 (18.5<N<25); 4/4 patients), et l'habitus marfanoïde (scoliose (4/4), habitus longiligne (4/4), arachnodactylie (3/4), une anomalie du pectus (2/4). Cette seconde région minimale localisée en 3q27.3q28 locus contient un gène candidat intéressant nommé *AHSG* codant pour une protéine secrétée impliquée dans la voie TGF béta.

En conclusion, nous rapportons un nouveau syndrome microdélétionnel associant une dysmorphie faciale reconnaissable, un habitus marfanoïde, des

troubles psychiatriques à type de psychose avec troubles de l'humeur, et une DI modérée à sévère.

2. *Discussion et perspectives*

Ce travail rapporte les 7 premiers patients porteurs de délétions affectant le locus 3q27.3. Initialement, nous avons eu l'occasion d'examiner une patiente avec une maigreur importante, une dysmorphie faciale marquée et des troubles psychotiques. La CGH-array réalisée chez cette patiente a identifiée une anomalie non rapportée dans la littérature. Grâce à la base de données Decipher, nous avons pu récupérer des patients additionnels, qui se sont ajoutés environ 2 ans après la soumission de notre patiente. Ce formidable outil a donc permis de constater la relative homogénéité des phénotypes rapportés et nous avons donc contacté directement les responsables des services cliniques. Ainsi, nous avons pu affiner les manifestations cliniques associées à cette microdélétion.

Il existe peu de syndrome microdélétionnel avec une forte pénétrance de troubles psychotiques et le contenu génique du locus 3q27.3 s'est révélé particulièrement intéressant. Le gène *SST* est apparu comme un bon candidat. Les hypothèses physiopathologiques de la schizophrénie, un type de psychose, plaident pour une dérégulation de la migration et de la fonction des interneurones corticaux. Il est supposé que les interneurones composant le néocortex dérivent de 3 populations cellulaires embryonnaires. L'une d'elle exprime SST comme marqueur spécifique (29).

Nous pensons que ce syndrome microdélétionnel rare est reconnaissable grâce à la dysmorphie faciale associée à l'habitus marfanoïde. Le gène *AHSG* compris dans l'intervalle semble être un bon candidat pour ce phénotype par son implication dans la voie du TGF béta. Un des patients rapporté présente ostéoporose sévère compliquée de fractures récidivantes. AHSG a également un rôle dans l'ossification de la plaque de croissance et la minéralisation osseuse en général. Il serait intéressant que les autres cas index rapportés bénéficient d'ostéodensitométrie afin d'évaluer l'indication d'un traitement médicamenteux. Parmi nos 5 cas index, 2 avaient eu une échographie cardiaque ne révélant pas de dilatation aortique.

Les approches génomes entiers telles que la CGH-array permettent la mise en évidence de variants rares à forte pénétrance. Dans le cadre de pathologies considérées comme multifactorielles, la mise en évidence d'anomalies génétiques pathogènes permet de déterminer des bases physiopathologiques, nécessaires à leur compréhension et prise en charge.

TITLE

3q27.3 microdeletional syndrome: a recognisable clinical entity associating dysmorphic features, marfanoid habitus, intellectual disability and psychosis with mood disorder.

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SHORT TITLE

New syndrome with psychotic disorders and marfanoid habitus: 3q27.3 microdeletion.

KEY WORDS

Array-CGH, 3q27.3 locus, neuropsychiatric troubles, marfanoid habitus, Decipher

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Abstract

Background: Since the advent of array-CGH, numerous new microdeletional syndromes have been delineated while others remain to be described. Although 3q29 subtelomeric deletion is a well-described syndrome, there is no report on 3q interstitial deletions.

Methods: We report for the first time seven patients with interstitial deletions at the 3q28q27.3 locus gathered through the Decipher database and suggest this locus as a new microdeletional syndrome.

Results: The patients shared a recognisable facial dysmorphism and marfanoid habitus, associated with psychosis and mild to severe intellectual disability (ID). Most of the patients had no delay in gross psychomotor acquisition, but had severe impaired communicative and adaptive skills. Two small regions of overlap were defined. The first one, located on the 3q27.3 locus and common to all patients, was associated with psychotic troubles and mood disorders as well as recognisable facial dysmorphism. This region comprised several candidate genes including *SST,* considered a candidate for the neuropsychiatric findings because of its implication in interneuronal migration and differentiation processes. A familial case with a smaller deletion allowed us to define a second region of overlap at the 3q27.3q28 locus for marfanoid habitus and severe ID. Indeed, the common morphological findings in the first four patients included skeletal features from the marfanoid spectrum: scoliosis (4/4), long and thin habitus with leanness (average BMI of 15 (18.5<N<25)) (4/4), arachnodactyly (3/4) and pectus excavatum (2/4)). This phenotype could be explained by the deletion of the *AHSG* gene, wich encodes a secreted protein implicated in bone maturation and the TGFb signalling pathway.

Conclusion: We report on a new microdeletional syndrome that associates with a recognisable facial dysmorphism and marfanoid habitus including scoliosis, neuropsychiatric disorders of the psychotic spectrum and moderate to severe intellectual disability.

INTRODUCTION

Following the advent of microarray based technology, novel submicroscopic micro-deletion and duplication syndromes have been identified and delineated[1]. Academic databases such as the Decipher database [see URLs] are a great resource for gathering rare overlapping chromosomal rearrangements for further accurate delineation of the clinical presentations [2 3].

Very recently, three unrelated cases carrying 3q26.3q27.2 microdeletions were reported. Their clinical presentation associated neonatal hypotonia, severe feeding problems, specific facial features, abnormal dentition, recurrent upper airway infections, developmental delay and severe growth impairment [4]. The three deletions did not overlap with the cases reported herein.

In this article we report on the clinical features of seven patients (five probands) carrying microdeletions encompassing the 3q27.3 locus. The patients presented clinically-recognisable dysmorphism, marfanoid habitus and neuropsychiatric problems suggesting bipolar disorder with psychosis.

METHODS

Patients

Following the diagnosis of a patient with a 3q27.1-3q28 microdeletion and a striking phenotype, and interesting candidate genes at the 3q27.3 locus, the Decipher database (see URLs) was interrogated for 3q27.3 microdeletions and revealed new cases in France (Patient 2), Denmark (Patient 3), Belgium (Patient 4) and Italy (Patients 5 to 7).

Molecular cytogenetics analysis

All microdeletions were detected using genomic microarrays. Array types included array-CGH (Agilent ® 44k and 150k), BAC-array (Patient 4), an SNP array study using the Affymetrix ® SNP6.0 (Patient 2). The deletions were confirmed on FISH studies, except for patient 1 whose deletion was confirmed by quantitative PCR. Parental analyses were performed using FISH analyses except for patient 1 for whom parental analyses were performed by quantitative PCR.

RESULTS

CLINICAL REPORTS

Pictures of the patients are shown in figure 1 and the clinical findings are detailed in Table 1.

Patient	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5
Familial history	None	Sister	None	None	Father and sister
Antenatal findings	IUGR Placenta previae	None	None	None	None
Neonatal history	Low weight gain	Low weight gain	Thin, Dry skin	NA	NA
Birth measurement (Weeks Gestation)	(39)	(38)	(36)	NA	(34)
OFC (cm)	34	34	NA	NA	NA
Length (cm)	48	54	53	NA	NA
Weight (kg)	3,20	2,88	3,20	NA	2,90
Growth parameter at last examination (years)	24	12	16	37	20
OFC (cm)	53 (-2 SD)	52 (-1,5 SD)	NA	58.5 (+2 SD)	63 (+3 SD)
Height (cm)	170 (M)	115.5 (<-3 SD)	165 (-1 SD)	202 (>+3 SD)	196 (>+3 SD)
Weight (kg)	37.8 (<-3 SD)	15.8 (<-3 SD)	47.5 (-2 SD)	72 (2 SD)	175 (>+3 SD)
BMI	13.0	11.9	17.4	18	46
Facial dysmorphism					
Triangular face	+	+	+	+	+
Absent/low fat	+	+	+	+	+
Enophtalmia	+	+	+	+	+
Downslanting palpebral fissures	+	+	+	+	+
High nasal bridge	+	+	+	+	+
Low setted columella	+	+	+	+	+
Short philtrum	+	+	+	+	+
Thin upper lip	+	+	+	+	+
Small ears	+	+	+	+	+
Crowded teeth	-	+	-	+	+
Skeletal findings					-

Scoliosis	+	Severe kyphosis	Kyphosis	+	-
Pectus	-	-	Excavatum	Excavatum	-
Upper limbs	Arachodactyly	-	Arachnodactyly	Arachnodactyly	-
Lower limbs	Flat feet	+	Pes valgus	-	-
Dolichostenomelia	-	-	-	+	-
Neurodevelopment					
Motor delay	-	+	-	+	-
Speech delay	+	+	+	+	-
Intellectual disability	Severe	Severe	Severe	Severe	Mild
Scholarship	Special care	Special care	Special care	Special care	Standard
Psychiatric disorders					
Psychosis	Hallucinations	Delusions	Hallucinations	NA	Delusions
Mood	Bipolar troubles	Depressive	Mood disorders	NA	Depressive
ASD	+	+	ADHD	Stereotypies	ADHD
Anxiety	+	+	Phobia	NA	Social phobia
Oppositional	-	+	+	NA	-
Eating	-	-	-	NA	Boulimia
Substance use	-	-	-	NA	-
Treatments	Neuroleptics Anxiolytics	Neuroleptics Anxiolytics Anti-epileptics	Neuroleptics	NA	Neuroleptics Anxiolytics
Paraclinical exams					
Brain MRI	Normal	Thick corpus callosum	NA	NA	Normal
Skeletal survey	Normal	NA	NA	NA	Normal
Echocardiography	NA	NA	NA	Mitral valve prolapse	Normal
Others	Atopic dry skin	Atopic and dry skin	Atopic and dry skin	ICD	
Array CGH Chromosomal mapping (Hg19) Familial segregation	Arr chr3q27.2 q27.3 chr3 (185445864-187832449)x1 *De novo*	Arr chr3q27.3q28 chr3 (185816939-192541120)x1 *De novo*	Arr chr3q27.3q28 chr3 (184355123-189587274)x1 *De novo*	Arr chr3q27.2q28 chr3 (185711163-190282110)x1 *Parents not analysed*	Arr chr3q27.3 chr3 (186461680-187863305)x1 *Inherited from the father*

Table 1: Clinical description of the five index cases (available data) with detailed mapping of the 3q27.3 diagnosed deletions.; + : present; - : absent, NA: Not Available; OFC: Occipito-Frontal Circumference; SD: Standard Deviation; M: Mean; ASD: Autism Spectrum Disorder; ADHD: Attention Deficit and Hyperactivity Disorder; Del: Deletion; ICD: Intra-cardiac Device, IUGR: Intra-Uterine Growth Retardation.

Patient 1

Patient 1 was the second child of non-consanguineous parents. Pregnancy was complicated by a placenta praevia and intra-uterine growth retardation. Post-term delivery was normal and birth measurements were: weight 3.2 kg, length 48 cm and occipito-frontal circumference (OFC) 34 cm. Low weight gain was noticed during the first days of life. Although food intake was normal, growth retardation was explored during the first years. Cystic fibrosis was ruled out, intestine and gastric biopsies were performed and were found to be normal. No delay in psychomotor acquisitions was noticed but behavioural disturbances appeared before 3 years of age. Special care schooling and neuroleptic therapy were initiated at 5 years of age. Schooling was interrupted after 16 years of age secondary to the development of psychotic symptomatology not compatible with schooling. At 18 years of age she developed severe depression after a death in the family. Melancholia was diagnosed. She remained silent and isolated for 6 months and the episode of melancholia lasted approximately 3 years. At about age 20, she had lost acquisitions such as reading, writing and washing. She developed anxiety disorders and presented one episode of visual and auditory hallucinations. No episodes of delusion were noticed. The parents reported mood swings with recurrent depressive moods. She is currently living in a residence for the disabled and is assisted for most of her daily needs. Growth was constant but abnormal despite normal food intake. At age 24, she measured 1.70 m (Mean) and weighed 37.8 kg (<-3 SD). Her BMI was calculated at 13, (18,5<N<24,5) and OFC measured 53 cm (-2 SD). Facial dysmorphism included a slender face, deep-set eyes, a broad nasal bridge, a low-implanted columella, a small mouth with a thin upper lip, short philtrum and prognathism (Figure 1). Comparisons with old photographs revealed that this dysmorphism increased with age (Figure 1b). The skeletal examination identified a long and thin habitus with arachnodactyly and flat feet. She had no sub-cutaneous fat. Investigations included normal brain MRI and CT-scan, metabolic screening with serum amino-acids and urine organic acid chromatography, homocysteinemia assay and standard chromosomal analysis. Further metabolic analysis included a normal lipid profile, as well as normal insulinemia, leptinemia and pituitary hormone assays. Serum adiponectin was assayed in the patient and her parents but did not show any difference (Patient 1: 909ng/ml, father: 2012 ng/ml, mother: 11314 ng/ml; laboratory norms: 865-21424ng/ml). Array-CGH revealed a 2.4 Mb *de novo* deletion of the 3q27.1q27.3 locus (Table 1).

