Inversion Region for Hypertension and Brachydactyly on Chromosome 12p Features Multiple Splicing and Noncoding RNA

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Abstract—Autosomal-dominant hypertension and brachydactyly (Online Mendelian Inheritance in Man 112410) is a prototype-translational research project. We used interphase fluorescent in situ hybridization and discovered complex rearrangements on chromosome 12p in 5 families but elucidated a common inverted region in the linkage interval. The inversion contains no known gene. However, we found 5 expressed sequence tags in databases. We used 5′- and 3′-Rapid Amplification of cDNA Ends PCR for elongation of the transcripts in phenotype-relevant tissue (fetal aorta, fetal brain, and fetal cartilage). We detected tissue-specific multiple splicing with different exon usage of 32 exons in the gene-related structure. These different transcripts lack both open reading frames and Kozak sequences. In vitro transcription/translation experiments did not identify any peptide-related molecules. We then performed quantitative RT-PCR to test for differential expression of the various spliced transcripts in the total fibroblast RNA of affected and nonaffected Turkish family members. Skin fibroblasts of affected individuals have a significantly increased proliferation rate compared with nonaffected individuals. Ten of 12 spliced exon combinations representing all of the spliced variants do not show a significantly different RNA expression rate. However, 2 RT-PCR products are exclusively expressed in nonaffected individuals. Both reverse transcription amplicons share 1 exon. This result is surprising because of the autosomal-dominant mode of inheritance of the trait. RNA secondary prediction of this single exon results in a stable stem-loop structure known to be essential for microRNA processing. We are pursuing the possibility of microRNA expression in affected patients that leads to complete down regulation of a spliced transcript. (Hypertension. 2008;51 [part 2]:426-431.)

Key Words: hypertension ■ genetics ■ Mendelian ■ chromosomal rearrangements ■ translational research

Translational research is a recently introduced neologism to emphasize the bench-to-bedside-and-back-again research process demanded of basic and clinician scientists, who focus on elucidating fundamental mechanisms of disease in patients who directly confront them. We embarked on such a project in 1994, when we first pursued autosomal-dominant hypertension with brachydactyly (Online Mendelian Inheritance in Man 112410), a condition initially described in the English-language literature by Bilginturan et al.1 Bilginturan et al1 introduced us to a large Turkish family residing on the Black Sea coast in northeastern Turkey. The family has since become a source of patients and friends as we work together with them. The mutual hope is that we can translate their condition into knowledge, which might be directly beneficial to them and to other patients with hypertension. All of the affected persons develop severe hypertension in childhood and, if left untreated, develop stroke by age 50 years. Affected persons also have type E brachydactyly and are ≈10 cm shorter than nonaffected persons. We mapped the gene locus to chromosome 12p.2

From Bedside to Laboratory Bench and Back Again, and Again, and Again, and Again
To elucidate the mechanisms contributing to the blood pressure elevation, we invited affected and nonaffected family members to our clinical research center in Berlin-Buch. We reasoned that salt-sensitivity would be likely, because all of the other Mendelian hypertension syndromes have involved sodium chloride homeostasis.3 We performed a standardized volume expansion and contraction protocol developed at Indiana University School of Medicine. Weinberger et al4–6 have verified the use and reproducibility of these maneuvers and assessed their long-term prognostic value. We found that affected persons were not salt sensitive.7
Furthermore, their plasma renin activity, plasma aldosterone, catecholamine values, renal artery morphology, and renal function were not different than in nonaffected persons. Thus, the routine clinical assessment suggested that the syndrome closely resembles essential hypertension, a state of affairs quite different from the other Mendelian forms of hypertension.

The linkage interval on the basis of our microsatellite markers was large (≈10 cM), and we were not aware of other families. However, a description in the literature of a Japanese child with a chromosome 12p deletion who also had type E brachydactyly and numerous other anomalies led to a collaboration that allowed us to map the deletion in the Japanese child. The overlapping segment from the deletion and our linkage interval reduced this interval by half. In the mean time, with keen detective work and help from colleagues in North America, we found 2 other non-Turkish families with the same syndrome.

