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Molekulárně genetická analýza vybraných kryptických přestaveb lidských chromosomů

Molecular Genetic Analysis of Selected Cryptic Rearrangements of Human Chromosomes

Disertační práce

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Motto

„CAUSA LATET, VIS EST NOTISSIMA.“
„Příčina je skryta, výsledek je všeobecně známý.“

Publius Ovidius Naso

Abstrakt

Předkládaná práce shrnuje výsledky výzkumu zaměřeného na studium kryptických přestaveb lidských chromosomů. Konkrétně se zaměřuje na tři základní oblasti výzkumu.

První oblast představuje výzkum kryptických přestaveb identifikovaných jako kauzální příčiny onemocnění u mentálně retardovaných pacientů s původně neznámou etiologií. Nejčastěji se jedná o tzv. mikrodeleční sydromy. Velká variabilita fenotypu a často překrývající se symptomy mikrodelečních syndromů vyžadují celogenomový přístup. V rámci výzkumu bylo vyšetřeno 64 probandů a u 10 (16 %) z nich byly nalezeny kryptické přestavby, jež byly dále analyzovány.

Druhou oblast představuje výzkum kryptických přestaveb asociovaných s pseudoautosomálním regionem 1 (konkrétně s oblastí genu *SHOX*), jež mohou být přirozenou součástí variability genomu i příčinami vzniku onemocnění. V rámci výzkumu bylo vyšetřeno 98 pacientů s Léri-Weillovou dyschondrosteózou nebo idiopatickým malým vzrůstem, přičemž kauzální mutace byla nalezena u 68,8 %, resp. 7,8 % probandů. Současně byla drobná delece, tzv. delece L05101 nalézající se v blízkosti regulačních oblastí genu *SHOX*, vyhodnocena jako populační polymorfismus bez zjevného fenotypového dopadu. Komparativní analýza duplikací detekovaných v souboru 250 zdravých jedinců v porovnání s rozsahem a umístěním duplikací u pacientů označila duplikace s vysokým patogenním potenciálem. Výsledky byly podpořeny metaanalýzou publikovaných duplikací ve sledované oblasti.

Třetí oblast představují přestavby chromosomálního regionu 8q24 a mutace v genu *TRPS1* asociované s velmi vzácným trichorhinofalangeálním syndromem. V rámci výzkumu bylo vyšetřeno 9 pacientů, u 7 byla nalezena pravděpodobná příčina onemocnění. Zvolená metodika umožnila rovněž analýzu rozsáhlých 5´ a 3´ netranslatovaných oblastí genu *TRPS1* a detekovala zde řadu polymorfismů.

Práce poukazuje na význam výzkumu kryptických přestaveb lidských chromosomů pro lepší porozumění strukturní variabilitě lidského genomu a pro pochopení vzniku určitých patologií s jednoduchým i komplexním genetickým pozadím.

Klíčová slova

kryptické přestavby lidských chromosomů; mentální retardace; gen *SHOX*; duplikace v oblasti *SHOX*; trichorhinofalangeální syndrom; gen *TRPS1*; molekulární cytogenetika člověka

Abstract

The presented dissertation summarizes the results of research focused on the study of cryptic rearrangements of human chromosomes. Specifically, it focuses on three core areas of research.

The first area is the research of cryptic rearrangements identified as causal causes of mental retardation in patients with previously unknown aetiology. The most common are the so-called microdeletion syndromes. The large variability of the phenotype and often overlapping symptoms of microdeletion syndromes require a whole-genome approach. Within the research, 64 probands were investigated and in 10 (16%) cryptic rearrangements were found and further analyzed.

The second area is the research of cryptic rearrangements associated with the pseudoautosomal region 1 (specifically with the *SHOX* gene region), which may be both natural components of population variability and the cause of the disease. Within the research, 98 patients with Léri-Weill dyschondrosteosis or idiopathic short stature were examined, with a causal mutation found in 68.8%, and 7.8% probands respectively. At the same time, the minor deletion (so-called L05101 deletion) was evaluated as a population polymorphism without an apparent phenotypic impact. Duplications with high pathogenic potential were identified by mean of comparative analysis of duplications detected in a set of 250 healthy individuals and patients. The results were supported by meta-analysis of published duplications in the observed area.

The third area is the research of rearrangements of chromosomal region 8q24 and *TRPS1* gene mutations associated with the rare trichorhinophalangeal syndrome. The probable cause of the disease was found in 7 patients out of 9. The chosen methodology also allowed analysis of extensive 5' and 3' untranslated regions of the *TRPS1* gene and detected a number of polymorphisms.

The dissertation highlights the importance of the research of cryptic rearrangements of human chromosomes in order to better understand the structural variability of the human genome and the origin of pathologies both with simple and complex genetic background.

Keywords

cryptic rearrangements of human chromosomes; mental retardation; *SHOX* gene; *SHOX* area duplications; trichorhinophalangeal syndrome; *TRPS1* gene; human molecular cytogenetics

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1. Koncepce a struktura práce

Předkládaná práce představuje shrnutí vědeckého výzkumu, na němž se předkladatel spolupodílel v průběhu svého postgraduálního doktorského studia. Práce není ve svém celku založena na řešení jednoho základního vědeckého problému, či na jednolitém hledání odpovědi na jednu zásadní vědeckou otázku, nýbrž je koncipována jako mozaika vícera dílčích vědeckých výzkumů, jež ovšem spojuje tématická souvislost. Z toho vychází i podoba práce coby souhrnné, obsahující jako nedílnou součást pětici původních vědeckých článků publikovaných v impaktovaných odborných časopisech (publikace I – V).

Středobodem předkladatelova vědeckého zájmu v kontextu této práce jsou kryptické strukturní aberace lidských chromosomů, jednak jako příčiny patologií a jednak jako součást přirozené lidské genetické variability. Za účelem užšího vymezení ji tedy lze tématicky klasifikovat do oboru molekulární cytogenetika člověka. Vlastní výzkum se konkrétně dotýká

a) kryptických přestaveb asociovaných s mentální retardací (publikace I)
b) kryptických přestaveb asociovaných s kostními dyspláziemi, konkrétně
    a. přestaveb pseudoautosomálního regionu 1 (publikace II, III a IV)
    b. přestaveb chromosomálního regionu 8q24 (publikace V).

