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Epigenetics

ISSN: 1559-2294 (Print) 1559-2308 (Online) Journal homepage: http://www.tandfonline.com/loi/kepi20

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To cite this article: Rita Genesio, Daniela Melis, Sole Gatto, Antonella Izzo, Valentina Ronga, Gerarda Cappuccio, Ambra Lanzo, Generoso Andria, Maurizio D'Esposito, Maria R. Matarazzo, Anna Conti & Lucio Nitsch (2011) Variegated silencing through epigenetic modifications of a large Xq region in a case of balanced X;2 translocation with Incontinentia Pigmenti-like phenotype, Epigenetics, 6:10, 1242-1247, DOI: 10.4161/epi.6.10.17698

To link to this article: http://dx.doi.org/10.4161/epi.6.10.17698

Epigenetics 6:10, 1242-1247; October 2011; © 2011 Landes Bioscience

Variegated silencing through epigenetic modifications of a large Xq region in a case of balanced X;2 translocation with Incontinentia Pigmenti-like phenotype

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Key words: X;autosome translocation, epigenetic silencing, incontinentia pigmenti, IKBKG, PEV-like heterochromatization

Molecular mechanisms underlying aberrant phenotypes in balanced X;autosome translocations are scarcely understood. We report the case of a de novo reciprocal balanced translocation X;2(q23;q33) presenting phenotypic alterations highly suggestive of Incontinentia Pigmenti (IP) syndrome, a genodermatosis with abnormal skin pigmentation and neurological failure, segregating as X-linked dominant disorder. Through molecular studies, we demonstrated that the altered phenotype could not be ascribed to chromosome microdeletions or to XIST-mediated inactivation of Xq24-qter. Interestingly, we found that the Xq24-qter region, which translocated downstream of the heterochromatic band 2q34, undergoes epigenetic silencing mediated by DNA methylation and histone alterations. Among the downregulated genes, we found the *inhibitor of kappa light polypeptide gene enhancer in B cells, kinase gamma* (*IKBKG/NEMO),* the causative gene of IP. We hypothesize that a mosaic functional nullisomy of the translocated genes, through a Position Effect Variegation-like heterochromatization, might be responsible for the proband's phenotypic anomalies. Partial silencing of *IKBKG* may be responsible for the skin anomalies observed, thereby mimicking the IP pathological condition. In addition to its clinical relevance, this paper addresses fundamental issues related to the chromatin status and nuclear localization of a human euchromatic region translocated proximally to heterochromatin. In conclusion, the study provides new insight into long-range gene silencing mechanisms and their direct impact in human disease.

Introduction

Balanced X;autosome translocations [t(X;A)] occur with a frequency of 1/30,000 live births.[1] The estimated risk for developing an aberrant phenotype is roughly 6.7%; however, microdeletions at the breakpoint level have been discovered and characterized in only about half of these cases. For the remaining anomalies associated with t(X;A), very few causes have been hypothesized and/or demonstrated. One of the possible mechanisms is ascribed to the XIST-mediated inactivation of the autosomal region translocated to the derivative X chromosome.[2] Another process, called Position Effect Variegation (PEV), has been described, mainly in *D. melanogaster*,[3] when genes are positioned within proximity of constitutive heterochromatin. Gene expression results in variegated silencing, mediated both by chromatin remodeling and RNA silencing.[4,5] This phenomenon is not well understood in humans. Finally, as chromosome position in the nucleus may affect gene expression, translocation of a gene to the vicinity of the nuclear periphery may likewise influence its expression.[6]

The process of X inactivation does not occur randomly in t(X;A). The selection of cells more suitable for the proliferation causes a skewed inactivation. Indeed, the abnormal X is more frequently inactivated in unbalanced translocations, whereas the normal X is inactivated in cases of balanced translocations.