Patient 2

Subject 2 was born at 38 weeks of gestation by vaginal delivery following the first uneventful pregnancy of young healthy non-consanguineous parents. Birth measurements were: weight 2.8kg, length 54 cm and OFC 34 cm. The neonatal period was marked by low food intake and insufficient weight gain. He presented chronic diarrhoea. Investigations for the low weight gain ruled out gluten intolerance, and revealed normal rectal biopsies and abdominal US. Gross psychomotor acquisitions were delayed with sitting at 1 year of age and walking at 2 years of age. Speech was delayed. He presented seizures at 6 years of age, successfully treated without recurrence. The clinical evaluation did not reveal a neurological deficit. Neuroleptic therapy was begun in childhood because of behavioural disorders. At 8 years old, he was not toilet-trained and could neither run nor bicycle. His size was normal (115.5 cm; +1 SD), but weight gain was severely impaired (15.8 kg; -3 SD). His OFC measured 52 cm (-1.5 SD) and BMI was calculated at 11.9 (18.5<N<24.5). The clinical examination revealed facial dysmorphism with a slender face, enophtalmia with downslanting palpebral fissures, high nasal bridge, small low set ears and dolichostenomelia (Figure 1a). The facial dysmorphism, became more specific with age (Figure 1b). The parents reported atopic skin. Severe, progressive scoliosis required orthopaedic and surgical treatment. Between 15 and 18 years of age, three arthrodesis operations were necessary and resulted in arthrodesis from C1 to L5. Follow-up showed frequent fractures of both femoral diaphyses as well as several fingers and toes. Osteodensitometry revealed severe osteoporosis. At 22 years old, his size was 1.75m (Mean), weight 40kg (< -3 SD). A recent psychiatric evaluation confirmed behavioural problems including severe anxiety with sleeping disorders, greatly impaired communicative skills, and verbal and gestural stereotypies. He displayed a depressive mood and had episodes of delusion and obsessive troubles, sometimes complicated by auto and hetero-aggressive behaviour. He is currently treated with anti-psychotic neuroleptics, anxiolytics and anti-epileptic drugs. He is currently living in a home for the disabled and is not autonomous for most of his daily needs. The brain MRI revealed a thick corpus callosum. The electromyogram and muscular biopsy were normal. Biological investigations showed normal levels for plasma amino-acids and urine organic acid assays, transferrin isoelectrofocalisation, creatine kinase and aldolase, thyroid hormones, IGF1, blood formula, and ferritinemia. The standard chromosomal analysis was normal and other genetic investigations excluded Angelman and fragile X syndrome.

BAC array identified a 4.2Mb *de novo* 3q28q27.3 deletion mapped between the markers RP11-110C15 and RP11-279P10, and confirmed by FISH analysis (Table 1).

Patient 3

Patient 3 was the first child of non-consanguineous healthy parents. The pregnancy was normal. The mother did not take any medication. The patient was born after 40 weeks of gestation. Available birth parameters were weight 3.2kg (25-50e percentile), length 53cm (25-50e percentile). No microcephaly was reported. The infant showed a thin, dry atopic skin during the neonatal period. No hypotonia was noticed and food intake was normal. Gross psychomotor acquisitions were normal for smile response, head control, sitting, crawling and walking. He was toilet-trained at around 3 years of age. Speech acquisition was delayed. Small sentences were uttered at 7 years of age. At the same age, audition was normal, and horizontal nystagmus was noticed. He never had seizures. Schooling was rapidly interrupted and he was oriented towards a school for special needs. At the age of 16 years, he could not read or write and he is currently living in an establishment for the disabled. Measurements were 47.5 kg (-2 SD) for weight, 165 cm (-0.5 SD) for height and 55,5 cm for OFC. BMI was below normal at 17.4 (18.5<N<24.5). Facial dysmorphism included enophtalmia, downslanting palpebral fissures, small ears, a high nasal bridge with a hooked nose and a short anteverted philtrum. He displayed skeletal features such as kypho-scoliosis, mild pectus excavatum, gynecomasty, arachnodatyly and pes valgus, with visible veins on the chest. Apart from the morphological findings, the patient had psychiatric symptoms including mood disorders, psychosis with episodes of hallucination and delusion, anxiety and opposing defiant disorder (see details in Table 1). He was treated with successive neuroleptic medications. Autonomy was limited, and he required help for washing, dressing, cooking and using the phones and public transport. He was able to eat and take his medications alone. Routine laboratory tests, standard chromosomal analysis and fragile-X investigations were normal. Affymetrics Genome-wide SNP array 6.0 identified a 5.2 Mb *de novo* 3q27.1q28 deletion, confirmed by qPCR (Table 1).

Patient 4

Patient 4 is the second child of non-consanguineous parents, and was referred to the clinic as an adult. He had a healthy elder sister. The pregnancy was uneventful. There was no information about

the neonatal and childhood periods. Delayed psychomotor acquisitions and specialised schooling since nursery school were reported. At 37 years of age, his measurements were: weight 72kg (+1SD), height 202cm (+3SD) with an arm span of 205 cm, and OFC 58.5cm (+2SD). BMI was calculated at 18 (18.5<N<24.5). The clinical examination revealed facial dysmorphism with a triangular face, downslanting palpebral fissures, retrognathism, small and low-implanted ears, a high arched palate and dental crowding. Skeletal findings included scoliosis, pectus excavatum and arachodactyly with positive thumb sign but without the wrist sign. He also had a fragile skin with skin striae and delayed wound healing. During the medical interview, gestural stereotypies were noticed. Paraclinial examinations were performed and echocardiography revealed mitral valve prolapse, while abdominal ultrasound showed kidney stones. Patient 4 was living in a residential home for the disabled. He was autonomous for most of the activities of everyday life. He had no psychiatric evaluation but was diagnosed with attention-deficit disorder together with intellectual disability. In early adulthood, the patient presented a heart rhythm disorder that required an ICD implant. Genetic tests included normal Fragile-X testing and standard karyotyping. Array CGH identified a 4.5Mb deletion of the 3q27.3q28 locus, confirmed by FISH analysis (Table 1). Parental analysis could not be performed.

Patients 5, 6 and 7

Patient 5 is the second child of non-consanguineous parents. He was born pre-term at 38 weeks of gestation, after an uneventful pregnancy. Birth weight was 2.9 kg. There were no concerns during the neonatal period. Psychomotor acquisitions, including speech acquisition, were within the normal range. Behavioural and psychotic troubles were diagnosed, but with the appropriate treatment, schooling was possible in a normal school. At 14 years of age, a De Raven non-verbal progressive matrices test was performed and the patient scored 38, showing mildly impaired cognitive abilities (N>=41). Psychiatric disturbances progressively invaded his everyday life and limited his cognitive abilities. However, he completed his normal education. He was referred for a clinical consultation at 20 years old because of a familial presentation of psychiatric troubles. His measurements were 175kg (>+3 SD), 196cm (>+3 SD). OFC was 63cm (+3 SD). The clinical examination revealed no skeletal anomalies, and the neurological examination was normal except for behavioural disorders in keeping with the psychiatric presentation. He had an inguinoscrotal hernia that was not treated surgically. Facial dysmorphism included deep-set eyes, a high nasal bridge with a hooked nose and a short

philtrum on a thin upper lip. The psychiatric evaluation revealed complex psychosis with symptomatology developing on a schizoid personality associated with depressive mood disorder and delusions. Hyperactivity and attention-deficit disorder was noticed, without autism spectrum disorder. Anxiety disorder with episodes of panic attacks, social phobias, obsessive-compulsive disorder and bulimia nervosa were also noticed. He has been treated with neuroleptics for many years. He is now living in the family home. He is autonomous in daily life except for telecommunication, transportation and drug observance. Paraclinical examinations included a normal echocardiography, a normal lipid profile and standard chromosomal analysis. Array CGH revealed a 1.4Mb microdeletion of the 3q27.3 locus (Table 1). FISH analysis confirmed this deletion, inherited from the father (patient 6) and also found in his symptomatic sister (patient 7).

Patient 6, the father of patient 5 was affected by adult-onset depression at 39 years old, followed by progressive dementia. He is now living in a home. On family photographs (no consent available), he had a very similar facial gestalt to patient 5. No further information could be obtained.

Patient 7, the sister of patient 5 had a severe psychotic disease complicated by crises of delirium. Her psychiatric presentation was associated with borderline cognitive disability. She also has a similar facial gestalt to patient 5. No further information was available on this case.

Array CGH results

In all patients, a small interstitial deletion involving chromosomal band 3q27.3 was identified with molecular karyotyping. Chromosomal locations of deletions are detailed in Table 1. Alignment of the diagnosed deletions is shown Figure 2 using the UCSC browser (see URLs). The smallest region of overlap, common to all patients and named SRO1 contained five genes referenced in OMIM (*MASP1, SST, ADIPOQ, ST6GAL1* and *BCL6)* and three non-referenced genes (*RPL39L, RTP1* and *RTP4*). The patients presented with psychosis and facial dysmorphism. Given that patients 5, 6 and 7 displayed different clinical features, a second region (SRO2) was defined by the exclusion of patients 5, 6 and 7 who carried the smaller deletion. SRO2 included seven genes (*FETU8, KNG1, HRG, DGKG, TBCCD1, AHSG* and *CRYGS*) (Figure 2). The four patients with a deletion overlapping with SRO2 presented with ID, leanness and marfanoid habitus (Figure 1, Table 1).

DISCUSSION

We report here on the first five index cases with interstitial microdeletions of the 3q27.3q28 locus. The deletions were either *de novo* or inherited. Patients shared a common recognisable phenotype that included facial dysmorphism, neuropsychiatric disorders of the psychotic spectrum and mild to severe ID. Four of the seven patients also displayed skeletal features belonging to the marfanoid habitus.

The five index patients shared similar facial dysmorphism (Figure 1), which included a slender face, deep set eyes, a high nasal bridge and a hooked nose above a short philtrum and a thin upper lip surrounding a small mouth, and small low-set ears. Prognathism was noticed in 3/5 patients. Teeth were reported as crowded in 2/5 patients. When old photographs were available for comparison, the majority of these dysmorphic features appeared to evolve with age (figure 1b). Four of the index patients also displayed a strikingly long, thin habitus (patients 1 to 4), which included sometimes severe scoliosis, requiring surgery in one case (patient 2). Arachnodactyly was found in 3/4 patients, a pectus anomaly was reported in 2/4 patients, and foot anomalies were also found in 2/4 patients. One patient presented dolichostenomelia. Interestingly, 1 patient had a mitral valve prolapse.

The major concern of the patients and families seemed to be the psychiatric manifestations. Motor acquisitions were within the normal ranges for 3/5 patients and speech was delayed in 4/5 patients. Four of the five index patients had to be oriented to a school for special needs from primary school onwards. Overall, 6/7 patients developed dysthymia, diagnosed as depression or melancholia in 4/7 patients. Symptoms from the psychotic lineage, namely delusion (3/7) or hallucination (2/7) were reported. These psychiatric features were suggestive of psychosis associated with mood disorders in 6/7 patients. Regarding patient 4, behavioural troubles from the autism spectrum disorder associating stereotypies and low interactive skills were present. Anxiety was frequently associated (5/7) and sometimes complicated by social phobias (2/7) or eating disorders (2/7). In this report, four of the five index cases required medication that associated anti-psychotic neuroleptics with anxiolytics because of their psychiatric illness. Severe intellectual disability was reported in 4/7 patients. However, we could not determine whether this was due to regression or secondary to the development of the psychiatric illness. We suggest that the psychiatric presentation of the psychosis spectrum associated with mood disorders associated with this facial gestalt could be a recognizable feature of a 3q27.3 microdeletion syndrome.

Given the genotype-phenotype results, our hypothesis was the existence of a contiguous gene syndrome with one gene in the SRO1 deleted in all patients that was responsible for the psychotic troubles, and a second gene deleted in the SRO2 that was responsible for the marfanoid habitus. The first SRO was associated with the neurobehavioural and psychiatric presentation in all seven patients. This deleted region encompasses five genes previously associated with human diseases (*MASP1, ADIPOQ, ST6GAL1, SST* and *BCL6)* (See URLs). Among these genes, the responsibility of the *MASP1* gene could be ruled out since it is responsible for the autosomal recessive 3MC syndrome [5]. *BCL6* is a gene that codes for a protein promoting B-lymphocyte differentiation, and associated in human pathology with malignant haemopathies[6]. In contrast, the *SST* (somatostatin) gene could be a good candidate for the psychiatric presentation of our patients. Indeed, the *SST* gene is expressed in migrating neurons and further acts as a neuropeptide in early embryogenesis[7]. The SST protein is particularly expressed in the dendritic GABAergic spines interneurons. These GABAergic inhibitory sub-types of neurons are thought to be involved in schizophrenia and psychotic diseases, and SST was reported to be under-expressed in the frontal lobes of schizophrenic patients[8 9]. However, no mutations in this gene were reported in a large cohort of schizophrenic patients screened by exome sequencing[10]. The absence of truncating mutations in this gene in a series of over 6500 healthy exomes from the NHLBI cohort could argue in favour of the pathogenicity of haploinsufficiency of this gene [see URLs]. Finally, the *ADIPOQ* gene was first considered a good candidate for the long, thin habitus. *ADIPOQ* codes for a ubiquitous diffusible protein that binds the ADIPOQ receptors. This hormone synthesised in the post-prandial status regulates the activity of the AMPK cellular energy sensor. Rare variants as well as common variants of the *ADIPOQ* are associated with insulin resistance and metabolic syndrome in diabetic patients[11]. However, the serum concentration of ADIPOQ protein was assayed in patient 1 and her parents, but the results were not informative. The responsibility of the gene in the skeletal phenotype could also be ruled out since it was also deleted in patient 5, and his affected family members were overweight.