Předkládaná práce tedy sestává ze souhrnu výzkumné problematiky (v jehož rámci bude představena odborná tématika, a to včetně začlenění v práci prezentovaného vědeckého bádání do kontextu aktuálních trendů v rámci disciplíny), ze shrnutí a charakteristiky výstupů výzkumu, to vše doplněno o plné znění pěti odborných publikací[1].

[1] V případě citování těchto publikací bude v rámci odkazu v textu uvedeno i označení publikace v rámci této práce (P-I až P-V), např. (Solc et al., 2014, P-III).

2. Souhrn výzkumné problematiky

2.1 Kryptické přestavby

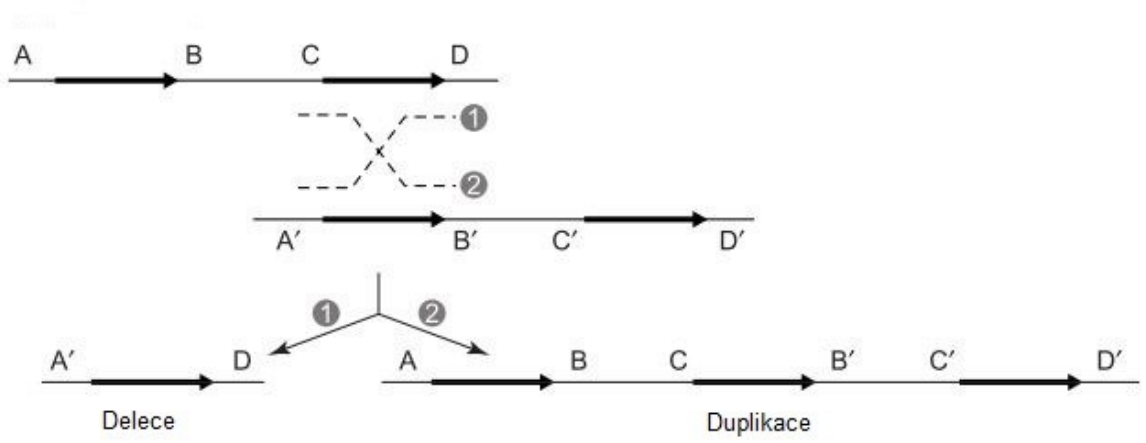
Jako „kryptické přestavby“ se obvykle označují takové strukturní aberace chromosomů, které není možné detekovat za využití metod klasické cytogenetiky, tedy za využití rozličných pruhovacích technik aplikovatelných na metafázní chromosomy. Kryptické přestavby se od klasických neodlišují kvalitativně, jedná se o kvantitativní kontinuum, přičemž rozhraní je relevantní čistě z metodologického hlediska. S ohledem na to nelze ani stanovit jednoznačnou obecnou velikostní hranici mezi kryptickými a klasickými přestavbami, neboť nejnižší rozlišovací schopnost pruhovacích technik se mírně liší v závislosti na různých chromosomových oblastech. Obecně se dá říci, že za kryptické lze považovat přestavby menší než 3 – 5 Mb (Gijsbers et al., 2009, Salman et al., 2004). Co do typu se může jednat o přestavby různého druhu – delece, duplikace, translokace atd. Označují se pak většinou předponou „mikro“, tedy jako „mikrodelece“, „mikroduplikace“ apod.

Kryptické přestavby lze detekovat za využití technik molekulární genetiky a molekulární cytogenetiky. Tyto metody můžeme pro přehlednost rozdělit do tří vývojových skupin. První skupinu tvoří metody postavené na hybridizaci – jako příklad jmenujme metody FISH (fluorescent *in situ* hybridization), m-FISH (multicolour FISH), SKY (spectral karyotyping) a nejpokročilejší CGH (comparative genomic hybridization), resp. aCGH (array CGH, tzv. mikročipy). Druhou skupinu pak metody odvozené od metody PCR (polymerase chain reaction), zejména qPCR (quantitative PCR). Třetí skupinu pak představují metody kombinující oba předchozí mechanismy do jednoho celku – MAPH (multilocus amplifiable probe hybridization) a MLPA (multiplex ligation-dependent probe amlification).

Asi 12 % genomu tvoří relativně stabilní CNV (copy number variations). Jedná se o přirozeně se vyskytující strukturní variabilitu představovanou drobnými delecemi a duplikacemi, přičemž variabilní úseky mívají velikost od 1 kb až po několik Mb (Stankiewicz et Lupski, 2010; Watson et al., 2014). V současné době se na CNV soustředí pozornost výzkumu. Cílem je jak odhalit CNV s možným funkčním významem (mající

podíl na populační variabilitě fenotypu jedinců), tak vybrat CNV bez fenotypového efektu jako další složku systému pro identifikaci osob a jejich původu.

Mikrodelece a mikroduplikace mohou vznikat kdekoli v genomu, ale některá místa jsou k jejich vzniku náchylnější (Ballif et al., 2007; de Vries et al., 2003; Weise et al., 2012). Jedná se o oblasti tzv. LCR (low copy repeats; 10^2 – 10^3 kb) (Bailey et al. 2002; Mefford et Eichler 2009) a TAR (telomere associated repetition; 10^3 kb) (Flint et al., 1997). Rozvoj LCR, neboli segmentálních duplikací, je jedním ze základních mechanismů evoluce. Duplikované úseky jsou často tandemově uspořádané, nacházejí se v genomu ve dvou nebo více kopiích a sdílí vysoký stupeň homologie (97 %) (Shaffer et al., 2007). Kromě homologie sdílejí bloky LCR i velikost typickou pro konkrétní region (přibližně od 10 do 400 kb) a vzdálenost mezi jednotlivými bloky repetic (Stankiewicz et Lupski, 2002).



Obrázek 1

Mechanismus vzniku delece a reciproké duplikace prostřednictvím LCR

– převzato z Lupski, 1998

Vysoký stupeň homologie umožňuje nealelickou homologní rekombinaci, tzv. NAHR (non-allelic homologous recombination). Segmentální duplikace mohou být uspořádány ve formě přímé či invertované repetice a k rekombinaci může docházet jak mezi homologními chromosomy, tak mezi sesterskými chromatidami, nebo dokonce i v rámci jedné chromatidy. Důsledkem NAHR je delece, případně reciproká duplikace chromosomálního úseku ležícího mezi sekvencemi účastnícími se rekombinace. Pokud

deletovaná oblast obsahuje geny, může být jejich ztráta spojena s patologickým fenotypem. V rámci jednoho repetitivního úseku se rekombinace odehrává často mezi dvěma určitými homologními úseky. Tímto způsobem vznikají opakující se charakteristické přestavby asociované s některými mikrodelečními syndromy (Gu et al., 2008; Shaffer et Lupski, 2000).