We have analyzed X inactivation patterns and chromatin remodeling in a case of de novo reciprocal balanced translocation X;2(q23;q33) presenting phenotypic alterations (such as medium-severe developmental delay, altered pigmentation and facial dysmorphisms). We proposed the following three hypotheses to explain the phenotype: (1) the presence of microdeletions/microduplications at critical breakpoint regions; (2) X inactivation of the derivative X causing both functional disomy of Xq24-qter and monosomy of 2q35-qter; (3) abnormal silencing of the translocated Xq24-qter region.

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Submitted: 06/28/11; Accepted: 08/10/11
DOI: 10.4161/epi.6.10.17698

Array comparative genomic hybridization (a-CGH) together with X inactivation and replication timing studies excluded the first two hypotheses. Indeed, we demonstrated that genes mapping to the translocated Xq24-qter in the proband's skin fibroblasts are downregulated. Therefore, we evaluated whether the silencing of X translocated genes might be chromatin-mediated. Among the silenced genes, *IKBKG/NEMO* gene (inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase gamma), mapping to Xq28, is particularly interesting because mutations impairing its function cause altered pigmentation and mental retardation in patients affected by Incontinentia Pigmenti (IP; OMIM 308310).[7] Based on these findings, we hypothesize a functional correlation between changes in *IKBKG* gene expression and altered phenotype.

Results

Xq24-qter translocated region shows variegated silencing of gene expression. High resolution karyotype and DNA FISH confirmed the 46,XX,t(X;2)(Xpter->Xq23::2q35->2qter;2pter->2q34::Xq24->Xqter) de novo translocation in all the analyzed fibroblasts and lymphocytes of the proband (**Fig. 1A**).

Copy number analysis by Affymetrix SNP array 6.0 at 10 Kb resolution did not reveal any variation for chromosomes 2 and X, demonstrating that no deletion or duplication occurred at breakpoints. Only polymorphic variants were detected in the entire genome, thus excluding the occurrence of other rearrangements (data not shown). Fluorescent BrdU assay, combined with whole chromosome 2 FISH (wcp2), demonstrated that the normal X chromosome was late replicating and, therefore, presumably inactivated in 100% metaphases from lymphocytes, thus excluding autosome inactivation (**Fig. 1B**). The same analysis, performed in skin fibroblasts, showed that the Xq region translocated to chromosome 2q was late replicating in 60% metaphases, suggesting that, at least in skin fibroblasts, gene silencing occurs in this region (**Fig. 1B**).

Since histone H3K27me3 marks the inactive X chromatin state, we analyzed it by immunofluorescence. Results confirmed the partial inactivation of the translocated Xq24-qter region in fibroblasts, as 60% of the proband nuclei showed a double hybridization spot (**Fig. 1C**).

Expression analysis of translocated X region in proband vs. controls showed evidence for downregulation. qRT-PCR analysis revealed downregulation of eight genes located in the translocated Xq24-qter trait (*WDR44, IL13RA1, ELF4, MCF2, DKC1, G6PD, IKGKB* and *VBP1).* Conversely, the *TSPAN6* gene (located in the Xq22 band upstream the translocation) appears to be normally expressed; therefore, the aberrant silencing is specific for the translocated genes (**Fig. 1D**).