The second SRO contained seven genes (*FETU8, KNG1, HRG, DGKG, TBCCD1, AHSG* and *CRYGS*). Among these, four had previously been associated with human pathology and referenced in the OMIM database. The causality of three of them could be ruled out, since *KNG1* [MIM#228960] and *HRG* [MIM#613116] genes are implicated in haematological diseases and *CRYGS* is implicated in a dominant form of cataract[12]. However, the *AHSG* gene, which codes for the FETUIN-A protein,

appeared to be a good candidate gene for the skeletal phenotype and/or intellectual deficiency for different reasons: i) several rare variants of this protein were shown to be associated with leanness [13] [14]; ii) serum levels of AHSG were suggested to correlate positively with insulin sensitivity and weight gain [15]; iii) the null mice presented improved insulin sensitivity and limited weight gain[16] [17]; iv), the AHSG protein was implicated in accelerated growth plate mineralisation in bone[18]; v) the AHSG protein is a matricial protein that binds to the TGFb superfamily of proteins and inhibits certain TGFb1 functions[19]; vi) this gene was not included in the smaller deletion found in obese patient 5. The TGFb pathway is strongly implicated in matricial signalling, and many components of this signalling pathway are implicated in marfanoid syndromes[20].

To conclude, we report here a new 3q27.3 microdeletional syndrome based on the description of seven patients (five index cases). The clinical hallmarks of this syndrome may include recognisable facial dysmorphism, highly penetrant neuropsychiatric disturbances suggestive of psychosis with mood disorders with, intellectual disability and a marfanoid habitus. Genotype-phenotype correlations revealed two SRO, and highlighted the possible role of *SST* in the neuropsychiatric phenotype and *AHSG* in the skeletal phenotype.

URL

Decipher: www.decipher.sanger.ac.uk

UCSC browser: www.genome.ucsc.edu/

OMIM: www.omim.org

EVS http://evs.gs.washington.edu/EVS/

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COMPETING INTERESTS

None

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PATIENT CONSENT

Obtained.

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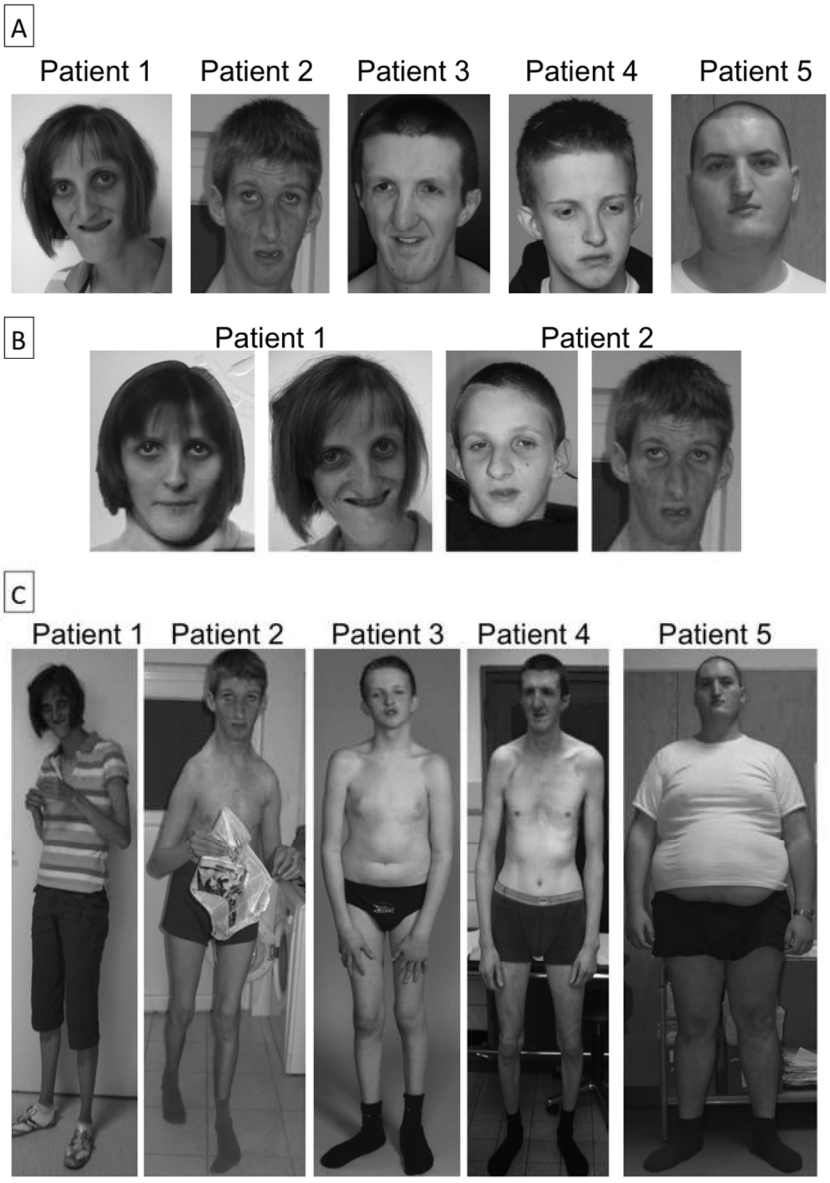
LEGENDS TO THE FIGURES

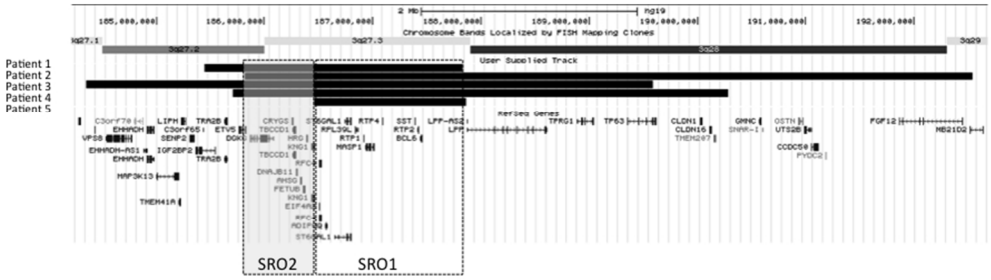
Figure 1: Photographs of the patients. A. Note the common facial gestalt associating a slender face with deep-set eyes, a broad nasal bridge, a hooked nose with a short philtrum, a small mouth with a thin upper lip and relative prognathism. The ears are small and low-set. B. Evolution of the facial dysmorphism in patients 1 and 2. C. Long and thin habitus, with variable features of the Marfan spectrum, except for patient 5.

Figure 2: Alignment of the microdeletions diagnosed in the 5 patients. Two small regions of overlap were defined. SRO1 was common to all five index cases and was systematically associated with facial dysmorphism and neurobehavioural problems suggesting bipolar disorder with psychosis. Among the deleted genes, *SST* appeared to be a good candidate. SRO2 was common to four index cases who presented a low BMI and marfanoid habitus. The *AHSG* gene is thought to be a candidate gene for leanness and the marfanoid habitus.



317x453mm (72 x 72 DPI)

Figure 2



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I. ETUDE D'UNE COHORTE HOMOGENE DE PATIENTS PAR SHD-E : IDENTIFICATION DE LA CAUSE MOLECULAIRE DU SYNDROME DE SHPRINTZEN-GOLDBERG

In-Frame Mutations in Exon 1 of SKI cause Dominant Shprintzen-Goldberg Syndrome

1. *Résumé de l'article*

Le syndrome de Shprintzen-Goldberg (SGS) est caractérisé par un habitus marfanoïde sévère, une DI, des limitations articulaires, une dysmorphie faciale typique et une craniosténose. En utilisant une stratégie de séquençage d'exome sur une famille présentant un SGS, nous avons identifié une délétion hétérozygote respectant le cadre de lecture du premier exon du gène *SKI*. Le séquençage direct de *SKI* a permis d'identifier une autre délétion partiellement chevauchante et 10 mutations différentes affectant de manière récurrente certains résidus chez 18 des 19 cas séquencés. Ces individus comprenaient une famille avec récurrence dans la fratrie dont la mère était porteuse d'une mosaïque somatique. Aucune mutation n'a été retrouvée dans une cohorte de 11 patients supplémentaires présentant une association de syndrome marfanoïde avec DI et craniosténose.

Les mutations étaient concentrées dans le domaine d'interaction avec les R-SMAD du premier exon de SKI. L'interaction de SKI avec SMAD2/3 et SMAD4 régule la voie de signalisation du TGF béta. Le modèle murin de knock-out pour le gène *Ski* est consistant avec les manifestations cliniques du SGS. Ces résultats démontrent que le SGS fait partie des pathologies associées à la voie TGF béta.

2. *Discussion et perspectives*

Grâce au recrutement international de nombreux patients présentant un syndrome marfanoïde avec DI, le Professeur Faivre a pu réunir la plus grande cohorte de patients suspects cliniquement de SGS. Malgré le nombre d'échantillons de patients atteints de SGS séquencés en SHD-E et la taille de la cohorte de réplication que nous avions à exploiter l'identification de *SKI* a été difficile.

Une première série de patients a été séquencée avec une première capture ne couvrant pas la plupart des premiers exons du chromosome 1. Ainsi, parmi les 2 trios envoyés initialement, aucun variant *de novo* n'affectait un gène partagé par les 2 cas index. Après plusieurs mois de vérifications par Sanger, une nouvelle série d'exomes a été envoyée. L'ADN d'un nouveau trio et 2 apparentés extrêmes d'une famille de SGS ont été capturés avec un kit améliorant la couverture des régions riches en GC. Il n'y avait pas non plus dans cette série de variant de bonne qualité partagé affectant un gène commun à ces 3 cas index. Une délétion de 12 bases respectant le cadre de lecture affectait un gène répondant aux critères de notre filtre biologique de la voie du TGF béta. En recherchant les variants de faible qualité, il a été identifié chez le cas index du trio de la 2^{e} série un variant faux sens *de novo* affectant le même gène. Ces variants ont été secondairement vérifiés en Sanger chez ces patients puis dans la cohorte de réplication. Le séquençage de cette région extrêmement riche en GC a requis une combinaison d'approches afin d'identifier les mutations recherchées.

La découverte du gène responsable du SGS a été réalisée en parallèle d'un grand groupe américain travaillant sur la maladie de Marfan et les syndromes marfanoïdes coordonné par Hal C Dietz. Leur groupe a étudié un trio en SHD-E, qui a permis la mise en évidence d'une mutation faux sens *de novo* en aval de la région riche en GC. Le gène a été retrouvé muté dans une cohorte de réplication constituée de 10 patients avec SGS (36).

Depuis la publication de l'article, le séquençage du gène est proposé par le laboratoire de génétique moléculaire du CHU de Dijon à titre de recherche, et plus de 10 nouveaux cas ont maintenant été séquencés. Un travail complémentaire de description clinique est en cours afin d'affiner les critères de recherche de mutation de *SKI*. Ce travail permettra également de préciser le spectre des manifestations cliniques associées au SGS et leur évolution. Des études fonctionnelles sont également en cours en collaboration avec l'équipe de Ghent (Anne De Paepe, Bert Calleweart) afin de tester l'hypothèse haploinsuffisance versus dominant négatif, par comparaison à des lignées de patients ayant une délétion 1p36.

3. Article

In-Frame Mutations in Exon 1 of *SKI* Cause Dominant Shprintzen-Goldberg Syndrome

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Shprintzen-Goldberg syndrome (SGS) is characterized by severe marfanoid habitus, intellectual disability, camptodactyly, typical facial dysmorphism, and craniosynostosis. Using family-based exome sequencing, we identified a dominantly inherited heterozygous in-frame deletion in exon 1 of *SKI*. Direct sequencing of *SKI* further identified one overlapping heterozygous in-frame deletion and ten heterozygous missense mutations affecting recurrent residues in 18 of the 19 individuals screened for SGS; these individuals included one family affected by somatic mosaicism. All mutations were located in a restricted area of exon 1, within the R-SMAD binding domain of *SKI*. No mutation was found in a cohort of 11 individuals with other marfanoid-craniosynostosis phenotypes. The interaction between SKI and Smad2/3 and Smad 4 regulates TGF-β signaling, and the pattern of anomalies in *Ski*-deficient mice corresponds to the clinical manifestations of SGS. These findings define SGS as a member of the family of diseases associated with the TGF-β-signaling pathway.