In the mean time, the “bedside,” or perhaps the “patient-side,” aspects of the condition led us to arrange therapy for all of the affected members of the Turkish family. The family permitted us to perform a prospective, randomized, double-blind, crossover trial of 6 different regimen classes in affected persons at 2 doses for 4 weeks with a 2-week washout period between treatments. The study was performed with ambulatory blood pressure monitoring, humoral, and urine determinations. Hydrochlorothiazide, angiotensin-converting enzyme inhibition, β-blockade, calcium channel blockade, and central imidazole receptor inhibition were all similarly effective and lowered blood pressure ≈8 mm Hg compared with placebo. Renin, aldosterone, and electrolyte responses were as expected with the individual regimens. Thus, pharmacological phenotyping also supported the notion that affected persons had a hypertension closely resembling essential hypertension. This study was performed in northeastern Turkey and was logistically a challenge.

We sequenced all of the genes in the linkage interval and found no mutations. One particular challenge was the transcription factor L-Sox5. We were made aware of the fact that Sox5 has a long splice variant (L-Sox5), that the transcription factor regulates collagen II, and that L-Sox5 is expressed in the digit tips and vertebrae of developing mice. We sequenced the entire 500,000-bp L-Sox5 gene and used each encountered single nucleotide polymorphism for linkage disequilibrium mapping. Several single nucleotide polymorphisms showed recombinations, indicating that L-Sox5 lies immediately outside of our linkage interval.

Meanwhile, on the patient side, we followed a lead suggested by an altered baroreflex function in affected compared with nonaffected subjects on the basis of continuous beat-by-beat blood pressure and heart rate measurements. These changes caused us to pursue the idea that affected persons could exhibit neurovascular contact at the brain stem area involved in baroreflex regulation, which might result in hypertension. Jannetta et al first suggested this notion. Naraghi et al performed an early case-control study and, indeed, they convinced us to pursue this issue further. MRI was performed at the University of Trabzon, not far from where the family resides. Fifteen hypertensive subjects had neurovascular contact involving the posterior-inferior cerebellar artery and the ventrolateral medulla, whereas none of 12 normotensive family members had such structures. The challenge now was to transfer this hypothesis to the patients in terms of mechanisms.

We next arranged for the second clinical research center visit for affected and nonaffected family members in Berlin. This time, we performed extensive autonomic nervous system testing, including microneurography. We relied on complete autonomic ganglion blockade with trimethaphan. These studies showed that affected persons had, if anything, less active sympathetic nerve traffic to muscle than nonaffected persons. Nevertheless, their blood pressure response to phenylephrine was markedly accentuated and was not affected by ganglion blockade. Nonaffected persons were able to buffer blood pressure–elevating effects of phenylephrine, whereas affected persons could not. Thus, baroreflex blood pressure buffering was markedly impaired in affected, compared with nonaffected, individuals.

Seeking Alternative Translational Hypotheses

By this time, we had sequenced all of the known genes in the linkage interval and found no mutations. We then followed the hypothesis that a chromosomal rearrangement between the candidate genes PDE3A/SUR2/KCNJ8 for hypertension and SOX5 for the skeletal phenotypes, separated by several megabases, could have caused position effects acting on both genes. We used bacterial artificial chromosomes (BACs) that spanned our linkage interval. These BACs can be investigated with fluorescent probes, allowing the order of the BACs to be tested visually under a fluorescent microscope. We were able to determine that the BACs of affected persons were not in the correct order on 1 of the chromosome 12p copies. The “rearrangement” was complex and involved an inversion, a deletion, and a reinsertion. The chromosomal confusion was adjacent to 3 potential hypertension genes, encoding for phosphodiesterase 3 (PDE3A), an ATP-dependent potassium channel (KCNJ8), and the sulfonyl-urea receptor 2 (SUR2), a regulatory component of that same potassium channel. These findings called for a whole new dimension in patient-oriented research.