***IKBKG* promoter is hypermethylated on both alleles and is depleted in H3K4me3.** The downregulated *IKBKG* gene data appears to be the most interesting, because the proband's clinical signs were those typical of IP patients.[7] Thus, we analyzed the epigenetic features of the *IKBKG* promoter[8] (**Fig. 2A**). We examined the CpG island DNA methylation pattern by bisulfite analyses[9,10] of patient's and control's fibroblasts. Most of the CpGs were hypermethylated in roughly 50% of the clones in control cells, representing the inactivated allele, while the other half of clones was hypomethylated representing the active X allele (**Fig. 2B**). These data indicate that the experiment was not biased for either methylated or unmethylated strands at this locus. The same analysis in patient's cells revealed a high level of methylation in all clones, suggesting an aberrant DNA hypermethylation of both *IKBKG* alleles. To widen the study of *IKBKG* promoter epigenetic features we performed ChIP assays for H3K4me3 (**Fig. 2C**). We analyzed the genomic region surrounding the *IKBKG* promoter, amplifying by qRT-PCR the CpG island (**Fig. 2A**, #2) and two regions 3 Kb upstream and downstream (**Fig. 2A**, #1 and #3 respectively). As a negative control, we amplified an intergenic region 13 Kb downstream of the start site (**Fig. 2A**, #4). We found significant H3K4me3 enrichment at all CpG islands in control cells and a much less enrichment in patient's cells. Neither upstream nor downstream regions were enriched in either control or patient cells (as well as the furthest region in both cell lines). As this histone modification is specifically correlated to active chromatin domains, we can infer that in control female cells the H3K4me3 enrichment is due to the transcribed active X allele. The low H3K4me3 association in patient's cells suggests that the *IKBKG* translocated allele loses this permissive mark and acquires characteristic repressive chromatin status.

Following the same hypothesis, we extended the ChIP analysis to the downregulated genes *WDR44, ELF4* and *DKC1*, located in Xq24, Xq26.1 and Xq28, respectively (**Fig. 3A**). For each gene we analyzed H3K4me3 enrichment in the region spanning the CpG island and a non-coding sequence (**Fig. 3A**, #2, 3, 6 and #1, 4, 5 respectively). We found significant H3K4me3 enrichment at CpG islands in control versus patient cells, while no enrichment was found in non-coding regions.

It is well known that chromosome position in the nucleus may affect gene expression.[6,11] We next examined the nuclear positioning of the translocated region Xq24-qter. We performed interphase dual-DNA-FISH using wcpX and wcp2 (**Fig. 3B**). In fibroblasts, the translocated Xq24-qter is located peripherally in the 80% of observed nuclei, suggesting that gene silencing at the translocated region is associated to its nuclear compartmentalization.

Discussion

The present study analyzes the X inactivation pattern and chromatin remodeling in a case of de novo reciprocal balanced translocation X;2(q23;q34), occurring in a female with an IP-like phenotype. We showed a variable downregulation of several genes mapping to the Xq24-qter translocated region. To unravel the molecular mechanisms responsible for the downregulation of these genes, the epigenetic features of *IKBKG* promoter were analyzed. In patient's cells, the *IKBKG* promoter is hypermethylated, depleted in H3K4me3 and enriched in H3K27me3, indicating that DNA methylation and histone changes mediate the aberrant gene repression we report.

It is known that *IKBKG* mutations are responsible for IP.[7] The *IKBKG* gene product affects the activation of the transcription nuclear factor κB (NFκB). The latter is essential in immune,