Shprintzen-Goldberg syndrome (SGS [MIM 182212]) has been described as being associated with intellectual disability (ID), marfanoid habitus (including arachnodactyly, pectus deformity, scoliosis, and pes planus with foot deformity), camptodactyly, and facial dysmorphism (including hypertelorism, exophthalmos, downslanting palpebral fissures, and maxillary and mandibular hypoplasia). The hallmark of this syndrome, although inconsistent, is the presence of craniosynostosis[1,2] (see Web Resources). Other findings include mitral valve prolapse, recurrent hernias, loss of subcutaneous tissue, and thin translucent skin. Infantile hypotonia, severe scoliosis, and obstructive apnea are common features as well. It is not known whether individuals with SGS display an aortic risk because some rare cases have been described with aortic dilatation[1,2] (see Web Resources). We assumed that SGS is an autosomal-dominant disorder on the basis of previous descriptions of simplex cases (although recurrence in siblings has been reported).[3] Because of the clinical overlap with Marfan syndrome (MFS [MIM 154700]) and Loeys-Dietz syndrome (LDS1A [MIM 609192], LDS1B [MIM 610168], LDS2A [MIM 608967], LDS2B [MIM 610380], LDS3 [MIM 613795], and LDS4 [MIM 190220]), mutations in *FBN1* (MIM 134797), *TGFBR1* (MIM

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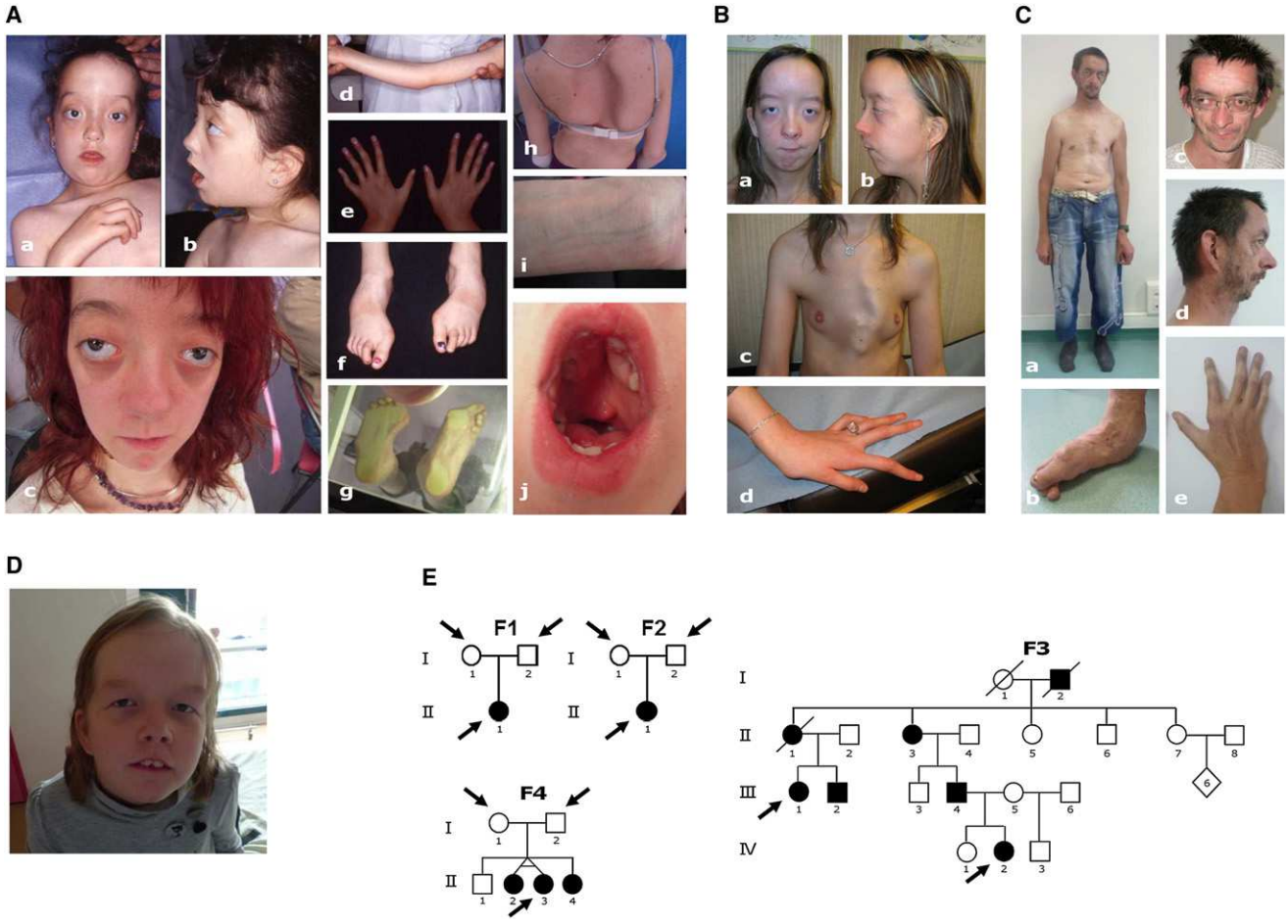

Figure 1. Clinical Presentations and Pedigrees of Subjects with SGS and Mutations in *SKI*
(A) Photographs of affected individual II-1 (from family 2), who has a *SKI* de novo c.94C>G variant. Note the hypertelorism, proptosis, downslanting palpebral fissures, maxillary and mandibular hypoplasia, low-set ears (Aa–Ac), joint contractures (Ad), arachnodactyly and camptodactyly (Ae), deformed feet (Af–Ag), severe scoliosis (Ah), translucent skin (Ai), and hypertrophy of the palatal shelves (Aj).
(B) Photographs of affected individual 14 (family 8), who has a *SKI* de novo c.103C>T variant. Note the dysmorphic features in favor of SGS (Ba–Bb), severe pectus carinatum (Bc), arachnodactyly, and camptodactyly (Bd).
(C) Photographs of affected individual III-4 (from family 3), who has a c.280_291delTCCGACCGCTCC variant in exon 1 of *SKI*. Note the dysmorphic features and habitus in favor of SGS (Ca, Cc, and Cd), foot deformity (Cb), and hand deformity with camptodactyly (Ce).
(D) Photographs of affected individual IV-2 from family 3 (child of individual III-4 in C).
(E) Pedigrees of families 1 (F1), 2 (F2), 3 (F3), and 4 (F4) studied by exome sequencing. Individuals studied are shown by an arrow.

190181), and *TGFBR2* (MIM 190182) should be excluded.[4] We hypothesized that SGS is a clinically distinct entity resulting from heterozygous mutations of other gene(s) involved in the TGF-β-signaling pathway.

We recruited a cohort of 19 SGS-affected individuals originating from six European countries and Australia. The cohort included five related individuals from a family consistent with autosomal-dominant inheritance (family 3), another family with recurrence in siblings (family 4)[3] (Figure 1 and Table 1), ten simplex cases (including one previously published individual),[5] and one probable autosomal-dominant case. We also additionally assembled a second cohort of 11 individuals with marfanoid habitus and craniosynostosis; these individuals did not present with the dysmorphic features of SGS (Table S1, available online). Informed consent for research investigations was obtained from the affected individuals, legal representatives, or relatives. The research protocol was approved by the local ethics committees. The 30 individuals were first screened for *FBN1*, *TGFBR1*, and *TGFBR2* mutations by direct sequencing and multiplex ligation-dependent probe amplification and for chromosomal rearrangements by 180K or 244K Agilent array comparative genomic hybridization. We identified simplex heterozygous missense mutations in *FBN1* (c.3761G>A [p.Cys1254Tyr]; RefSeq accession number NM_000138.4), *TGFBR1* (c.734A>G [p.Glu245Gly]; RefSeq NM_004612.2) and *TGFBR2* (c.1583G>A [p.Arg528His]; RefSeq NM_003242.5) in three individuals from the second cohort (Table S1 and Figure S1).

First, we used the Nimblegen SeqCap EZ Exome v.2.0 kit to perform exome sequencing in two trios (families 1 and 2; Figure 1) with simplex SGS according to standard procedures; we used 8 μg of DNA from affected individuals

Table 1. Detailed Clinical Features of SGS Individuals and Summary of the Detected Mutations in *SKI*

	Family 1	Family 2	Family 3					Family 4			Family 5	Family 6	Family 7	Family 8	Family 9	Family 10	Family 11	Family 12	Family 13	
	II-1	II-1	III-4	IV-2	II-1	III-1	III-2	II-2	II-3	II-4	11	12	13	14	15	16	17	18	19	Total
Sex	F	F	M	F	F	F	M	F	F	F	M	M	F	F	F	F	M	M	M	12F and 7M
Age (years)	21	20	42	11	44[a]	13	14	22	22	20	18	16	5	21	10[a]	11	32	20	26	–
Craniosynostosis	+	+	–	–	–	–	–	+	+	+	+	+	–	+	+	+	–	+	+	12/19
Arachnodactyly	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	18/19
Pectus deformity	+	+	+	–	–	–	–	+	+	+	+	+	+	+	+	+	+	+	+	15/19
Scoliosis	–	+	+	+	+	–	+	+	+	+	+	+	–	+	+	+	+	+	+	16/19
Joint contractures	+	+	+	+	+	+	+	+	+	+	–	+	–	+	+	+	–	+	–	15/19
Camptodactyly	+	+	–	–	–	–	–	–	–	–	–	+	–	+	+	+	+	+	+	9/19
Foot malposition	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+	–	17/19
Scaphocephaly or dolichocephaly	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	18/19
Hypertelorism	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	18/19
Proptosis	+	+	+	–	+	+	–	+	+	–	+	–	+	+	+	+	+	+	+	15/19
Downslanting palpebral fissures	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	–	–	16/19
Micrognathia or retrognathia	+	+	+	–	+	–	–	+	+	+	+	+	+	+	+	–	+	+	+	15/19
Intellectual disability	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	19/19
Hernias	–	–	–	–	–	–	–	+	+	+	+	–	+	+	+	+	+	+	+	11/19
Loss of subcutaneous fat	+	–	–	–	–	–	–	+	+	–	–	+	–	+	+	–	–	+	–	7/19
Valvular anomalies	–	–	–	–	–	–	–	–	–	–	MVP	–	–	MVP	MVP, MI	MVP	MVP, MI	–	–	5/19
Aortic root dilatation	–	–	–	–	–	–	–	–	–	–	+[b]	–	–	–	UNL	+	+	–	–	3/19
Myopia	–	–	+	+	+	+	–	+	+	N/A	–	–	–	–	+	–	+	–	–	8/18
SKI mutation	c.100 G>T	c.94 C>G	c.280_291del TCCG ACCG CTCC	c.280_291del TCCG ACCG CTCC	c.280_291del TCCG ACCG CTCC	c.280_291del TCCG ACCG CTCC	c.280_291del TCCG ACCG CTCC	c.101 G>T	c.101 G>T	c.101 G>T	c.104 C>A	c.94 C>G	c.283_291del GACC GCTCC	c.103 C>T	c.95 T>C	c.100 G>A	c.94 C>G	c.92 C>T	–	–
Amino acid substitution	p.Gly 34Cys	p.Leu 32Val	p.Ser 94_Ser 97del	p.Ser 94_Ser 97del	p.Ser 94_Ser 97del	p.Ser 94_Ser 97del	p.Ser 94_Ser 97del	p.Gly 34Val	p.Gly 34Val	p.Gly 34Val	p.Pro 35Gln	p.Leu 32Val	p.Asp 95_Ser 97del	p.Pro 35Ser	p.Leu 32Pro	p.Gly 34Ser	p.Leu 32Val	p.Ser 31Leu	–	18/19
Inheritance	de novo	de novo	AD	AD	AD	AD	AD	AD, SM	AD, SM	AD, SM	de novo	father N/A	de novo	de novo	de novo	parents N/A	parents N/A	parents N/A	AD	–

The following abbreviations are used: AD, autosomal dominant; F, female; SM, somatic mosaicism; M, male; MVP, mitral valve prolapse; MI, mitral insufficiency; N/A, not available; and UNL, upper normal limit.
[a]Affected individual 5 died of respiratory insufficiency. Affected individual 15 died suddenly, and an autopsy showed severe mitral valve dysplasia with calcifications of the mitral annulus.
[b]Aortic dilatation requiring surgery at 16 years of age (aortic root dilatation with Z score = 7.014). He also has vertebrobasilar and internal carotid tortuosity and a dilated pulmonary-artery root.

and both parents (Figure S1). The resulting exome-capture libraries underwent two 75 bp paired-end sequencing runs on an Illumina HiSeq 2000. Reads were aligned to the human reference genome (GRCh37/hg19) with the Burrows-Wheeler Aligner,[6] and potential duplicate paired-end reads were removed with Picard v.1.22 (see Web Resources). The Genome Analysis Toolkit (GATK) v.1.0.57 was used for base quality-score recalibration and indel realignment,[7] as well as for single-nucleotide-variant and indel discovery and genotyping with the use of standard hard-filtering parameters.[7] Variants with a quality score < 30, allele balance > 0.75, sequencing depth < 4, quality-to-depth ratio < 5.0, length of homopolymer run > 5.0, and strand bias > -0.10 were flagged and excluded from subsequent analyses. We used the GATK Depth of Coverage tool to assess coverage by ignoring reads with a mapping quality < 20 and by ignoring bases with a base quality < 30. In total, 92% of the primary target was covered at least four times in all individuals (Table S2). All variants identified in the affected individuals were annotated with SeattleSeq SNP annotation (see Web Resources). We focused on de novo heterozygous exonic variants (missense, nonsense, and splice-site variants and coding indels). Candidate mutational events were then inspected with the Integrative Genomics Viewer (see Web Resources).[8] The resulting variants were excluded when the frequency was over 1/1,000 in the National Heart, Lung, and Blood Institute (NHLBI) Exome Variant Server (EVS) (see Web Resources). After applying variant calling filters, we failed to identify any candidate de novo mutations. Indeed, none of the variants identified in family 1 were confirmed by Sanger sequencing, and two de novo variants were confirmed in family 2. The first de novo variant was a *COL4A4* (RefSeq NM_000092.4) missense mutation (c.4423G>T [p.Asp1475Tyr]) that is likely to cause benign hematuria only. The second de novo variant was a *C1ORF54* (RefSeq NM_024579.3) frameshift mutation (c.272delT [p.Val91Glufs*3]). Because *C1ORF54* is predicted to encode a brain-, smooth-muscle-, and skin-secreted extracellular protein, the gene was sequenced in the rest of the cohort, but no pathogenic mutation was identified (conditions are available on request).