Dalšími chromosomálními oblastmi, v nichž často dochází k přestavbám, jsou subtelomerické sekvence, které jsou obecně bohaté na geny (Mefford et Trask, 2002; Saccone et al., 1992). Nachází se zde tzv. TAR, které bezprostředně sousedí s vlastními telomerami. Různé chromosomy mohou sdílet vysoce homologní TAR sekvence a umožňují tak opět vznik NAHR (Flint et al., 1997). Pokud v důsledku NAHR, nebo z jiného důvodu, dojde ke ztrátě telomery, mohou se v průběhu buněčného dělení vytvářet můstky mezi sesterskými chromatidami. Při rozestupu během anafáze pak dochází k náhodnému roztržení můstku, přičemž jedna chromatida obsahuje koncovou část duplikovanou a druhá chromatida tuto část postrádá. Než dojde k opětnému pokrytí poškozeného konce chromosomu novou telomerou, může se u rychle se dělící buňky uvedený cyklus opakovat (tzv. breakage-fusion-bridge cyklus) a dochází ke vzniku komplexních přestaveb (Selvarajah et al., 2006).

Významnou roli při vzniku chromosomálních přestaveb hrají mechanismy, které se účastní opravy dvouvláknových zlomů. V průběhu dělení mohou být opraveny pomocí homologní rekombinace s využitím sesterské chromatidy. Mimo fázi dělení se uplatňuje nehomologické spojení konců, tzv. C-NHEJ (classic non-homologous end joining). Jedná se o evolučně velmi starý a energeticky nenáročný proces, kdy jsou jednoduše ligací spojeny dva sousedící dvouvláknové konce. Za normálních okolností je tento proces velmi rychlý (cca 30 min). Právě rychlost zaručuje, že v drtivé většině bude tím nejbližším sousedním dvouvláknovým koncem opravdu navazující chromosomální fragment. Za určitých okolností, které nejsou zatím objasněny, buňka zvolí alternativní cestu, tzv. A-NHEJ. Vzhledem k tomu, že při této cestě předchází ligaci volných konců vyhledávání mikrohomologií, trvá A-NHEJ mnohem déle (až 20 hodin). Dlouhá časová prodleva mezi vznikem zlomu a jeho opravou může vést ke spojení nesprávných konců a vzniku chromosomální přestavby (Karran, 2000).

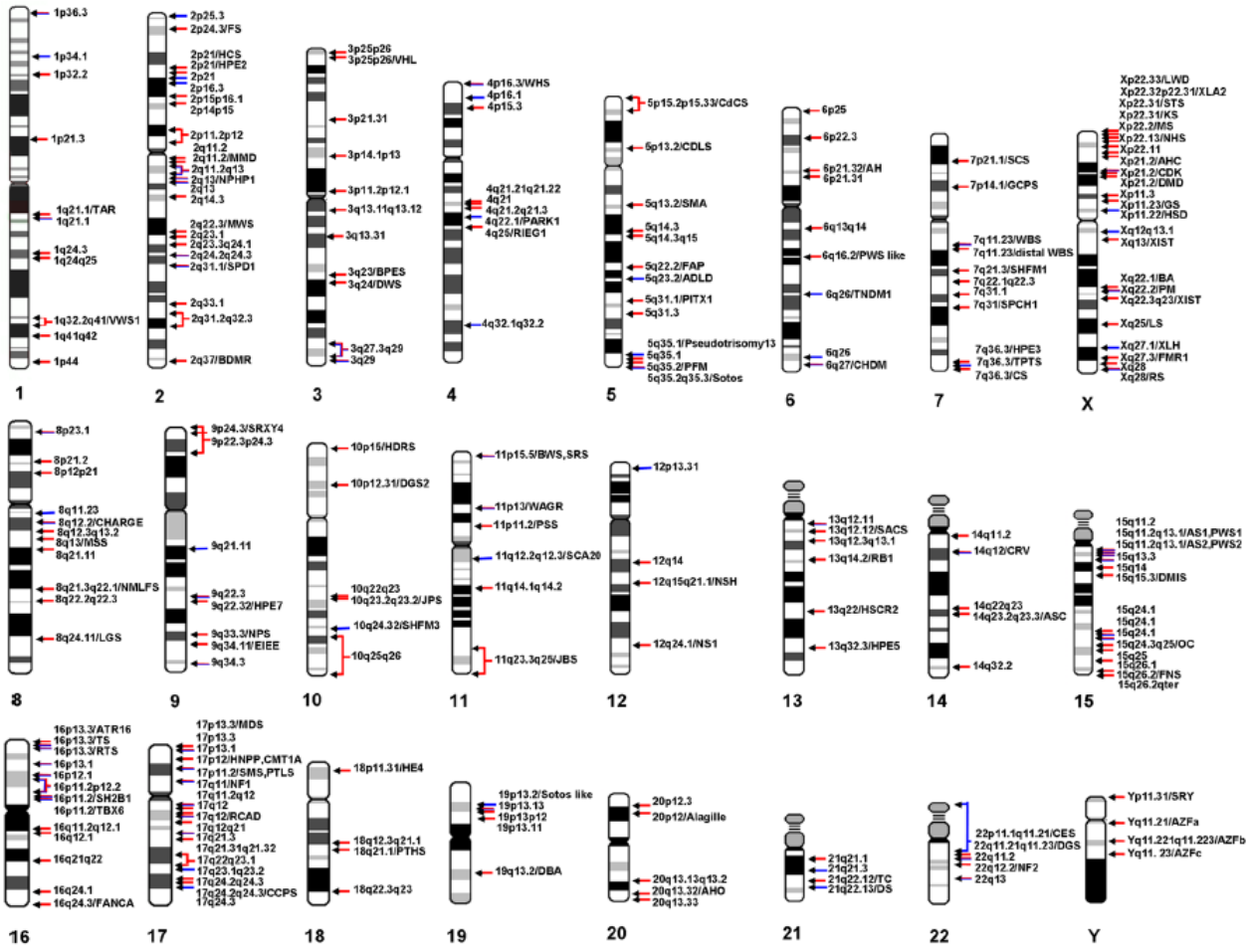
Další mechanismus založený na využití mikrohomologie nealelních oblastí, který vede k tvorbě chromosomálních přestaveb je tzv. FoSTeS (fork stalling and template switching) uplatňující se během replikační fáze (Gu et al., 2008).