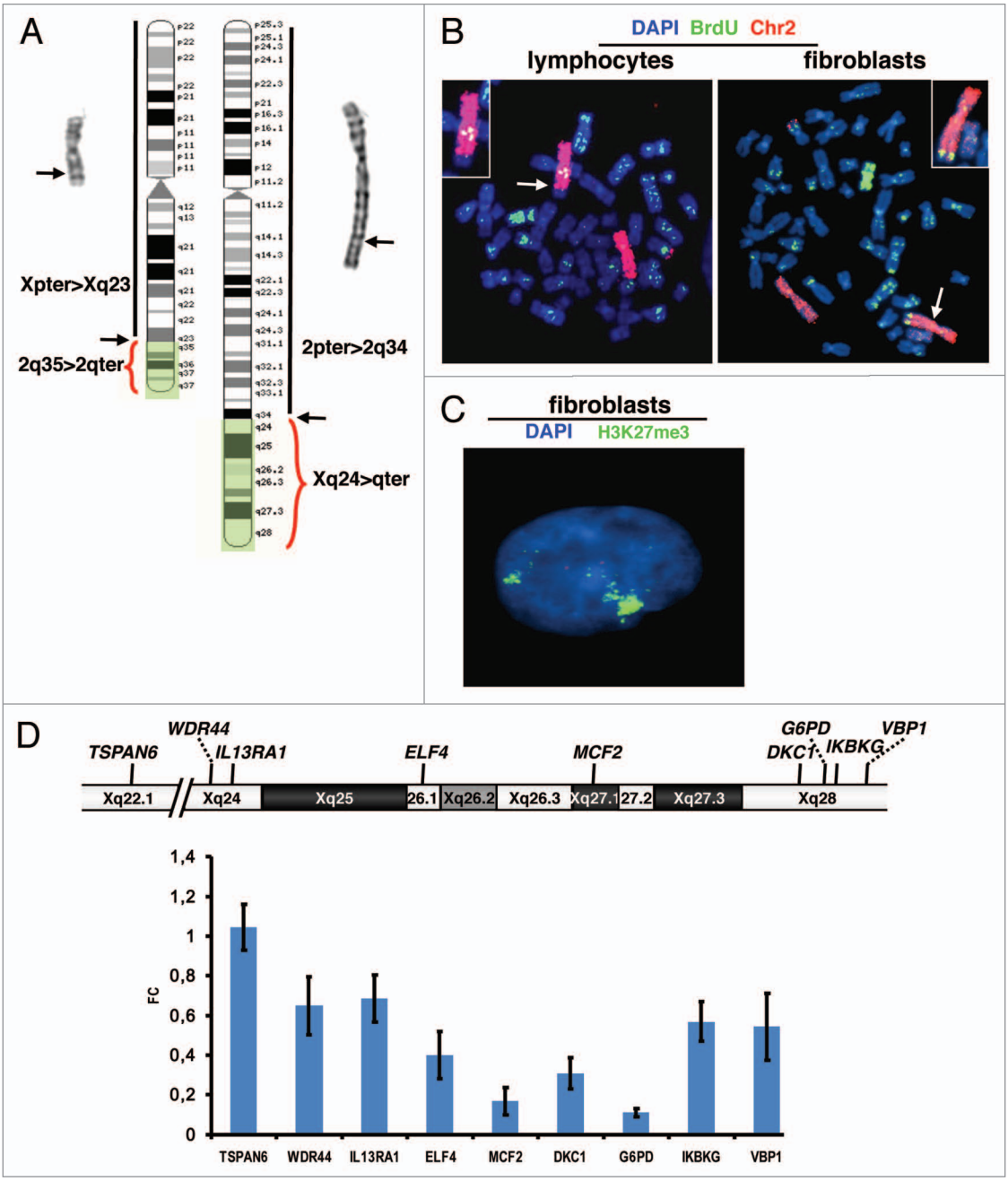

Figure 1. (A) Derivative chromosomes from the de novo translocation 46,XX,t(X;2)(Xpter->Xq23::2q35->2qter;2pter->2q34::Xq24->Xqter) and their schematic representations. The arrows indicate the breakpoint. (B) Immuno-FISH of lymphocytes and fibroblasts nuclei of the proband to detect late-replicating chromatin. BrdU incorporation is marked in green and the whole chromosome 2 painting is red. Derivative chromosomes 2 pointed by the white arrows are enlarged in the small inset. The normal X chromosome is late-replicating (green) in 100% analyzed cells (right and left parts). The Xq24-qter region translocated to chromosome 2 is also late replicating (green) in 60% of the metaphases only in fibroblasts (right part). (C) Immunofluorescence of the proband fibroblasts with anti-H3K27me3 showing enrichment on the inactive X chromosome. The second hybridization spot corresponds to the translocated Xq24-qter region. (D) Gene expression analysis by qRT-PCR of eight genes mapping in the Xq24-qter and one gene mapping outside the translocation (Xq22.1).