Subsequently, we performed exome sequencing by using Nimblegen Exome v.3.0 capture in the two most distant affected relatives (individuals III-1 and IV-2, who share 1/32 of their genomes) from family 3 and in one (individual II-3) of the three affected siblings from family 4, as well as in all of their unaffected parents (Figure 1), in accordance with the manufacturer's recommendations. After mapping the raw sequencing reads against the reference genome and applying the same variant calling filters, we searched for heterozygous variants present in both distant relatives in family 3 and for heterozygous variant calls present in one sibling but absent in both parents in family 4 according to the gonadal-mosaicism hypothesis (Table S2). We first identified 314 variants shared by the two distant affected individuals from family 3. Considering the hypothesis of the implication of the TGF-β pathway, we set up a biological filter with the EMBL-EBI reactome,[9] and 42 partners were listed (Table S3). When we filtered against EVS data and the TGF-β-signaling biological filter, only *SKI* was revealed (Table S2). Indeed, we found a 12 bp deletion (c.280_291delTCCGACCGCTCC [p.Ser94_Ser97del]) in a highly conserved region of exon 1 of *SKI* (RefSeq NM_003036.3) (Figure 2). Remarkably, in the first attempt of exome analysis, no *SKI* mutation was detected in family 1 or 2, but we noted that exon 1 (and therefore the mutational hotspot) was not covered by v.2.0 of Nimblegen SeqCap EZ Exome capture. By reducing the quality filters and the number of reads in family 4, we detected suggestive evidence of a missense mutation (c.101G>T [p.Gly34Val]; two reads) in *SKI* (RefSeq NM_003036.3). Sanger sequencing confirmed this mutation and the segregation in favor of a somatic mosaicism given that we found a lower level of the mutant allele in the asymptomatic mother. We performed PCR reactions on genomic DNA by using primers designed to amplify all seven exons and intron-exon boundaries of *SKI* in the remaining individuals from the SGS and non-SGS marfanoid-craniosynostosis cohorts (Table S5). After a variant was identified, the parents were secondarily studied when available. It was difficult to set up PCR conditions for the amplification of *SKI* as a result of GC-rich regions, particularly in exon 1. Because of the size of exon 1, it was necessary to design three pairs of primers (E1-1, E1-2, and E1-3), and a new pair of primers was necessary for achieving the sequencing of the hot-spot region (named E1-ATG, Table S5). PCR fragments were purified with the multiscreen Vacuum Manifold system (Millipore). Sequencing was performed with the ABI BigDye Terminator Cycle Sequencing kit (v.3.1) (Applied Biosystems) in ABI 3130 sequencer 7 (Applied Biosystems) according to the manufacturer's instructions. Sequence data were analyzed with SeqScape v.2.7 (Applied Biosystems). The pathogenicity of missense mutations was tested with PolyPhen-2 and SIFT online software (see Web Resources).[10] Screening of our entire cohort of SGS individuals revealed a total of ten de novo missense mutations, including somatic mosaicism in a family with recurrence in siblings and two overlapping in-frame deletions (one of them was dominantly inherited in a large family) (Table 1 and Tables S4 and S5), accounting for 18 of 19 cases tested. All mutations were found in the R-SMAD binding domain, affecting five conserved residues. Familial segregation and in silico prediction models were in favor of their pathogenicity (Table S4). A three-dimensional protein modeling was realized with Phyre^2 software (see Web Resources). An automatic modeling script with standard parameters in the Phyre^2 pipeline was used for generating the Protein Data Bank file of the protein. Overall, 83% of residues were modeled at >90% confidence, and 104 residues were modeled ab initio. A detailed description of the protein-modeling results is provided in Figure 3. We also sequenced *SKI* in the second cohort of individuals

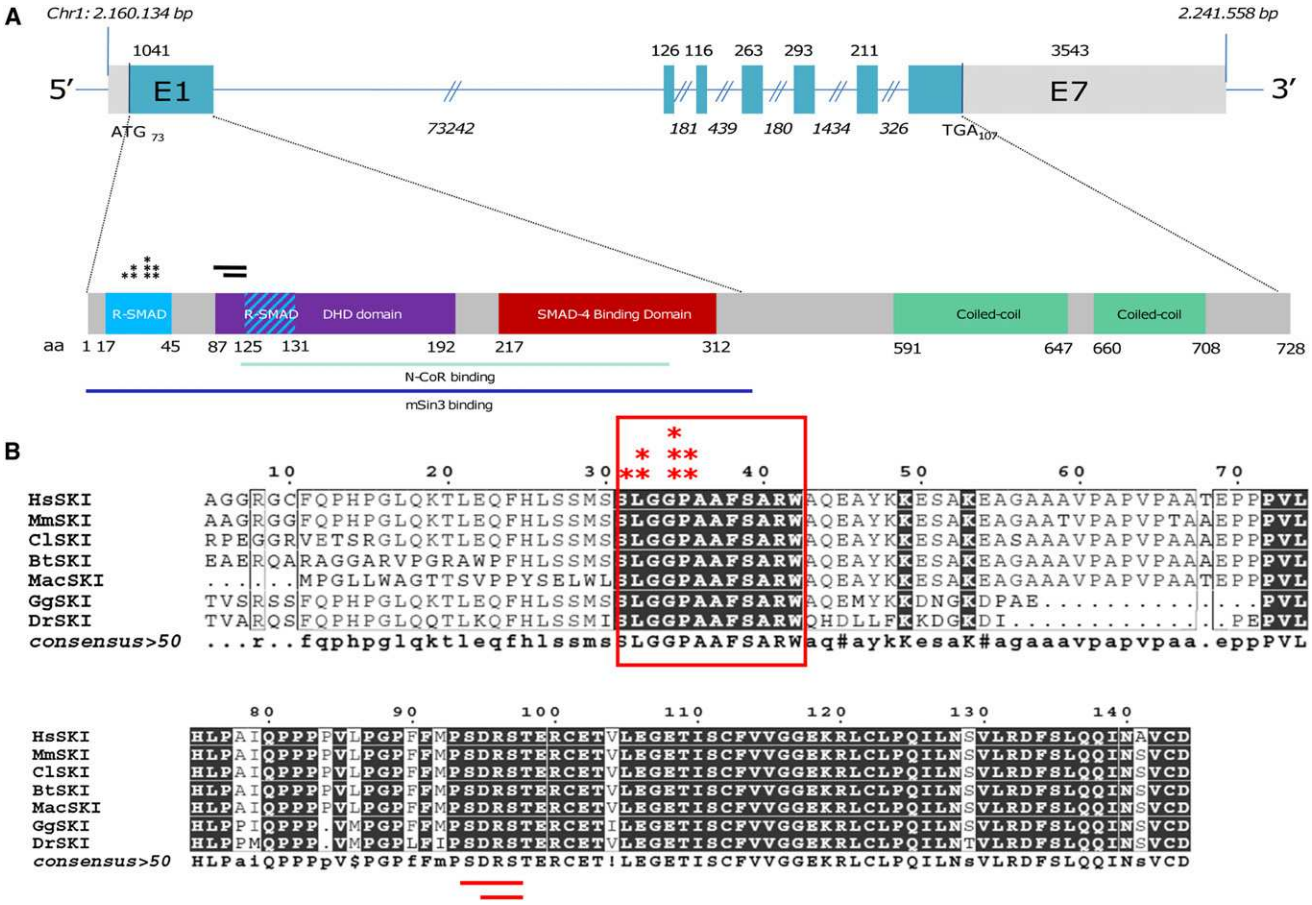

Figure 2. Location of SGS-Associated Mutations in *SKI*

(A) Schematic representation of the seven coding exons of *SKI* (top). The 5′ and 3′ UTRs are denoted in light gray. Exon 1 encodes the N-terminal R-SMAD- and SMAD- binding domains (blue and red box, respectively, at the bottom) and the DHD domain (purple box), and the remaining exons encode the C terminus with its two coiled-coil domains (green boxes at the bottom). Sites for interaction with N-CoR and mSin3 are also shown as light blue and dark blue lines, respectively. All mutations (asterisks for missense variants and lines for deletions) are located in the R-SMAD binding domain.

(B) Highly conserved amino acid residues (indicated in dark boxes) are conserved in vertebrates. All mutations affect highly conserved residues. The following abbreviations are used: Hs, *Homo sapiens*; Ms, *Mus musculus*; Cf, *Canis familiaris*; Bt, *Bos Taurus*; Mac, *Macropus eugeneii*; Gg, *Gorilla gorilla*; and Dr, *Danio rerio*.

with a marfanoid-craniosynostosis phenotype incompatible with SGS but found no variant, thus further highlighting the phenotypic and genetic specificity of SGS.

Here, we report the identification of heterozygous exon 1 *SKI* mutations in 18 cases presenting with the characteristic features of SGS. The identification of recurrent heterozygous mutations in a specific area of exon 1 will facilitate genetic screening and help genetic counseling. Our results also feature information useful in clinical care because three individuals of the SGS cohort presented with aortic dilation; one such individual had vertebrobasilar and internal carotid tortuosity and a dilated pulmonary-artery root, further highlighting the overlap between SGS and Loeys-Dietz syndrome (Table 1).[11] Therefore, a transthoracic echocardiogram, as well as imaging by computed tomography or magnetic resonance imaging of the neck, thorax, abdomen, and pelvis, can be justified. All of the individuals with *SKI* mutations had intellectual disability, supporting the hypothesis that SGS and Furlong syndrome should be separate.[12] Given the absence of *SKI* mutation from the second cohort with non-SGS marfanoid craniosynostosis, we can conclude that other gene(s) remain to be determined for other types of marfanoid-craniosynostosis syndromes.