We next developed techniques for buttocks biopsies with generous help from others. We included gene expression, patch clamp, small vessel myography, forearm plethysmography, and systemic regulatory studies in our repertoire. A buttocks microvessel section from a 25-year–old affected (not very hypertensive) subject and a sibling are shown (Figure 1). The photomicrographs are qualitative and not quantitative. Ambulatory blood pressure monitoring comparisons are not available; however, casual blood pressures were barely different. Nevertheless, the robust hyperplasia of the affected person would at least suggest that the Folkow hypothesis warrants testing. The in vitro and in vivo studies testing the 3 genes resulted in an innocent verdict for all. We have continued our search with the understanding that rearrangement syndromes are not trivial and provide extremely difficult challenges.

In the mean time, we accrued encouragement through a collaboration with colleagues from China and work done in...
Methods

Three-Color Interphase Fluorescent In Situ Hybridization

We prepared air-dried slides from lymphoblastoid cell lines of affected and nonaffected family members from 5 families as interphase preparations. Nineteen BACs (CTIB library and RP11 library), namely, 604C2, 684M17, 405A12, 184C8, 465D4, 96K9, 12D15, 73M14, 359J14, 268P4, 449P1, 114G22, 284C17, 681I19, 686N4, 345P1, 153K16, 115C18, 444N1, and 1 P1 artificial chromosome (Genome Systems, Inc), namely, 134P11, were labeled by nick translation with either FluoroX (green), Cy3 (red), or both (yellow) (Genome Systems, Inc), namely, 134P11, were labeled by nick translation with either FluoroX (green), Cy3 (red), or both (yellow) to generate 500 bp fragments. Denatured probes were hybridized as combinations of 3 differentially colored BAC/P1 artificial chromosome to slides overnight at 37°C. After washing and staining with 4',6-diamidino-2-phenylindole, the slides were examined under a fluorescence microscope. For each combination, ≈20 chromosomes were scored for every individual. In total, 64 different combinations were performed. A rearrangement was assumed when the 3' end of one chromosome was adjacent to the 5' end of a neighboring chromosome.

5' and 3' Rapid Amplification of cDNA Ends

We performed 5' rapid amplification of cDNA ends (RACE) and 3' RACE using the GeneRacer kit (Invitrogen) according to the manufacturer’s instructions. Database sequence information of expressed sequence tag (EST) AK094733 and sequence information of newly identified exons were used for primer design (primer sequences available on request). 5' RACE was carried out from exons 13 and 22 according to our exon classification of the gene-related structure. 3' RACE was conducted from exons 19, 20, and 24. Briefly, total RNA from human fetal aorta, fetal brain (Stratagene), and fetal cartilage (cartilage 2: hand and foot cartilage of a 14-week-old embryo; cartilage 3: tibia growth plates of a 14-week-old embryo) was used as starting material. Nested 5' RACE and 3' RACE products generated with a nested universal primer and the universal primer, which binds to the universal tag sequence and an oligo-dT primer that carries a universal tag sequence on the 5' end. We confirmed the rearrangement using a universal tag sequence on the 5' end. For both polyadenylation and cDNA reaction we used the NCCode First-Strand cDNA Synthesis Kit (Invitrogen). The cDNA served as template for the subsequent PCR analysis of miRNA using universal primer, which binds to the universal tag sequence and an miRNA-specific primer. The total PCR volume was 25 μL. The PCR mix contained 10 mmol/L of Tris-HCl (pH 8.3), 50 mmol/L of KCl, 2.5 mmol/L of MgCl2, 250 μmol/L of each 2'-deoxynucleoside 5'-triphosphate (dATP, dCTP, dGTP, and dTTP from Pharmacia), 25 ng of cDNA, and 0.6 U of Ampli Taq Gold DNA Polymerase (Applied Biosystems). The PCR conditions were as follows: 95°C for 10 minutes, 40 cycles consisting of a heat-denaturation step of 15 seconds at 94°C, an annealing step of 20 seconds at 58°C, and an extension step of 30 seconds at 72°C. The PCR amplifications were performed in a 9600 Thermocycler (Applied Biosystems).

Isolation of Alternative Splice Variants by RT-PCR

To investigate alternative splicing and different exon usage, RT-PCR was performed with primers localized in exon 13 and exon 24, exon 1/6, and exons 15, 18, 19, and 20, respectively (primer sequences available on request). We used total RNA from human fetal aorta, fetal brain (Stratagene), and fetal cartilage. Reverse transcription was performed using a RevertAid First Strand cDNA Synthesis kit (Fermentas) and hexanucleotides according to the manufacturer’s instructions. Cloning and sequencing were carried out as described above.