2.2 Kryptické přestavby asociované s mentální retardací

První oblast vědeckého zájmu této práce představují kryptické strukturní aberace chromosomů asociované s mentální retardací. Kryptické přestavby chromosomů, zejména pak mikrodelece, mohou vést ke vzniku komplexních onemocnění (syndromů). K symptomům u řady těchto syndromů patří mimo jiné právě i mentální retardace.

Obecně vzato postihují různé formy mentální retardace přibližně 1 – 3 % populace (Vissers et al., 2010). Etiologie mentální retardace je velmi heterogenní, přičemž se může vyskytovat jak izolovaně, tak v komorbiditě s jiným onemocnění, či jako součást syndromů zahrnujících v klinickém projevu např. malformace a dysmorfie. U přibližně poloviny pacientů zůstává příčina mentální retardace neznámá. Genetické příčiny (zahrnující mutace genomové, chromosomální i genové, případně další poruchy na molekulární úrovni) lze najít (podle různých studií) u 17 – 41 % případů (Bernardini et al., 2010; Rauch et al., 2006). U přibližně 5 – 20 % jedinců (ze všech osob s mentální retardací) je kauzální příčinou kryptická chromosomální aberace (de Vries et al., 2003; Liang et al., 2008; Hirschfeldova et al., 2011, P-I).

Jak již bylo řečeno, kryptické přestavby mohou vést ke vzniku komplexních onemocnění (syndromů). Nejčastěji se jedná o tzv. mikrodeleční syndromy, které jsou způsobeny kryptickými delecemi, vedle toho je stále větší pozornost věnována tzv. mikroduplikačním syndromům, jež jsou způsobovány mikroduplikacemi. Detailní studie Weise et al. (2012) uvádí, že přestože ke vzniku reciprokých mikrodelecí a mikroduplikací (a tudíž i syndromů) by mělo docházet ve stejné míře, bylo v době vzniku studie popsáno 211 mikrodelečních syndromů, ale pouze 79 mikroduplikačních syndromů, z čehož pouze v 56 lokusech byly popsány jak mikrodelece, tak mikroduplikace (tedy reciproké přestavby) (viz obrázek 2). Vedle toho studie ukazuje, díky metanalýze publikovaných odborných prací, že zatímco mikrodeleční syndromy jsou v odborné literatuře popisovány (ve vzrůstající míře) již od roku 1990, mikroduplikační syndromy až od roku 2004. Autoři tuto nerovnováhu interpretují jednak jako možný následek nerovnoměrného selekčního tlaku proti aberantním gametám v průběhu gametogeneze a jednak jako možný následek skutečnosti, že mikroduplikace vedou v obecné rovně, ve srovnání vůči mikrodelecím, k mírnějším (až žádným) klinicky relevantním fenotypovým projevům. Navíc mikroduplikace se pojí s mnohem variabilnějším fenotypem (dokonce i mezi členy jedné rodiny nesoucími stejnou aberaci).



Obrázek 2

Schématický přehled všech chromosomálních regionů, jejichž klinicky významná mikrodelece či mikroduplikace byla popsána alespoň dvakrát (v době vyjití publikace). Červené šipky představují zachycené mikrodelece, modré šipky zachycené mikroduplikace a smíšené šipky zachycené reciproké mikrodelece i mikroduplikace.

– převzato z Weise et al., 2012

Většina syndromů patří mezi tzv. syndromy přilehlých genů („contiguous gene syndromes“), což znamená, že klinický projev je ve své sumě způsoben aberací několika sousedních genů, přičemž aberace každého z nich vede ke konkrétní součásti patologického fenotypu. Variabilita v rozsahu i přesné lokalizaci přestavby pak souvisí s poměrně vysokou mírou variability klinických projevů (k níž ovšem přispívá i genetické pozadí a negenetické faktory). Fenotyp dalších syndromů je pak úplně nebo ve výrazně převažující míře důsledkem aberace jednoho konkrétního genu, zatímco ostatní aberované

geny se na klinickém obrazu pacienta neprojevují nijak výrazně. Výsledný klinický projev se pak podobá následkům bodových mutací daného genu s majoritním působkem. Fenotypová variabilita syndromu se pak týká zejména oněch minoritně přispívajících genů, ovšem nejvýznamnější fenotypové rysy bývají přítomny vždy.

Svou roli hraje i povaha postižených genů. Pokud se jedná o geny kódující strukturní proteiny, pak je obvykle postižena jen určitá tkáň či tkáně a klinický projev nemusí být nijak závažný i v případě rozsáhlé delece. Naopak i malá delece genu, který kóduje transkripční faktor, jehož exprese je přísně regulována, může mít za následek postižení mnoha tkání, protože sekundárně jsou jeho nízkou hladinou postiženy exprese velkého množství genů v různých tkáních.

S přihlédnutím k velké variabilitě a vzájemnému překryvu fenotypů řady mikrodelečních a především mikroduplikačních syndromů můžeme předpokládat, že záchytnost těchto syndromů v populaci je stále podhodnocena. S rozvojem metody aCGH a díky detekci CNV pomocí sekvenování nové generace (NGS) nalezených mikroduplikací a mikrodelecí neustále přibývá. Především u mikroduplikací je však třeba velká ostražitost při interpretaci kauzality nalezené přestavby ve vztahu ke sledovanému fenotypu probanda. Nápomocné pak mohou být různé databáze, jako je např. DECIPHER.

Předkládaná ***publikace I*** (Hirschfeldova et al., 2011, P-I) shrnuje výsledky výzkumu provedeného na 64 probandech s diagnostikovanou idiopatickou mentální retardací. Publikace popisuje kryptické přestavby nalezené u 10 probandů (16 %), z nichž některé patří mezi dobře charakterizované a spojené s určitým syndromem, zatímco jiné nikoli. Dále předkládá rozbor fenotypových projevů pacientů a diskutuje jejich molekulárně genetické pozadí.