Several lines of evidence implicate the TGF-β pathway in marfanoid habitus, and SGS-affected individuals present with severe marfanoid habitus, allowing us to apply a biological filter strategy to select variants in the TFG-β pathway.[9] *SKI* is an outstanding candidate gene because it encodes a ubiquitous transcription factor with a precise pattern of spatiotemporal constitutional expression and is implicated in promoting differentiation and maturation of chondrocyte cells and inhibiting proliferation of cells. SKI is implicated in the repression of TGF-β signaling, mainly through inhibition of SMAD2 phosphorylation, and competes with pSMAD3-SMAD4 binding and recruiting transcriptional repressor proteins such as N-CoR and mSIN3 (Figures 2 and 3 and Figure S2).[13–16] Mutations

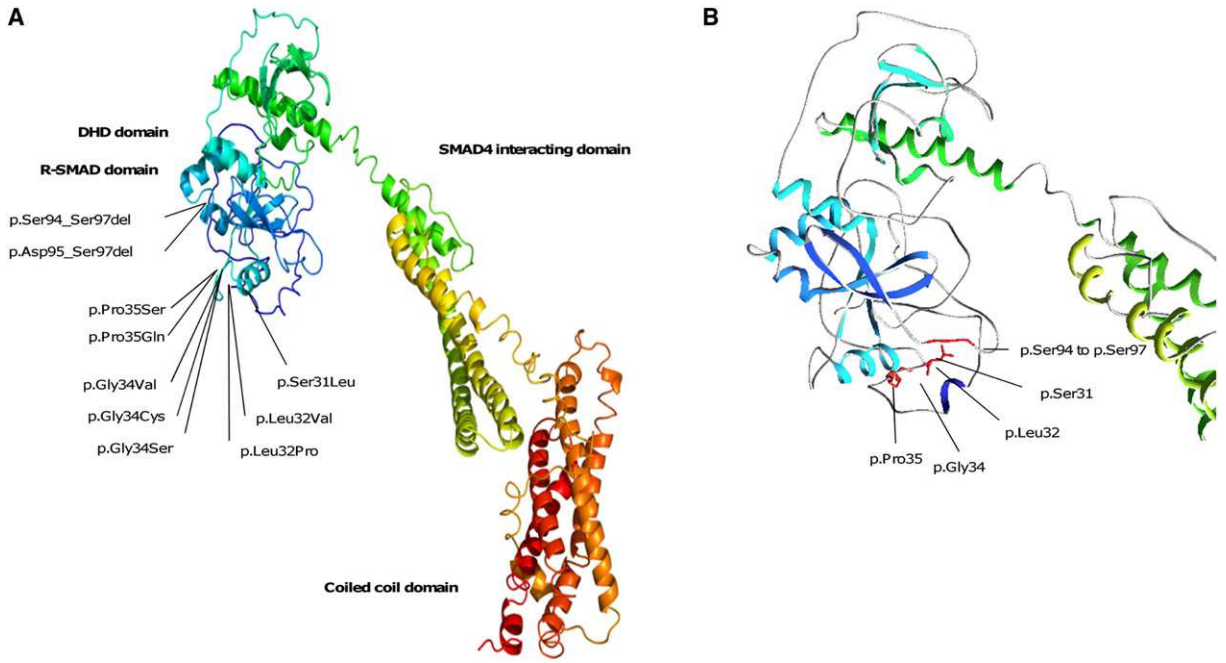

Figure 3. Three-Dimensional Modeling of SKI

(A) Functional domains of wild-type protein composed of an N-terminal DNA transcriptional regulating domain (dark blue) including R-SMAD (light blue) and DHD domains, a central SMAD4-interacting domain (greenish yellow), and a C-terminal coiled-coil domain (red).

(B) Enlargement of the region affected by all the mutations. The in-frame deletions shorten a loop (between residues 92 and 97). The missense mutations disrupt a flexible region (residues 31–35). All the mutations are localized on the same surface of the R-SMAD-binding domain.

found in the reported individuals affect the SMADs interacting domain and the transcription regulation domain DHD. All the mutated residues induce polarity changes and are located on the same structural surface, suggesting modification of the binding properties of SKI to the SMADs. The identified mutations in the SMAD interacting domains could lead to abnormal transcriptional repression of the downstream TFG-β signaling.[16] Furthermore, *Ski*$^{-/-}$ mice display a lethal phenotype with associated midline facial cleft, a depressed nasal bridge, eye anomalies, skeletal muscle defects, and digital anomalies.[17] Besides the mouse knockout model, the other major argument linking the SGS phenotype with *SKI* mutations is the role of SKI in the TGF-β pathway, a role which has been implicated in marfanoid habitus. It has been shown that the regulation of TGF-β signaling by SKI plays an important role in chondrocyte differentiation and maturation.[18] Because the SMAD4-SKI complex modulates the transcription of genes regulated by TGF-β signaling, missense mutations within SMAD-interacting domains could lead to abnormal transcriptional repression of the downstream TGF-β-signaling genes in SGS (Figure 2 and Figure S2).

Interestingly, the hallmark of most diseases with defects in the TGF-β pathway is the high risk of developing thoracic aortic aneurysms (TAAs), although aortic complications seem less frequent in the SGS cohort.[1] However, this finding could be explained by the young age of the reported individuals. Recently, functional studies in SMAD3 mutants raised the hypothesis that the ERK noncanonical TGF-β pathway could be implicated in TAAs. A crucial pathophysiologic distinction between canonical and noncanonical pathway activation points to the importance of the chronic activation of the noncanonical TGF-β pathway in the development of vascular symptoms in marfanoid syndromes.[19] In Myhre syndrome (MIM 139210), *SMAD4* mutations in the mad homology 2 domain protect mutant SMAD4 complexes from ubiquitination and impair the expression of TGF-β-driven target genes.[20,21] Accordingly, the increased accumulation of SMAD4 in Myhre syndrome results in developmental delay and short stature and has no known risk of TAAs.[21] Further studies would be useful for better understanding this aspect of the disease.

Myhre syndrome and SGS are the only TGF-β-pathway-related syndromes associated with ID. This feature can be explained in SGS given that *SKI* is necessary for neuronal proliferation and maturation and has been designated as a critical gene for ID in 1p36 telomeric deletion. Indeed, expression of SKI has been reported to be regulated by axon-Schwann-cell interactions and to be a crucial signal in Schwann cell development and myelination.[22] The SKI/SnoN domain of *Drosophila melanogaster* and *Caenorhabditis elegans* was shown to be necessary for the proper cellular differentiation of neuronal progenitors.[23] Moreover, Baranek et al. also showed that SKI, as a repressor of the TGF-β pathway, modulates its action during cortical

development through recruitment of the Sin3/HDAC complex to SMADs and thereby fine tunes the balance between proliferation and differentiation of progenitor cells.[24]

In conclusion, our findings show that in-frame mutations in exon 1 of *SKI* cause SGS. Additional studies are necessary for elucidating the region-specific and tissue-specific consequences of defective SKI-mediated TGF-β signaling. Furthermore, because SKI mutations could not be identified in a cohort with non-SGS marfanoid craniosynotosis, mutations in yet-to-be-identified genes are most likely responsible for other types of marfanoid-craniosynostosis syndromes.

Supplemental Data

Supplemental Data include two figures and five tables and can be found with this article online at http://www.cell.com/AJHG.

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Web Resources

The URLs for data presented herein are as follows:

Broad Institute Integrated Genomics Viewer, http://www.broadinstitute.org/igv/
Ensembl, http://www.ensembl.org/tools.html
ESPript, http://espript.ibcp.fr/ESPript/ESPript/
NHLBI Exome Sequencing Project (ESP) Exome Variant Server, http://evs.gs.washington.edu/EVS/
GeneDistiller 2, http://www.genedistiller.org/
GeneReviews, Greally, M.T. (1993). Shprintzen-Goldberg Syndrome, http://www.ncbi.nlm.nih.gov/books/NBK1277/
Genoscope, https://www.genoscope.cns.fr/
International HapMap Project, http://hapmap.ncbi.nlm.nih.gov/
MultAlin, http://multalin.toulouse.inra.fr/multalin/
Online Mendelian Inheritance in Man (OMIM), http://www.omim.org/
Open Astex Viewer, http://openastexviewer.net/web/
Phyre^2, www.sbg.bio.ic.ac.uk/phyre2/
Picard, http://picard.sourceforge.net/
PolyPhen-2, http://genetics.bwh.harvard.edu/pph2/
Primer3, http://frodo.wi.mit.edu/primer3/
SeattleSeq Annotation 131, http://snp.gs.washington.edu/SeattleSeqAnnotation131/
SIFT, http://sift.bii.a-star.edu.sg/

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Supplemental data to:

In-frame mutations in exon 1 of *SKI* cause dominant Shprintzen-Goldberg syndrome

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Figure S1: Design of the study.

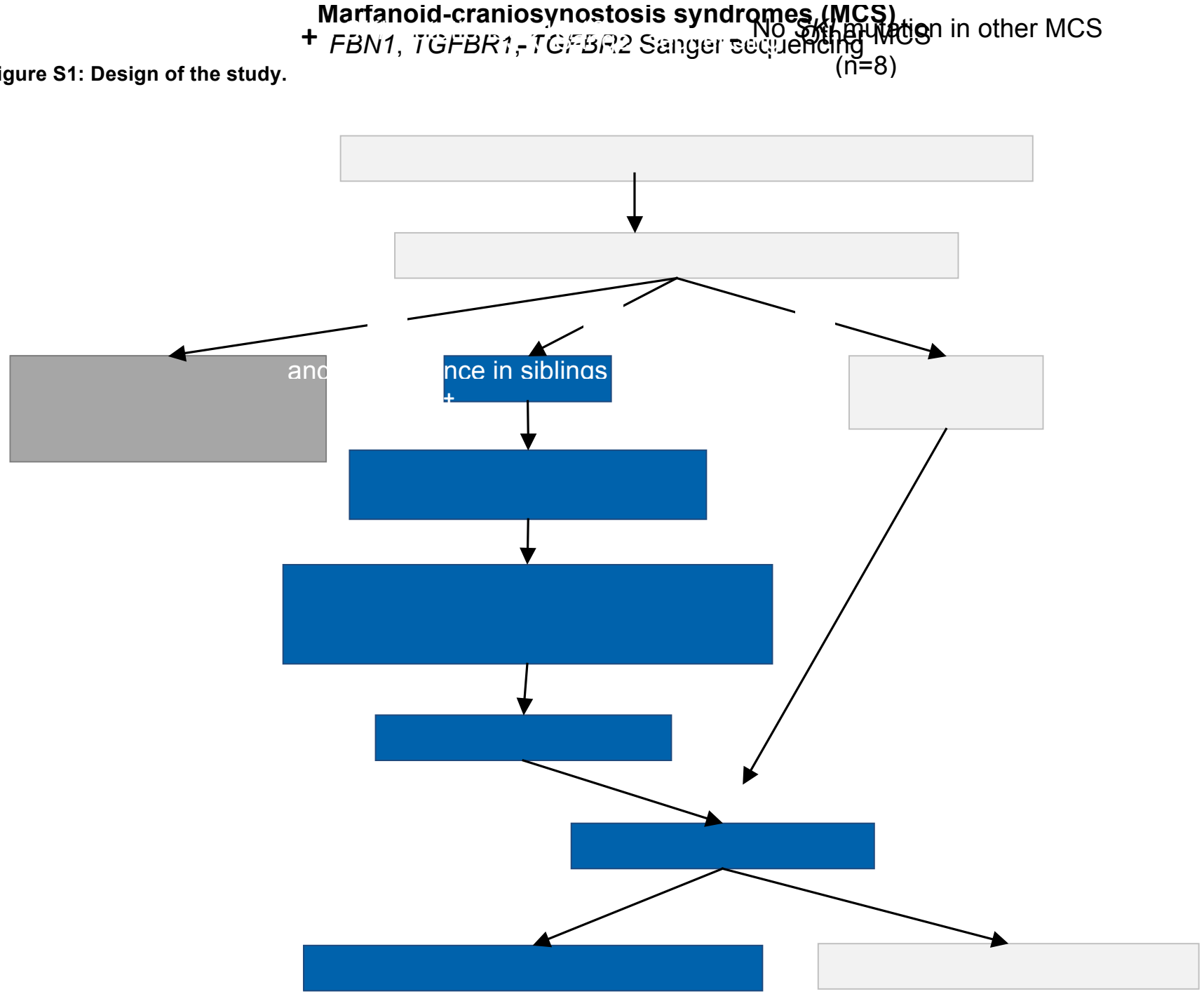


Table S3: Strategy for application of a biological filter (cellular TGF-β pathway).

HGNC gene symbol	OMIM number	OMIM reference
E2F4	600659	E2F TRANSCRIPTION FACTOR 4
E2F5	600967	E2F TRANSCRIPTION FACTOR 5
FKBP1A	186945	FK506-BINDING PROTEIN 1A
FOXH1	603621	FORKHEAD BOX H1
FOXO3	602681	FORKHEAD BOX O3A
HDAC1	601241	HISTONE DEACETYLASE 1
MEN1	131100	MULTIPLE ENDOCRINE NEOPLASIA, TYPE I
MTMR4	603559	MYOTUBULARIN-RELATED PROTEIN 4
NEDD4L	606384	UBIQUITIN PROTEIN LIGASE NEDD4-LIKE
NR1I2	603065	NUCLEAR RECEPTOR SUBFAMILY 1, GROUP I, MEMBER 2
PARD6A	607484	PARTITIONING-DEFECTIVE PROTEIN 6, C. ELEGANS, HOMOLOG OF, ALPHA
PARP1	173870	POLY (ADP-RIBOSE) POLYMERASE 1
PMEPA1	606564	TRANSMEMBRANE PROSTATE ANDROGEN-INDUCED RNA
PPA1	179030	PYROPHOSPHATASE, INORGANIC, 1
PPM1A	606108	PROTEIN PHOSPHATASE, MAGNESIUM-DEPENDENT, 1A
PPP1R15A	611048	PROTEIN PHOSPHATASE 1, REGULATORY SUBUNIT 15A
RBL1	116957	RETINOBLASTOMA-LIKE 1
RHOA	165390	RAS HOMOLOG GENE FAMILY, MEMBER A
RNF11	612598	RING FINGER PROTEIN 11
RNF111	605840	RING FINGER PROTEIN 111
SKI	164780	V-SKI AVIAN SARCOMA VIRAL ONCOGENE HOMOLOG
SKIL	165340	SKI-LIKE
SMAD2	601366	MOTHERS AGAINST DECAPENTAPLEGIC, DROSOPHILA, HOMOLOG OF, 2
SMAD3	603109	MOTHERS AGAINST DECAPENTAPLEGIC, DROSOPHILA, HOMOLOG OF, 3
SMAD4	600993	MOTHERS AGAINST DECAPENTAPLEGIC, DROSOPHILA, HOMOLOG OF, 4
SMAD7	602932	MOTHERS AGAINST DECAPENTAPLEGIC, DROSOPHILA, HOMOLOG OF, 7
SMURF1	605568	SMAD-SPECIFIC E3 UBIQUITIN PROTEIN LIGASE 1
SMURF2	605532	SMAD-SPECIFIC E3 UBIQUITIN PROTEIN LIGASE 2
SP1	189906	TRANSCRIPTION FACTOR Sp1
STRAP	605986	SERINE/THREONINE KINASE RECEPTOR-ASSOCIATED PROTEIN
STUB1	607207	STIP1 HOMOLOGOUS AND U BOX-CONTAINING PROTEIN 1
TFDP1	189902	TRANSCRIPTION FACTOR DP1
TFDP2	602160	TRANSCRIPTION FACTOR DP2
TGFB1	190180	TRANSFORMING GROWTH FACTOR, BETA-1
TGFBR1	190181	TRANSFORMING GROWTH FACTOR-BETA RECEPTOR, TYPE I
TGFBR2	190182	TRANSFORMING GROWTH FACTOR-BETA RECEPTOR, TYPE II
TGIF1	602630	TRANSFORMING GROWTH FACTOR-BETA-INDUCED FACTOR
TRIM33	605769	TRIPARTITE MOTIF-CONTAINING PROTEIN 33
UCHL5	610667	UBIQUITIN CARBOXYL-TERMINAL HYDROLASE L5
USP15	604731	UBIQUITIN-SPECIFIC PROTEASE 15
WWTR1	607392	WW DOMAIN-CONTAINING TRANSCRIPTION REGULATOR 1
ZFYVE9	603755	MADH-INTERACTING PROTEIN