Quantitative RT-PCR Expression

RNA was extracted from primary fibroblasts of affected and nonaffected family members following the TRIzol reagent (Invitrogen) protocol. Reverse transcription was performed using the RevertAid First Strand cDNA Synthesis Kit (Fermentas) and hexanucleotides according to the manufacturer’s instructions. RT-PCR conditions for TaqMan were optimized, and standardized conditions (annealing temperature: 60°C; 50 cycles) were applied for all of the reactions (ABI PRISM 7700 Sequence Detector). Briefly, 300 nM of PCR primers, 200 nM of probe, 10 to 50 ng of cDNA, and qPCR Master Mix (Eurogentec) were used. Probes were designed to cover a splice junction between neighboring exons (primer sequences available on request). Data were analyzed using SDS 7000 software and ΔΔCt comparative analysis as described by Applied Biosystems.

MicroRNA Detection

MicroRNA (miRNA) was isolated with the mirVana miRNA Isolation kit (Ambion) using RNA from primary fibroblasts. Isolated miRNA was polyadenylated with poly-A-polymerase. The polyA-tailed miRNA was used for synthesis of first-strand cDNA with an oligo-dT primer that carries a universal tag sequence on the 5' end. For both polyadenylation and cDNA reaction we used the NCCode First-Strand cDNA Synthesis Kit (Invitrogen). The cDNA served as template for the subsequent PCR analysis of miRNA using universal primer, which binds to the universal tag sequence and an miRNA-specific primer. The total PCR volume was 25 μL. The PCR mix contained 10 mmol/L of Tris-HCl (pH 8.3), 50 mmol/L of KCl, 2.5 mmol/L of MgCl2, 250 μmol/L of each 2'-deoxynucleoside 5'-triphosphate (dATP, dCTP, dGTP, and dTTP from Pharmacia), 25 ng of cDNA, and 0.6 U of Ampli Taq Gold DNA Polymerase (Applied Biosystems). The PCR conditions were as follows: 95°C for 10 minutes, 40 cycles consisting of a heat-denaturation step of 15 seconds at 94°C, an annealing step of 20 seconds at 58°C, and an extension step of 30 seconds at 72°C. The PCR amplifications were performed in a 9600 Thermocycler (Applied Biosystems).

Results

Fine Mapping of Chromosomal Rearrangements by Interphase Fluorescent In Situ Hybridization

We extended our interphase fluorescence in situ hybridization (FISH) cytogenetic investigations by increasing the number of genomic probes (20 BACs/P1 artificial chromosome) and by including more families, namely from Turkey, France, United States, Canada, and an isolated case from South Africa. In total, 64 different combinations of probes were performed. Two affected subjects and 1 nonaffected subject from each family and a normal unrelated individual were characterized.

The interphase FISH results revealed that all of the families and the isolated subject from South Africa had rearrangements, albeit all slightly different (Figure 2). We confirmed the inversion, deletion, and the reinsertion in the Turkish family. The inversion boundaries were determined at BAC 449P1 and 345P1. The American family showed an inversion between BACs 268P4 and 153K16. In the Canadian family, we found an inversion extended from BAC 268P4 to 345P1. In the French family, we observed an inversion that appeared

Figure 1. Vessel sections obtained from buttocks biopsies of affected (left) and nonaffected people, both in their early 20s. Casual blood pressure measurements were barely different. The marked difference in the vessel wall raises the notion that remodeling could be a primary event. However, this observation is qualitative and subjective.
to be larger, including BACs 12D5 and 115C18. Nonetheless, a common pattern was discernible as labeled in the figure involving an inverted area of ~450 kb. We covered the entire rearrangement interval with rare cutting enzymes (PmeI, SfiI, AclI, and SalI), separated the digested DNA of affected and nonaffected subjects of all families with pulse field gel electrophoreses, and performed Southern blots in an attempt to detect breakpoint fragments. However, no blot revealed a breakpoint-specific pattern (data not shown). However, a pulse field gel electrophoreses–based Southern blotting technique may be too crude to enable us to detect the rearrangement. Therefore, we are currently conducting Southern blots using frequent cutting enzymes.