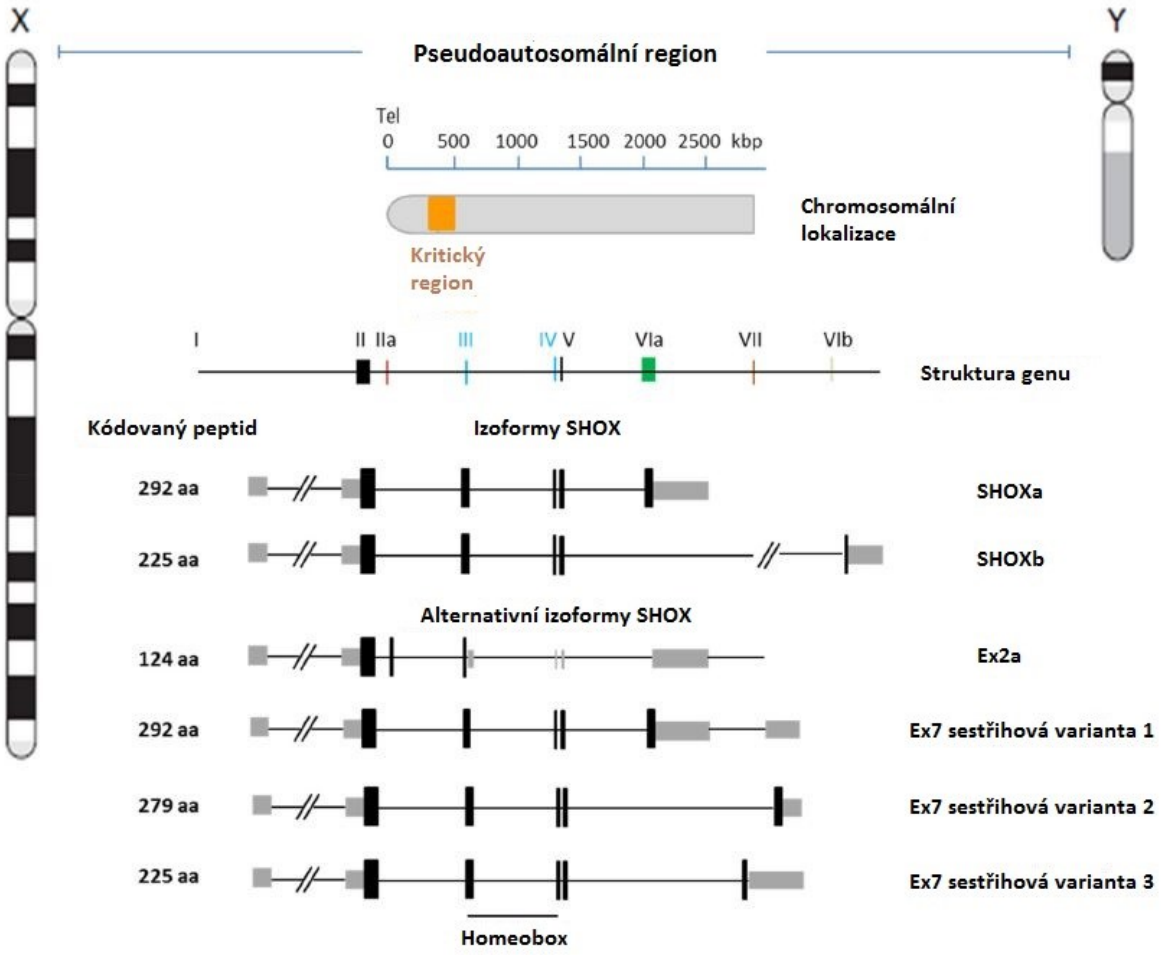
2.3 Kryptické přestavby pseudoautosomálního regionu 1

Druhou a nejrozsáhlejší oblastí vědeckého zájmu této práce, jsou mikrodelece a mikroduplikace lokalizované do pseudoautosomálního regionu 1 (PAR1; Xp22 a Yp11), konkrétně do oblasti genu *SHOX* a jeho známých regulačních elementů. Tento gen kóduje významný transkripční faktor, který hraje zásadní úlohu při regulaci růstu, zejména růstu dlouhých kostí. V sousedství genu se nalézá velké množství regulačních elementů s povahou zesilovačů. Aktivita některých z nich je natolik významná, že jejich ztráta v důsledku delece vede, i přes zachování neporušeného genu *SHOX*, k patologickému fenotypu. Vzhledem k tomu, že nelze fenotypově odlišit jedince s delecí genu *SHOX* od jedinců nesoucích sekvenční mutaci, byla současně provedena i mutační analýza tohoto genu. Výše uvedené (zejména strukturní) varianty či mutace byly analyzovány jak z pohledu klinického, tak z perspektivy přirozené variability lidského genomu.

Gen *SHOX* (short stature homeobox-containing gene; GeneID #6473), patřící mezi tzv. homeoboxové geny, poprvé definovali Rao et al. (1997) a paralelně nezávisle Ellison et al. (1997). Rozprostírá se po oblasti o velikosti přibližně 40 kb. Původní charakterizace uváděla, že gen je tvořen sedmi exony, přičemž exony 6a a 6b podléhají alternativnímu sestřihu, a kóduje tedy dvě izoformy proteinu SHOXa (o velikosti 292 aminokyselin) a SHOXb (o velikosti 225 aminokyselin). Další studie však rozšířily počet exonů o další čtyři (2a, 7-1, 7-2 a 7-3), a tudíž rozšířily i paletu izoforem proteinu (Durand et al., 2011) (viz obrázek 3). V rámci genu byla identifikována sekvence tzv. homeoboxu, kódující tzv. homeodoménu, vazebnou doménu o velikosti 60 aminokyselin, typickou pro transkripční faktory účastnící se embryonálního vývoje (Ellison et al., 1997; Rao et al., 1997). Gen *SHOX* má vysokou míru sekvenční podobnosti s dalším lidským genem *SHOX2* a také s jeho myším orthologem *Shox2*. Byl nalezen u většiny druhů obratlovců včetně šimpanze, psa, slepice, žáby a ryby, s pozoruhodnou výjimkou hlodavců, kteří gen v průběhu evoluce ztratili (Clement-Jones et al., 2000).