HGNC : HUGO Gene Nomenclature Committee ; OMIM : Online Mendelian Inheritance in Man.

Supplemental Tables

Table S1. Detailed clinical features of non-SGS marfanoid craniosynostosis patients.

Family	F14	F15	F16	F17	F18	F19	F20	F21	F22	F23	F24	
Affected Individual	20	21	22	23	24	25	26	27	28	29	30	Total
Sex	M	M	F	F	F	M	M	F	M	M	F	6M/5F
Age (years)	15	6	8	22	22	13	23	20	19	1	21	
Craniosynostosis	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	+	**11/11**
Arachnodactyly	+	+	+	+	+	+	+	+	+	+	+	11/11
Pectus deformity	+	-	+	+	-	+	-	+	-	-	+	6/11
Scoliosis	+	+	+	+	+	+	-	-	-	-	+	7/11
Joint contractures	-	-	-	+	-	+	-	-	+	-	-	3/11
Camptodactyly	-	-	-	-	+	+	-	-	-	-	+	3/11
Foot malposition	+	+	+	+	+	+	+	-	NA	-	+	8/10
Scapho/dolichocephaly	+	+	+	+	-	+	+	NA	-	+	+	8/10
Hypertelorism	-	+	+	-	-	-	-	+	+	-	+	5/11
Proptosis	-	-	-	+	+	-	-	+	+	+	+	6/11
Downslanting palpebral fissures	-	+	+	-	-	+	-	NA	NA	-	-	3/9
Micro/retrognathia	-	-	-	-	-	-	-	NA	-	+	+	2/10
Intellectual disability	-	-	-	-	-	+	+	NA	+	+	-	4/10
Hernias	+	+	+	+	+	-	-	NA	-	+	-	6/10
Loss of subcutaneous fat	-	-	-	-	-	-	-	NA	-	-	-	0/10
Valvular anomalies	+	+	+	+	-	-	+	NA	-	NA	+	6/9
Aortic root dilatation	+[a]	+[a]	+[a]	+	-	+	-	NA	-	NA	+	6/9
Myopia	-	+	-	-	NA	NA	-	NA	-	NA	-	1/7
SKI mutation	-	-	-	-	-	-	-	-	-	-	-	0/11
Other mutations	*FBN1* p.Cys1254Tyr	*TGFBR1* p.Glu245Gly	*TGFBR2* p.Arg528His	-	-	-	-	-	-	-	-	3/11
Inheritance	*de novo*	*de novo*	*de novo*	Sporadic	Sporadic	Sporadic	Sporadic	Sporadic	Sporadic	Sporadic	Sporadic	

F : Female ; M : Male ; NA : Not available.

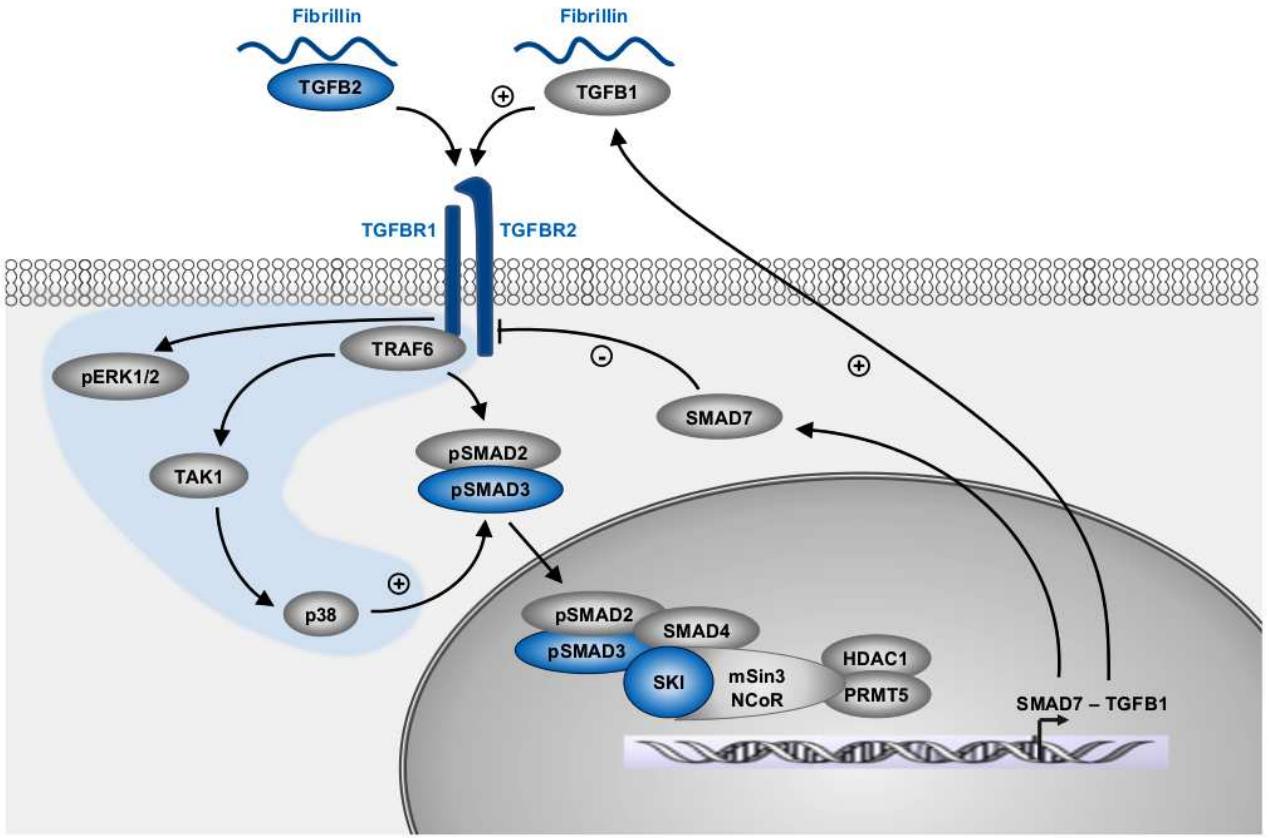
[a] Aortic dilatation required surgery in childhood

Table S2: Next generation sequencing statistics.

	Project 1 (2011)						Project 2 (2012)				
Family	F1	F1	F1	F2	F2	F2	F4	F4	F3	F3	F3
Individual	II-1	I-1	I-2	II-1	I-1	I-2	IV-2	III-1	II-4	I-1	I-2
NB_READS	152 949 574	188 373 140	153 270 902	164 636 014	172 788 840	188 524 030	140 793 700	123 309 166	124 054 811	110 402 922	120 007 422
PERCENT_MAPPED_READS	88,34%	88,62%	91,31%	90,08%	90,54%	88,29%	98,38	98,26	97,87	97,77	97,97
PERCENT_PAIRING	98,54%	98,95%	98,83%	98,82%	98,77%	98,93%	99,63	99,62	99,49	99,43	99,49
PERCENT_DUPLICATE_READS	7,11%	7,58%	8,44%	6,20%	6,99%	6,04%	12,76	10,94	12,77	14,13	13,36
Capture kit	Nimblegen SeqCap EZ Exome v2.0						Nimblegen exome V3				
PERCENT_NT_30X	88%	90%	88%	90%	90%	91%	88,32	87,77	86,22	84,87	86,16
PERCENT_REGIONS_0x	1%	1%	1%	1%	1%	1%	0,69	0,72	0,81	0,60	0,82
PERCENT_REGIONS_FULL_COVER	98%	98%	98%	97%%	98%	98%	97,30	97,23	96,94	97,04	96,91
MEAN_COVER	177,65	220,17	173,83	195,78	206,05	229,30	140,79	130,14	125,12	110,29	122,25
MEDIAN_COVER	142,00	177,00	139,00	158,00	179,00	187,00	111,00	103,00	99,00	87,00	97,00
Exonic heterozygous call	12 851	12 765	12 619	12 732	12 811	12 872	14 694	14 585	13 973	14 356	14 528
De novo Trio analysis	84			51			na		71		
Intrafamilial call segregation study	na			na			12 067		na		
Absent from local Exome database	50			30			314		31		
EVS6500 <1/1000 variant filter	32			18			46		24		
TGF-beta pathway filter	0			0			1		0		

na : not appropriate.

Supplementary Figure S2: SKI within the TGF-β pathway (adapted from Holm et al., 2011[19]).



This diagram highlights the genes implicated in marfanoid syndromes (in blue).

Table S4: Prediction of identified *SKI* variants.

Family	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12
Affected individual	**II-1**	**II-1**	**II-1, III-1, III-2, III-4, IV2**	**II-2, II-3, II-4**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**
Genomic location (bp), Hg19, Chromosome 1	2160305	2160299	2160485-2160497	2160306	2160309	2160299	2160488-2160497	2160308	2160300	2160305	2160299	2160297
Codon change	GGC/TGC	CTG/GTG	TCCGACCGCTCC/-	GGC/GTC	CCG/CAG	CTG/GTG	GACCGCTCC/-	CCG/TCG	CTG/CCG	GGC/AGC	CTG/GTG	TCG/TTG
cDNA position	c.100G>T	c.94C>G	c.280_291del TCCGACCGCTCC	c.101G>T	c.104C>A	c.94C>G	c.283_291del GACCGCTCC	c.103C>T	c.95T>C	c.100G>A	c.94C>G	c.92C>T
Protein position	p.Gly34Cys	p.Leu32Val	p.Ser94_Ser97del	p.Gly34Val	p.Pro35Gln	p.Leu32Val	p.Asp95_Ser97del	p.Pro35Ser	p.Leu32Pro	p.Gly34Ser	p.Leu32Val	p.Ser31Leu
Ensembl VEP	Missense	Missense	12-bp in-frame deletion	Missense	Missense	Missense	9-bp in-frame deletion	Missense	Missense	Missense	Missense	Missense
Polyphen2	Probably damaging	Possibly damaging	NA	Probably damaging	Probably damaging	Possibly damaging	NA	Probably damaging	Benign[a]	Probably damaging	Possibly damaging	Possibly damaging
SIFT[b]	Damaging	Damaging	NA	Damaging	Damaging	Damaging	NA	Damaging	Damaging	Damaging	Damaging	Damaging
EVS	-	-	-	-	-	-	-	-	-	-	-	-
dbSNP	-	-	-	-	-	-	-	-	-	-	-	-
In house exomes	-	-	-	-	-	-	-	-	-	-	-	-

NA : Not Available ; [a] Specificity : 0.44 ; Sensitivity : 0.98 ; [b] Median Information Content : 2.58. *SKI* genomic accession number: NC_000001.10; SKI transcript accession number: NM_003036.3.

Table S5: Sequences of primers used to sequence all coding *SKI* exons.