inflammatory and apoptotic pathways and acts by protecting cells from apoptosis in response to tumor necrosis factor-alpha. In the skin, NFκB regulates cell growth and apoptosis in the stratified epithelium.[12] It may also play a role in the maintenance of blood vessel architecture.[13] The proband phenotype is highly suggestive of the syndrome arising from *IKBKG* insufficiency. IP is a genodermatosis with abnormal skin pigmentation and neurological failure, is prenatally lethal in males and segregates as an X-linked dominant disorder. In affected females, it causes highly variable abnormalities of the skin, hair, nails, teeth, eyes and central nervous system. Based on our results, *IKBKG* down-regulation in fibroblasts could explain some clinical symptoms in

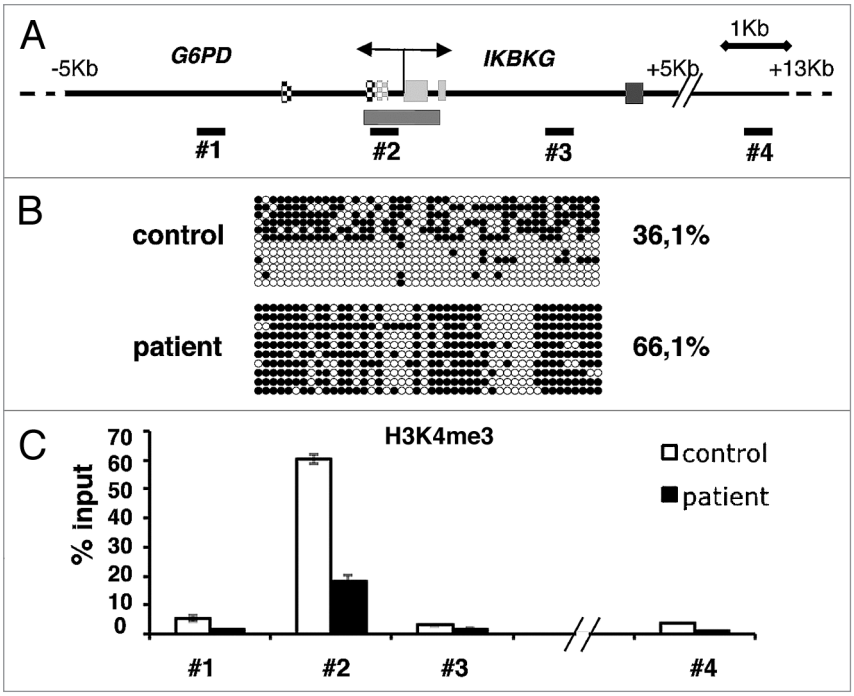

Figure 2. Epigenetic characterization of *IKBKG* promoter. (A) Representative scheme of *IKBKG* gene regulatory region. Grey boxes represent gene promoters and exons, the CpG island is in grey and the analyzed regions by ChIP assay are shown in black with serial labels (#1, #2, #3, #4). (B) DNA methylation is of IKBKG CpG island by bisulfite genomic sequencing comparing skin fibroblasts from the patient and the control. Circles indicate the methylation status of 46 cytosines from nucleotide positions-42 to 409 in *IKBKG* CpG island: filled circles, methylated CpGs; open circles, unmethylated CpGs. Each row of circles corresponds to one clone; at least 10 clones were analyzed for each cell type. Percentage of total CpG methylation for all samples is reported. (C) Histone H3K4me3 enrichment of *IKBKG* genomic region. After ChIP assay four regions (black bars) were amplified by qRT-PCR: the CpG-rich region (#2), two regions positioned 3 Kb upstream and downstream (#1 and #3, respectively) and an intergenic region, 13 Kb downstream of the start site as negative control (#4).

the proband's skin. The reduced (but still detectable) H3K4me3 enrichment in the *IKBKG* promoter confirms that the translocated allele is not completely silenced, but still retains some function (and probably does not present the same inactivation pattern in all the cells of the organism). The epigenetic analysis was extended to other downregulated genes mapping to the translocated Xq24-qter. Again, we demonstrated a chromatin-mediated gene silencing of the region.