Investigation of a Gene-Related Structure Within the Inverted Interval

The minimal region affected by the inversion contains no known genes. The University of California Santa Cruz (http://genome.ucsc.edu/cgi-bin/hgGateway) Genome Browser yielded 4 ESTs within our inverted interval, namely AK094733, BX098242, AL517295/AL517294, and AK058180. We have elucidated these ESTs with 5’ and 3’ RACE PCR starting with 5’ RACE from EST AK094733 (exon 13 of our exon classification) and 3’ RACE from exon 24. We identified 5’ RACE products from a fetal brain consisting of exon 11, exon 12, and the reverse primer binding exon 13. The transcription start points within exon 11 varied. In contrast, 5’ RACE products from fetal aorta extended from exons 1, 6, 7, 8, 12, and 13; as a second variant without exon 7; or as third variant from exons 11 and 12 to 13. Blat alignment (http://genome.ucsc.edu/cgi-bin/hgBlat?command=start) of the different RACE variants resulted in exon sharing of variants 2 and 3 to exons of EST BX098242. 3’ RACE from exon 24 confirmed this exon as the last exon of a transcript. 3’ RACE starting from exon 19 uncovered 4 differently spliced transcripts expressed in fetal aorta, fetal brain, and tibia growth plates of a 14-week-old embryo (cartilage 3). Five different splice variants from the RNA of fetal aorta and fetal brain and hand and feet cartilage of a 14-week–old embryo (cartilage 2) were found with 3’ RACE from exon 20. Some exons of these 3’ RACE fragments are shared with exons of ESTs AL517295/AL517294 and AK058180. Exons 22 and 23 were newly discovered at that time (data not shown but available on request). After updating the University of California Santa Cruz human EST database, EST AI935068 matched to exons 22 and 23. Complete sequencing of the underlying clone IMAGE:2329245 led to identification of exons 15 and 16. 5’ RACE, based on reverse-primer binding in exon 22, resulted in identification of the upstream transcript consisting of exons 1, 6, 7, 11, 12, 13, 17, 18, 19, 20, and 22 in fetal aorta, and no 5’ RACE product could be detected in the other used tissues. For further investigation of exon usage and differentiation of splice variants, RT-PCR with varying primer combinations was carried out. In total, in addition to the 4 known ESTs, 16 differently spliced partial transcripts were discovered by RACE experiments, and 12 RT-PCR products with variable exon usage were detected. Recently, antisense transcripts CD299222 and BC039484 were found in the database. In summary, the minimal inverted region in affected persons with the syndrome encodes a gene-related structure with ~32 possible exons. We identified multiple transcripts arising from alternative splicing in fetal aorta, fetal brain, and fetal cartilage (data not shown but available on request), which argues for tissue-specific transcription and splicing regulation. However, none of the splice variants has an open reading frame longer than 89 amino acids, and we found no Kozak sequences. The sequences are not homologous to known genes. In vitro transcription/translation experiments did not identify any peptide-related molecules (data not shown). This state of affairs strongly suggests that we are dealing with a gene (or genes) that do not code for protein.
Quantitative Splice Variant Expression
Fibroblasts of affected and nonaffected subjects of the original Turkish family were cultured from skin punch biopsy specimens. We observed a significantly faster cell growth rate in affected than in control subjects, a subphenotype of the syndrome that has also been described in spontaneously hypertensive rats. To test for an altered expression level of the differently spliced transcript because of the inversion and the ensuing changed chromosomal environment, we used fibroblast total RNA and performed quantitative RT-PCR. We selected 12 TaqMan probes and primers representing all of the splice variants known thus far, namely, between exons /20/22 and exons 22/23, respectively, are never expressed in affected persons (Turkish family only; left). The sequence of exon 22 strongly resembles the structure of an miRNA (B).