Amplicon	Forward Primer sequence	Reverse Primer sequence	PCR conditions
E1-1	TCCAGCGGCGGGACCCCTT	GATGGTCTCGCCTTCCAGTA	PCR Touchdown 60-50°C 10+30 cycles Standard Taq GC-Rich protocol (Roche)
E1-ATG	ATGCCCATGACTTTGAGGAT	GATGGTCTCGCCTTCCAGTA	PCR Touchdown 65-55°C 10+30 cycles Standard Taq Phusion GC-Rich protocol (Thermo)
E1-2	CAGGAGGCCTACAAGAAGGA	GGCTGCTGTAGAGCTCGG	PCR fixed Tm 56°C 40 cycles Standard Taq GC-Rich protocol (Roche)
E1-3	CTCATCACCAAGACGGACG	TCGGAGACCAGAGCCTGTAG	PCR Touchdown 60-50°C 10+30 cycles Standard Taq Phusion GC-Rich protocol (Thermo) DMSO 3%
E2	AGTGCATGGGGCTCTGACT	CAAGGAGAAGGGCCCAGTA	PCR Touchdown 65-55°C 10+30 cycles Taq Gold (Applied)
E3	GGGACATGAAGTGGCTTGTT	ACCCAGCCTGCAGAAACAT	PCR Touchdown 65-55°C 10+30 cycles Taq Gold (Applied)
E4	GAGCACACCTAGAGCGTTCC	AGGGAGGAGGCACAGAAAG	PCR Touchdown 65-55°C 10+30 cycles Taq Gold (Applied)
E5	CGTCTCCCTGGTGTGGAG	GTTCACCTGGTGCAGGCT	PCR Touchdown 65-55°C 10+30 cycles Taq Gold (Applied)
E6	ATGGTGAGGGGTGTGCTG	CTGCTCCAAGGCCTTTCC	PCR Touchdown 65-55°C 10+30 cycles Taq Gold (Applied)
E7	TGTCCTAGCAGGTGGAGGAG	TCTGAATTTCAAGTCTCCTTACTGG	PCR Touchdown 60-50°C 10+30 cycles Standard Taq GC-Rich protocol (Roche)

E: Exon

Overview of the Supplemental items

DISCUSSION

Ce travail de thèse visait à identifier des bases moléculaires et physiopathologiques de syndromes avec anomalies du développement et déficience intellectuelle. Deux approches complémentaires ont été envisagées : I) d'une part, des études de cas issus d'une cohorte de patients porteurs de DI, suivis à la consultation de génétique du CHU de Dijon, puis extension à partir d'une cohorte internationale. Les patients recrutés étaient porteurs de microréarrangements chromosomiques candidats pour la DI non rapportés dans la littérature. II) D'autre part, l'identification des bases moléculaires du syndrome de Shprintzen-Goldberg à partir d'une cohorte internationale homogène de patients.

Les résultats concernant la première partie de la thèse ont été obtenus grâce à de très étroites collaborations. D'abord avec les équipes locales de neuropsychologues, orthophonistes qui ont réalisé les évaluations cognitives, mais aussi avec les radiologues et médecins nucléaires ayant produit les imageries cérébrales anatomiques et fonctionnelles. Cette approche multidisciplinaire et transversale a permis une caractérisation indépendante et globale des patients. Une systématisation du phénotypage poussé de patients avec DI devrait permettre de séparer des groupes homogènes de patients présentant soit des anomalies génétiques communes, soit des manifestations phénotypiques communes. Ainsi, et comme dans d'autres domaines du neurodéveloppement, cette approche permettrait une meilleure compréhension des bases physiopathologiques de la DI. Le projet de Fédération Hospitalo-Universitaire TRANSLAD, soutenu par le CHU de Dijon et coordonné par le Pr Olivier-Faivre sera un modèle de prise en charge pluridisciplinaire et transversal des anomalies du développement avec DI.

Pour plusieurs des cas rapportés dans cette thèse, il a été possible de proposer des stratégies de prise en charge personnalisées. Dans le cas de la microdélétion 12p13.33, une rééducation orthophonique dédiée au traitement du CAS a été discutée et dans le cas de la microdélétion comprenant *SALM1/LRFN2* une remédiation neuropsychologique par entrainement de la mémoire de travail a

été conseillée. Dans ce dernier cas, une thérapeutique ciblée a même pu être discutée. Ces approches transversales et innovantes de diagnostics et de prises en charge sont cependant limités par le manque de personnel formé et/ou dédié à ces évaluations longues et spécialisées.

Le recrutement de patients supplémentaires a été possible grâce à l'existence de bases de données publiques. Le réseau national Achropuce et surtout la base de données de DECIPHER sont des outils formidables pour regrouper des données génétiques cliniques, cytogénétiques et bientôt moléculaires. Les nouvelles approches diagnostiques de génétique génèrent une masse de données importantes concernant chaque patient : en moyenne 3-5 CNV rares, bientôt 20 à 30 variants rares. L'utilisation de bases de données intégratives est le moyen actuel le plus performant d'abord pour séparer ce qui est pathogène des variations physiologiques et aussi pour préciser les conséquences d'une variation génétique. Des projets de grande envergure tels que le DDD (www.sanger.ac.uk/research/-areas/humangenetics/ddd/), le Gencodys (www.gencodys.eu) permettront une accumulation de données génétiques permettant de préciser le rôle chaque variation génétique. L'équipe GAD envisage le recrutement d'un chercheur spécialisé dans la physiologie neuronale. Avec son aide, nous espérons développer des outils de biologie cellulaire adaptés aux questions posées par certains patients vus en consultation.

En 2011, plus de 450 gènes étaient identifiés comme impliqués dans la DI. On estimait que 400 gènes étaient impliqués dans des formes de DI syndromique et environ 50 gènes dans la DI isolée (37). Depuis, le nombre de ces gènes est en perpétuelle augmentation grâce à l'utilisation du SHD. Des revues récentes ont fait état des connaissances génétiques sur les étiologies de DI (30,37). Cependant, les concepts évoluent rapidement et les données s'accumulent en masse. Le projet gene2synapse du Pr Rouleau de l'université de Montréal, débuté en 2008, est assez emblématique de cette période charnière. Il a fallu plus de 2 ans de travail à plusieurs personnes pour séquencer les premiers gènes du projet, ayant notamment conduit à l'identification de *SYNGAP1* dans la DI non syndromique (38). L'implantation du SHD a entraîné une réorientation immédiate du projet, permettant un achèvement bien plus rapide (39).

Les données génétiques à présent disponibles posent la question du spectre clinique associé aux mutations de gènes associés initialement à une pathologie neuro-comportementale particulière. Par exemple, le gène *SYNGAP1* a été rapporté initialement dans la DI sévère non syndromique (38). Récemment, ce gène a été retrouvé muté chez des patients porteurs d'encéphalopathies épileptiques, et d'autismes (40,41). A l'inverse, le gène *STXBP1*, initialement impliqué dans les encéphalopathies épileptiques infantiles précoces, a été retrouvé muté chez des patients avec DI isolée (42,43). Bien que ces phénotypes soient cliniquement sévères, l'évolution clinique semble difficile à prédire avec la seule information de l'élucidation de la cause génétique. Depuis 2 ans environ, les résultats de SHD démontrent les chevauchements phénotypiques provoqués par des gènes identiques. Dans le domaine des épilepsies, le consortium Epi4K a inclus 4000 patients atteints dans le but d'étudier les causes de ces pathologies par SHD. La première sous-population étudiée était constituée de 500 cas d'encéphalopathies épileptiques. Un SHD ciblé de 65 gènes d'épilepsies connus et candidats a permis le diagnostic 10% de patients. Parmi les gènes retrouvés mutés, 2 des 5 les plus fréquents ont été rapportés mutés chez des patients avec une DI isolée : *STXBP1* et *SYNGAP1* (40). De tels résultats avaient été rapportés avec les gènes de prédisposition à l'autisme (44). L'identification récurrente de gènes similaires dans des phénotypes différents a conduit à définir un spectre global de pathologies neurodéveloppementales.

Dans ces cohortes hétérogènes de patients, les gènes identifiés correspondent aux regroupements fonctionnels de gènes codants pour des facteurs synaptiques ou des facteurs de transcription de la différenciation neuronale. Considérant ces données, nous avons fait une demande de financement pour introduire en diagnostic le SHD-E dans les encéphalopathies épileptiques et DI profondes, en raison de la demande de conseil génétique majeure de ces familles. A mesure de l'accumulation des données de corrélations génotypes-phénotypes, le conseil génétique sur la pathologie diagnostiquée sera amélioré. L'hétérogénéité génétique importante dans la DI complique la constitution de cohortes locales de patients génétiquement homogènes. Au niveau national, chaque centre va pouvoir développer ses stratégies de SHD et les centres référents du diagnostic moléculaire de gènes de DI risquent à terme de perdre le ‘’monopole’’ de l'identification des mutations alors qu'ils bénéficient d'une expérience importante pour leur interprétation.

La seconde partie du travail présenté décrit la mise en évidence du gène en cause dans le syndrome de Shprintzen-Goldberg (SGS) à partir d'une cohorte homogène de patients. Une cohorte internationale de plus de 100 patients porteurs d'un syndrome marfanoïde avec DI a été constituée par le Professeur Olivier-Faivre. Au sein de cette cohorte, 19 patients présentaient un phénotype compatible avec un SGS. En particulier, 2 familles semblaient plus informatives. Dans la première, le SGS ségrégait de manière autosomique dominante sur 3 générations et dans la seconde, une récidive dans la fratrie était suggestive soit d'une mosaïque parentale soit d'un mode de transmission récessif autosomique. La stratégie de SHD-E s'est concentrée sur ces familles informatives pour identifier les variants candidats. Deux trios de cas sporadiques/parents ont également été envoyés en SHD-E. Un total de 6 patients atteints a été séquencé pour permettre la mise en évidence du gène *SKI*.

L'identification du gène *SKI* a été réalisée dans un contexte compétitif et a pu être publié online quelques jours après l'article de l'équipe dirigée outre-atlantique par Hal C Dietz et ayant identifié la quasi totalité des gènes de syndromes marfanoïdes jusque là. Leur publication apporte une démonstration fonctionnelle élégante de l'implication de *SKI* dans la voie du TGF béta et dans le SGS (36). Le séquençage du gène *SKI* est développé dans le laboratoire et de nouveaux patients ont pu être recrutés et séquencés. Des envois d'ADN de patients provenant du monde entier nous sont parvenus. Les données cliniques recueillies et confrontées aux résultats de génétique moléculaire permettront de déterminer le spectre clinique des manifestations cliniques du SGS, les critères diagnostiques faisant retenir le test moléculaire…

Il reste encore un grand nombre de gènes à identifier dans les syndromes marfanoïdes et bien que la CGH array soit d'un grand apport diagnostique, le SHD-E apportera sans doute de nouveaux éléments de réponse (45). De nombreux autres patients porteurs de syndromes marfanoïdes avec DI sont en cours de SHD-E et seront analysés au sein du laboratoire. La stratégie de priorisation fonctionnelle par appartenance à la voie du TGF béta pourra être envisagée comme un premier filtre de variants rares.

L'identification de *SKI* dans le syndrome de Shprintzen-Goldberg a été la première identification de gène de l'équipe GAD. Depuis, environ 20 gènes ont été identifiés dans des cadres physiopathologiques différents dont plusieurs dans la DI syndromique.

CONCLUSIONS ET PERPECTIVES

Cette thèse présente des résultats issus d'approches complémentaires, utilisant deux techniques pan-génomique disponibles actuellement : la CGH-array et le SHD-E.

Un groupe de patients issus de la consultation de génétique du CHU de Dijon a été étudiée par CGH-array. Les microréarrangements chromosomiques non publiés et contenant des gènes candidats codants pour des protéines synaptiques ou des facteurs de transcription promouvant la différenciation neuronale ont été sélectionnés. Les patients porteurs ont été évalués de manière globale et pluridisciplinaire. Des patients additionnels ont pu être recrutés au niveau international. Cette démarche a permis la description de 3 syndromes microdélétionnels et la caractérisation de 2 nouveaux gènes de DI. Les données clinico-biologiques apportées par l'étude de ces cas ont permis d'adapter la prise en charge de ces patients.

Ensuite, l'étude par SHD-E d'une cohorte de patients issus d'une collaboration internationale a permis d'identifier le gène responsable du SGS. Malgré l'apprentissage de l'interprétation de données d'exome et des difficultés techniques liées au type de capture utilisée, l'équipe a pu identifier les mutations de *SKI* chez les propositus. La cohorte de réplication séquencée a conduit à la découverte de mutations localisées dans un hot spot mutationnel chez 18 des 19 patients typiques, contrastant avec l'absence de mutation chez d'autres patients avec syndrome marfanoïde autre. Cette découverte confirme l'implication d'un gène de la voie du TGF béta dans le SGS, ce qui est consistant avec les données disponibles sur les autres syndromes marfanoïdes.

Les perspectives de ce travail sont représentées par la poursuite de la caractérisation d'anomalie de CGH rares par des évaluations pluridisciplinaires. Nous avons deux projets collaboratifs en cours concernant des familles de patients

porteurs d'anomalies cytogénétiques intragéniques (impliquant les gènes *DISC1* et *MYT1L*). Le développement en routine diagnostique du SHD-E dans les encéphalopathies épileptiques et DI profondes permettra de confirmer l'intérêt de cette technique dans ce spectre de pathologie réputé hétérogène. Enfin, un travail de corrélation génotype-phénotype nécessaire est en cours sur la cohorte de patients SGS.

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