This suggests that the gene regulation of the entire translocated portion is possibly affected by the repositioning of the translocated X tract in proximity of the chromosome 2q34 band, which is a G-positive heterochromatic region, through a PEV-like phenomenon. It is known that G-positive bands include gene-poor regions, even if it has been reported that they comprise tissue-specific genes that are transcribed only in selected cell types.[14,15]

The propagation of the repressive chromatin structure might occur in a discontinuous ("hopping") fashion, explaining why certain genes are not completely silenced. The mechanism by which the repositioning close to heterochromatic bands might cause gene silencing is not yet unraveled. One hypothesis is that it should be due to local differences in the binding affinity of heterochromatin complex proteins, determined by DNA sequence or epigenetic marks.[16]

Further profiling of the repressive histone modifications, including H3K9me3 and H4K20me3, in the translocated X tract and the nearest heterochromatin region could help to gain insight into this propagation mechanism.

Moreover, the peripheral nuclear position might cause the inactivation of the translocated portion of the active X. Both constitutive and facultative heterochromatin are typically enriched at nuclear periphery. Balanced chromosomal rearrangements may affect gene expression in the translocated portions by repositioning the derivative chromosomes.[17] In addition, relocation of human chromosomes can alter gene expression, although not every locus tethered to the periphery is silenced.[18,19] Additionally, lamin proteins might mediate epigenetic mechanisms involved in regulating gene silencing and heterochromatic spreading in PEV.[20]

In summary, the data presented in this work support the view that epigenetic silencing is a direct or indirect cause of mosaic functional nullisomy of translocated genes, leading to the phenotypic anomalies of the proband. Specifically, partial silencing of a key translocated gene, *IKBKG*, may be responsible for the phenotypic anomalies observed, mimicking the IP pathological condition. Further studies of the other downregulated genes should help clarify the complex phenotypic traits of the proband. Beyond its clinical relevance, this study contributes to our understanding of long-range gene silencing mechanisms (and their impact in human disease), by analyzing the epigenetic regulation of a euchromatic region proximal to a heterochromatin.

Material and Methods

Case report. The proband was a female, second child from non-consanguineous parents. She was born by C-section after an unremarkable pregnancy. APGAR scores were 5/7. A diagnosis of hemorrhagic hypoxic-ischemic encephalopathy was proposed.

She was referred to Pediatrics Clinical Genetics Unit of the University Federico II when 5 years old. The following clinical features were recorded: angiomas extending from the frontal hairline to the glabellar region and, in the nuchal region, a widespread hyperpigmentation on the right side of thorax and of abdomen and on the back of thigh and hip, cafè-au-lait spots, spear hypopigmentation on trunk, convergent strabismus, high-arched palate, dysmorphic facial features (small forehead, reduced bitemporal diameter, bilateral epichantal folds), scalp alopecia, distinctive hand (brachydactyly, tapering fingers, cutaneous