Evidence of Exon 22 Predicted miRNA Expressed in Primary Fibroblasts
We performed an analysis to search for appearance of the predicted exon 22 miRNA. We used isolated, small RNA fractions from fibroblasts of an affected and a nonaffected family member. After polyadenylation of the miRNAs, we synthesized the first-strand cDNA. The cDNA served as template for the subsequent PCR analysis of miRNA. We used specific primers for both plus and minus orientation of the predicted miRNA in exon 22 (Figure 3B). We used primers for miR146 expressed in fibroblasts as a control. Both the control reaction and the predicted plus exon 22 miRNA reaction showed a specific band on an agarose gel in nonaffected fibroblast RNA (Figure 4); however, affected fibroblasts showed only the control reactions. Minus-orientated miRNA was not detected in affected or in nonaffected fibroblast RNA.

Discussion
Our research identified a novel form of hypertension that is clinically characterized by a major defect in baroreflex buffering. We thereupon specifically investigated persons with essential hypertension in terms of their baroreflex buffering capacity and the existence of neurovascular contact. However, we found no clear relationship. As a consequence, we have been reluctant to recommend neurovascular contact surgery to lower blood pressure in any of our patients. We remain faced with a hypertensive condition resembling essential hypertension, aside from the fact that the condition causes a baroreflex alteration that has only been described in persons with complete autonomic failure. Replication of linkage to the same locus in persons with essential hypertension is encouraging. We have not tested the Chinese subjects in terms of possible rearrangements. Medications, generally 3 to 4 classes, have successfully lowered blood pressure.

Figure 3. A, Quantitative expression analysis of spliced exons 20/22 (top) and exons 22/23 (bottom) from total fibroblast RNA of affected and nonaffected Turkish subjects. Exons 20/22 and exons 22/23, respectively, are never expressed in affected persons (Turkish family only; left). The sequence of exon 22 strongly resembles the structure of an miRNA (B).

Figure 4. Detection of predicted exon 22 miRNA in fibroblasts. miRNA of exon 22 was only detectable in the nonaffected subject (B) in contrast to the affected subject (A). Minus-orientated miRNA was not detected in affected or in nonaffected fibroblast RNA. miR146 served as positive control. NC indicates no template negative control.
pressure in our patients, and none has died of stroke under treatment.

The novel data that we present here raise the provocative hypothesis that autosomal-dominant hypertension and brachydactyly, a multiphenotype Mendelian syndrome, are caused by a chromosomal rearrangement, rather than by a mutation in a single gene. Our ongoing EST analysis reveals a gene structure that does not seem to code for protein. Exons 20/22 or, rather, 22/23 are not expressed in cultured fibroblasts from affected persons and are predicted to produce an miRNA-like structure that is as yet unknown in humans or any other species. We are aware that the data presented here are preliminary and that several weaknesses remain in our presentation. The rearrangement must be verified by another method. Chromosome-orientation FISH and fiber FISH studies are planned, and a denser Southern blot analysis will be performed to characterize breakpoints. We have already conducted a comparative genomic hybridization analysis and plan a dense single nucleotide polymorphism analysis by microarray. We are also conducting long-range PCR and intend to sequence the entire chromosome 12p region in affected persons and their parents.

Perspectives

Should our hypothesis be confirmed, we will have identified a noncoding RNA responsible for a human disease. The targets of this construct could lie anywhere on the genome, and the mechanisms involved will lead us into “systems biology” by revealing new pathways involved in cardiovascular regulation. These pathways will undoubtedly lead us back to the “bedside” as we, along with basic and clinical scientists, seek to find our way together in a strange foreign environment. To date, we remain “lost in translation”; however, we are working hard to rectify this state of affairs.

Acknowledgments

We thank Astrid Mühl, Eireen Klein, Regina Uhlmann, and Irene Hollfinger for their technical assistance. We greatly appreciate the contribution of the patients and their families.

Sources of Funding

The Deutsche Forschungsgemeinschaft supported the study (BA 1773/4-1). M.K. received a scholarship from the Deutsche Hochdruckliga. The Investitionsbank Berlin supported the work of A.A.

Disclosures

None.

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_Hypertension_. 2008;51:426-431; originally published online December 17, 2007; doi: 10.1161/HYPERTENSIONAHA.107.101774

_Hypertension_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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