Studie genové exprese odhalily jasný rozdíl v rozložení exprese isoforem SHOXa a SHOXb. Zatímco převážně jsou obě exprimovány ve fibroblastech v kostní dřeni, což odpovídá jejich roli transkripčních faktorů regulujících růst zejména dlouhých kostí, isoforma SHOXa je navíc exprimována v několika dalších tkáních (Rao et al., 1997). Další studie prokázaly exprimaci SHOX i v řadě dalších embryonálních a fetálních tkáních (svaly, kůže, střeva, oko, mozek, mícha), stejně jako v adultních tkáních, přičemž

nejvýraznější exprimace byla zjištěna v placentě, kosterním svalstvu, kostní dřeni a tukové tkáni. V nízké míře byla zjištěna exprese SHOX také v některých oblastech fetálního i adultního mozku. Zejména byly v několika mozkových tkáních plodu a dospělých detekovány izoformy zahrnující nově identifikovaný exon 2a, v největší míře ve fetálním oku a mozku a v adultní kostní dřeni a kosterním svalstvu. Varianty obsahující některou formu exonu 7 byly detekovány ve větším množství pouze ve fetální mozkové tkáni. To naznačuje, že vedle regulace růstu může SHOX hrát roli i v regulaci vývoje nervové tkáně, což se zejména může týkat těch izoforem proteinu definovaných po dodatečném objevu čtyř exonů *SHOX*, jejich funkční specifika nejsou dosud uspokojivě rozluštěna (Durand et al., 2011; Marchini et al., 2016).



Obrázek 3

Gen SHOX. *Gen se vyskytuje přibližně 500 kb od telomery (Tel), zabírá oblast přibližně 40 kb a skládá se z devíti exonů kódující dvě hlavní izoformy SHOXa a SHOXb a přinejmenším čtyři další alternativní izoformy. Charakteristickou a funkčně nejvýznamnější tzv. homeodoménu kóduje tzv. homeobox.* – převzato z Marchini et al., 2016

Samotná exprese genu *SHOX* je regulována třemi základními mechanismy (viz obrázek 4). První regulaci představuje existence dvou alternativních promotorů, P1 a P2, které generují shodné mRNA s výjimkou nekódujících 5‘UTR sekvencí obsahujících různý počet AUG elementů. Zvýšený počet AUG vede následně ke snížení míry translace transkriptu. Dva promotory mohou být užívány v závislosti na aktuálních fyziologických potřebách, ovšem jejich rozličná aktivace není dosud vysvětlena (Blaschke et al., 2003; Marchini et al., 2016). Druhou formu představují regulační elementy (CNEs; conservative noncoding elements) ve fázi cis. Konkrétně byly v PAR1 popsány tři CNEs předcházející genu („upstream“) a čtyři CNEs následující gen („downstream“), jejichž zesilovací funkce („enhancer“) byla prokázána jak v lidských buňkách, tak v buňkách několika dalších živočichů (Benito-Sanz et al., 2012; Durand et al., 2010; Fukami et al., 2006; Sabherwal et al., 2007). Třetí regulační mechanismus představuje již zmiňovaný alternativní sestřih týkající se zejména 3‘UTR sekvencí, případně tzv. RUST (regulative unproductive splicing and translation), který tvoří díky alternativnímu sestřihu transkripty, které jsou kvůli přítomnosti předčasného terminačního kodónu degradovány systémem NMD (nonsense-mediatd mRNA decay), přičemž tvorba takových transkriptů je cílená a umožňuje rychlé přepnutí a tvorbu funkčního transkriptu v případě, že se v buňce objeví vhodný signál.

Obrázek 4

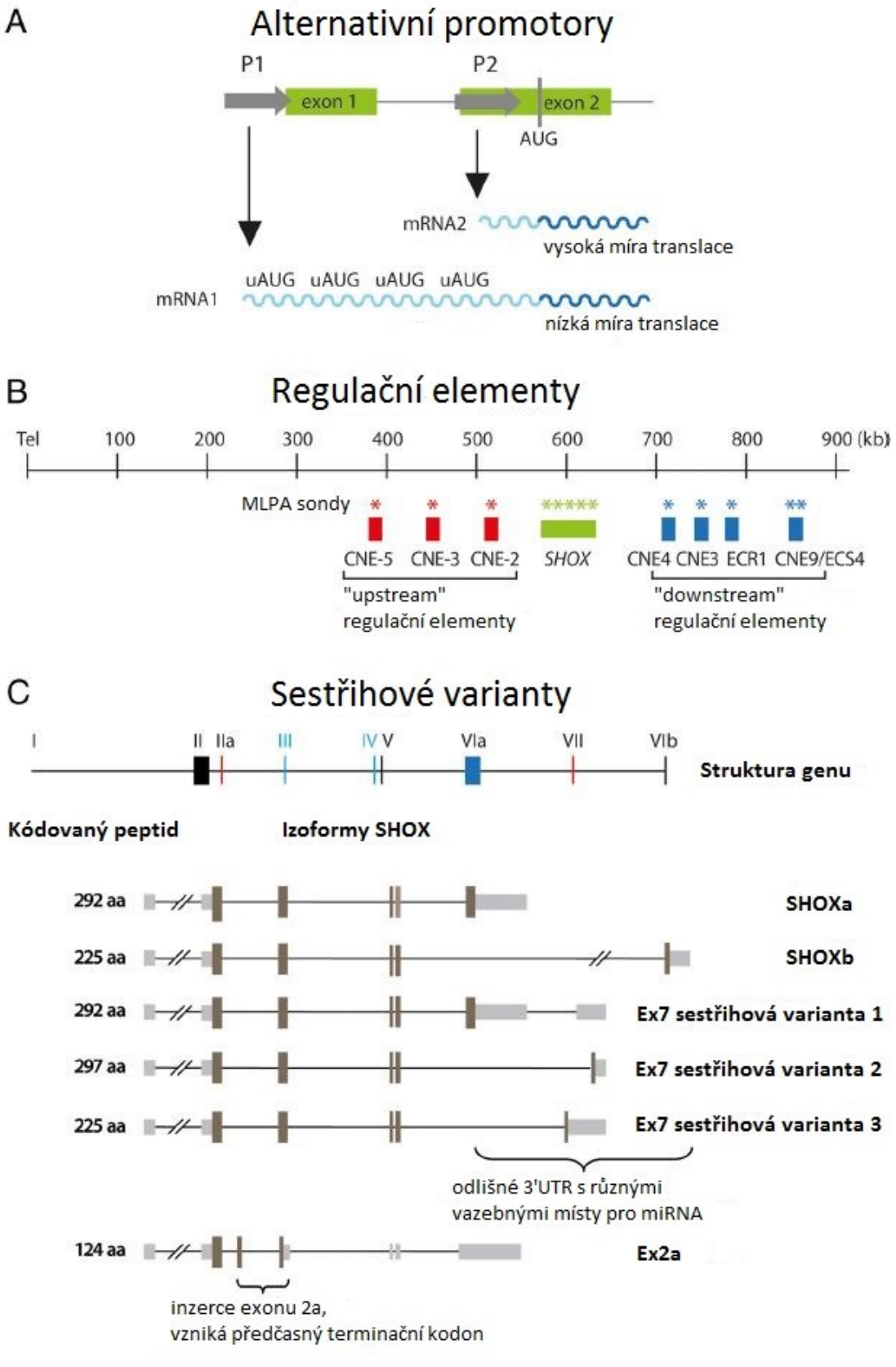
Mechanismy regulace exprese genu SHOX.