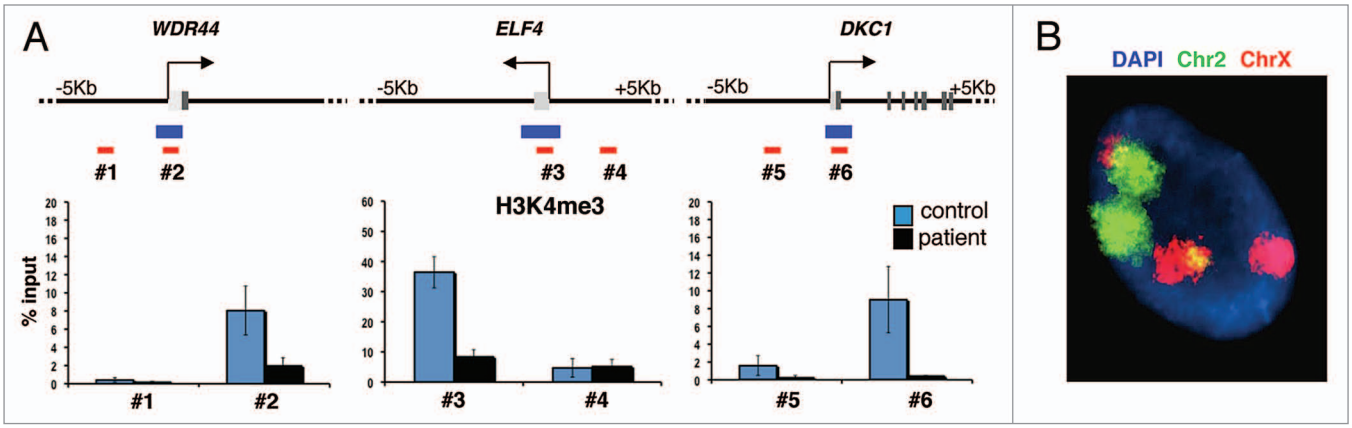

Figure 3. Chromatin structure and position of the translocated Xq24-qter. (A) H3K4me3 enrichment in three genomic regions (Xq24, Xq26.1 and Xq28) containing the three downregulated genes *WDR44, ELF4* and *DKC1*, respectively. For each of these genes we analyzed two regions (red bars), spanning the CpG island (blue bar) on the start site (#2, #3 and #6) and a non-coding region (#1, #4 and #5). Each result was calculated from two independent ChIP experiments. (B) Interphase dual DNA-FISH using both whole chromosome paint of X (red) and 2 (green) on proband's fibroblast nuclei showing the nuclear position of the translocated Xq24-qter region.

syndattily III-IV fingers, fetal pads) and feet (brachydactyly and cutaneous syndattily of II-III toes), bilateral clinodactily of the V finger, knock knees, right clubfoot, Achilles's tendon retraction. The ophthalmologic evaluation revealed a pale optic disc.

Neuropsychological evaluation: verbal IQ = 69, performance IQ = 49 and total IQ = 53, consistent with a mild mental retardation. Intellectual and executive functions, motor speed and strength were moderately retarded. Brain MRI showed asymmetrical lateral ventricular enlargement with left predominance. Trigoni were dilated and with festonated borders. The corpus callosum was prominently slimmed. Auditory brainstem response test showed a defect in the central conduction.

When she was 15 years old, new clinical elements were identified: skin pigmentation abnormalities including hypopigmented streaked areas on the anterior thorax, splash-like hypopigmented spots on anterior and posterior region of neck (**Fig. S1A**), other hypopigmented zones on left hemiabdomen and hemithorax, posteriorly on left leg (**Fig. S1B**), angiomas in the frontal, nucal and external thigh region, cafè-au-lait spots on inguinal folds and xifoombelical line, achantosys nigricans on the back of the neck, small and merging vesiculobullous skin eruptions on the right hip, few of them showing hyperpigmentation (**Fig. S1C**). All these features suggested a diagnosis of IP.

Karyotype. Peripheral blood from the proband was cultured for 72 h in Chromosome Medium (Euroclone). A primary culture of skin fibroblasts was set up in tissue culture plastic flasks (Falcon) using Chang medium A+C (Irvine Scientific). Metaphase chromosomes were analyzed by standard G-banding technique.

Comparative genomic hybridization (aCGH). CGH by Affymetrix SNP array 6.0, at 10 Kb resolution was performed on proband DNA according to Affymetrix protocols (www.affymetrix.com).

DNA FISH, immuno-FISH and immunofluorescence. FISH with whole-chromosome-probes (wcp) for chromosomes X and 2 was performed according to Vysis protocols. Late-replicating chromatin was detected as previously described in reference 21. Lymphocytes and fibroblasts of the patient were exposed to BrdU for 5 h prior to colcemid addition. The mouse monoclonal anti-BrdU has been detected with anti-mouse IgG-FITC, post-fixed in 4% paraformaldehyde and hybridized with the whole chromosome paint 2 (wcp2). Probe preparation, hybridization and post-hybridization washes were performed according to manufacturer's instructions. Slides were counterstained with DAPI/Antifade (Vector) and images captured using Olympus BX51 fluorescent microscope with Cytovision Software v.3.7. All FISH experiments were carried out analyzing about 200 nuclei for each cell type.