A) Alternativní promotory. Dva promotory (P1 a P2) řídí expresi SHOX *a produkují transkripty, které se liší ve svých 5‘UTR.*

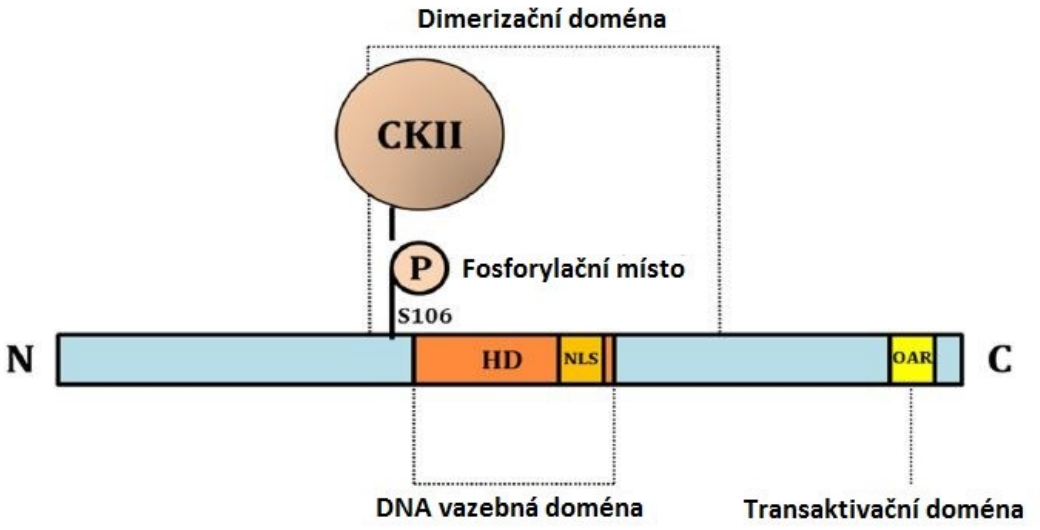
B) Regulační elementy. Horní vodorovná čára představuje fyzickou vzdálenost od telomer.

C) Sestřihové varianty. Různé isoformy jsou generovány genem SHOX *prostřednictvím alternativního sestřihu. Odlišné 3‘UTR mohou být podrobeny alternativní regulaci zprostředkované mikroRNA. Vložení exonu 2a vede ke vzniku předčasného terminačního kodonu v exonu 3, což by mohlo vést k degradaci mRNA. Exon III a IV obsahují homeobox. Světle šedá pole označují netranslatované oblasti; tmavě šedá pole představují otevřený čtecí rámec.*

– převzato z Marchini et al., 2016


A
Alternativní promotory
P1
P2
exon 1
exon 2
AUG
mRNA2
vysoká míra translace
uAUG uAUG uAUG uAUG
mRNA1
nízká míra translace
B
Regulační elementy
Tel
100
200
300
400
500
600
700
800
900 (kb)
MLPA sondy
CNE-5 CNE-3 CNE-2
SHOX
CNE4 CNE3 ECR1 CNE9/ECS4
"upstream"
regulační elementy
"downstream"
regulační elementy
C
Sestřihové varianty
I
II IIa
III
IV V
VIa
VII
VIb
Struktura genu
Kódovaný peptid
Izoformy SHOX
292 aa
SHOXa
225 aa
SHOXb
292 aa
Ex7 sestřihová varianta 1
297 aa
Ex7 sestřihová varianta 2
225 aa
Ex7 sestřihová varianta 3
odlišné 3'UTR s různými
vazebnými místy pro miRNA
124 aa
Ex2a
inzerce exonu 2a,
vzniká předčasný terminační kodon

V rámci proteinu SHOX, resp. v rámci nejčastěji se vyskytující izoformy SHOXa, lze popsat několik významných funkčních domén či míst (viz obrázek 5). Charakteristická pro homeoboxové geny je homeodoména, skládající se ze tří helikálních motivů, jež se přímo váže na molekulu DNA. Mutace vedoucí k záměně aminokyselin, či jiné poruše v této doméně, tuto schopnost oslabují, potažmo znemožňují (Gehring et al., 1994). Významný je jaderný lokalizační signál (sekvence aminokyselin definující směr transportu SHOX do jádra), jejíž mutace tento transport znemožňuje (Sabherwal et al., 2004). Dimerizační doména umožňuje vzájemnou interakci proteinů SHOX a vytváření funkčně efektivních dimerů (homodimerů i heterodimerů co do izoforem proteinu), kterážto schopnost může být opět narušena mutací (Schneider et al., 2005). Další významnou doménou je transaktivační doména, která zprostředkováva transkripci stimulující aktivitu proteinu. Tato doména je lokalizována poblíž C-konce proteinu a není součástí izoformy SHOXb – při stimulaci transkripce tedy SHOXb zřejmě funguje jako modulátor aktivity SHOXa v rámci dimeru (Rao et al, 2001). Specificky významné je pak fosforylační místo – jedná se o hojně fosforylovanou aminokyselinu serin na pozici 106 v rámci peptidu, přičemž fosforylace (prováděná casein kinázou II) slouží za nástroj regulace aktivity proteinu (Marchini et al., 2006).