Immunofluorescence with anti-H3K27me3 (Abcam) was performed according to standard protocols.[22] Ethidium bromide (1 mg/ml final concentration; BDH) and colcemid (KaryoMax Colcemid solution, 0.1 mg/ml final concentration; GIBCO) were added to the cell culture 2 h before cell harvesting to increase the proportion of extended metaphases. Trypsinized cells were rinsed in PBS and swollen in hypotonic solution (75 mM KCl) for 10 min at room temperature (RT). Cells were permeabilized by addition of KCM-T (10 mM Tris-HCl, pH 8.0, 120 mM KCl, 20 mM NaCl, 0.5 mM EDTA, 0.1% Tween 20) with 0.1% Triton X-100 and fixed in 4% paraformaldehyde in PBS, pH 7.4. Mouse monoclonal anti-H3K27me3 was diluted in KCM-T (1:200), applied for 2 h at RT and incubated with secondary antibody Alexa Fluor 488 goat-anti mouse (Molecular Probes) for 1 h at RT. Slides were counterstained with DAPI stain solution and analyzed as previously described.

Quantitative real-time PCR (qRT-PCR). Total RNA from proband and control skin was extracted using TRIzol reagent (Gibco/BRL Life Technologies, Inc., Gaithersburg, MD) and was reverse-transcribed using iScript cDNA Synthesis kit (Biorad). Real-time PCR was performed using iQ Supermix SYBR Green 2X (Biorad) on the Bio-Rad iCycler according to the manufacturer's protocols. PCR reactions were performed in triplicate. The primers (MWG Biotech, Ebersberg, Germany)

used for amplification are listed in **Table S1**. Fold change was calculated with $2^{-\Delta\Delta Ct}$ method using GAPDH as reference gene.

Bisulfite genomic sequencing (BGS). Bisulfite genomic sequencing was performed according to Epitect Bisulfite kit instruction (Qiagen). PCR primers for bisulfite analysis are listed in **Table S1**. Amplified DNA fragments were extracted from agarose gel and cloned by using TA cloning kit (Invitrogen, Carlsbad, CA). Sequencing of clones has been performed by using M13 forward primer (Primm Biotech).

Chromatin immunoprecipitation (ChIP). ChIP was performed as previously described in reference 23 and 24. Briefly, 5 x 10^6 cells were fixed with 1% formaldehyde. After cross-linking, chromatin was isolated and subjected to sonication, resulting in 200–1,000 bp DNA fragments. After immunoprecipitation with anti-H3K4me3 specific antibody (Upstate Biotech, 07-473), immunocomplexes were isolated by co-precipitation with protein A-Sepharose (Pharmacia). Anti-IgG was used as background control. The recovered DNA was measured and the immunoprecipitated DNA fragments were analyzed by quantitative Real Time PCR, by using SYBR Green quantitative PCR (iQ SYBR Green Supermix, Biorad) performed according to CFX96™ Real Time PCR Detection Systems. The enrichment of DNA was calculated in terms of % input = $2^{-\Delta Ct}$ x 100, where ΔCt (threshold cycle) is determined by $Ct_{IP\ sample}$ - Ct_{Input} and 100 refers to the input being 1% of the chromatin amount exposed to IP. The ChIP results derive from two independent experiments and Real Time PCR was carried out at least three times for each analyzed genomic region, in order to calculate the standard deviation, represented as the error bars.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We wish to acknowledge Dr. Mark Muller for critical reading and editing the manuscript. We also thank Gennaro Gaudioso for his technical help. We thank the patient and her family members for their participation in this study. This work was supported by grants from European Union (DISCHROM ITN 238242).

Note

Supplemental material can be found at:
www.landesbioscience.com/journals/epigenetics/article/17698

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