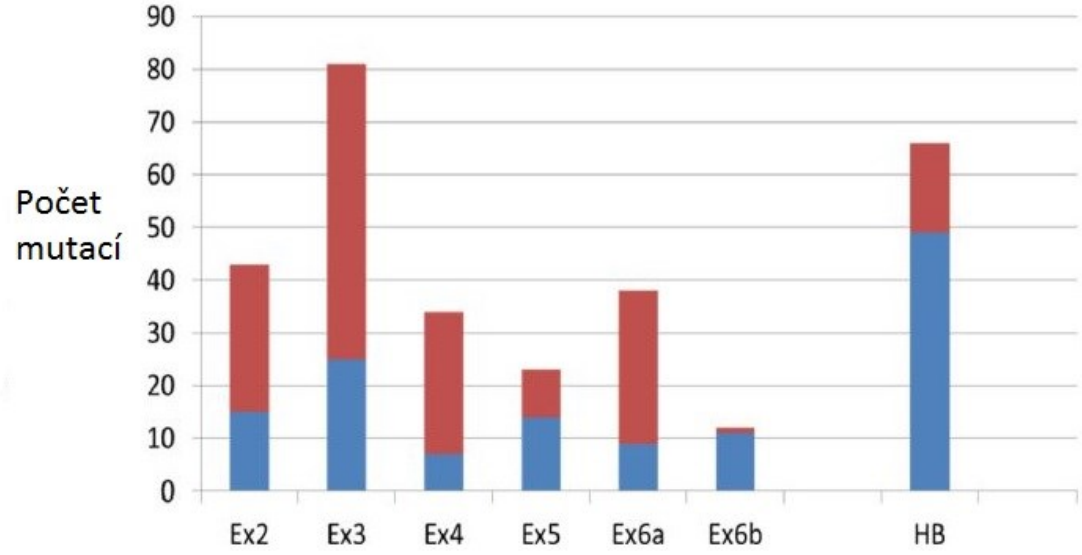


Obrázek 5

Protein SHOX, jeho významné domény a místa. (HD – homeodoména; NLS – jaderný lokalizační signál (nuclear localization signal); OAR – transaktivační doména (OAR – otp, aristaless and rax); N – N konec; C – C konec; P – místo fosforylace; CKII – casein kináza II)

– převzato z Marchini et al., 2016

Protein SHOX je hojně exprimován v buňkách růstových plotének, kde se zapojuje do regulace hypertrofie, proliferace a diferenciace chondrocytů. Zejména stimuluje tvorbu extracelulární matrix a ve vybraných buňkách spouští apoptózu. Interaguje s mnoha dalšími proteiny (včetně významného regulačního proteinu FGFR3 a genů rodiny SOX) a účastní se řady signálních drah (Marchini et al., 2016).



Obrázek 6

Shrnutí 230 mutací v exonech SHOX dle databáze SHOX (www.shox.uni-hd.de). Exon 2 obklopuje část 5'UTR a exony 6a a 6b část 3'UTR; exon 1 obsahuje pouze 5'UTR a často není zahrnut v diagnostických analýzách. Homeobox se nachází v exonu 3 a exonu 4. Nejčastějšími opakujícími se mutacemi jsou pArg147 v exonu 3 (sedmkrát) a pArg195 v exonu 5 (desetkrát). Modře jsou označeny unikátní mutace, červená představuje celkové množství mutací v daném exonu/oblasti. (Ex – exon; HB – oblast homeoboxu) – převzato z Marchini et al., 2016

Mutace genu *SHOX*, vedoucí k dysfunkčnosti či absenci proteinu SHOX, mohou vést k závažným klinickým důsledkům zahrnujícím malý vzrůst postavy, či obecněji patologický vývoj kostí. Klinicky významné jsou již mutace heterozygotní, což ukazuje na důležitost zachování genové dóze *SHOX*. Velmi přísná mnohaúrovňová regulace exprese umožňuje správné načasování tvorby poteinu. Zdá se ale, že množství vznikajícího proteinu SHOX vazané na jednu alelu není velké a v případě ztráty jedné alely není dostatečné k tomu, aby plně vykonával svou funkci. Přibližně 80 % detekovaných mutací

genu *SHOX* představují delece o různé velikosti zahrnující celý gen, nebo jeho regulační sekvence, dalších asi 5 % představují menší delece zasahující jen jeden, či několik exonů genu (Binder, 2011; Fukami et al., 2008; Chen et al., 2009). Delece v regulačních oblastech ovšem mohou být též součástí přirozené variability (Solc et al., 2014, P-III). Klinicky významné jsou též *missense* a *nonsense* bodové mutace, ke kterým dochází zejména v exonech 3 a 4 v místech kódujících homeobox, případně postihují transaktivační doménu kódovanou exonem 6a (Marchini et al., 2007) (viz obrázek 6). Zatímco u nalezených delecí je asociace s patologií zřetelná, u detekovaných duplikací je situace mnohem složitější. I když publikované práce ukazují, že duplikace jsou řazeny tandemově, je velmi obtížné stanovit, zda určitá duplikace výrazně narušuje expresi genu *SHOX*. Nicméně se zdá, že určité duplikace mají vysoký patogenní potenciál (Hirschfeldova et Solc, 2017, P-IV).

Poruchy genu *SHOX* jsou asociovány s Léri-Weillovým syndromem, Langerovým sydromem, idiopatickým malým vzrůstem a gonosomálními aneuploidiemi.

Léri-Weillův syndrom, neboli Léri-Weillova dyschondrosteóza (MIM 127300), vzniká v důsledku heterozygotní deficience genu *SHOX*. Jedná se o formu skeletální dysplazie s charakteristickou disproporčně malou postavou. Kromě malého vzrůstu patří mezi typické klinické projevy mesomélie (zkrácená předloktí a lýtka), cubitus valgus (vbočený loket), Madelungova deformita (zkrácená a ohnutá kost vřetení, dorsálně dislokovaná distální část kosti loketní) a zkrácené kosti metakarpální a metatarzální (Hintz, 2002). U přibližně 70 – 90 % pacientů s Léri-Weillovým syndromem byla prokázána delece genu *SHOX*, jeho části, nebo regulačních sekvencí (80 %), anebo bodová mutace (20 %) (Hirschfeldova et al., 2012, P-II; Kosho et al., 1999; Ogata et al., 2002).

Langerův syndrom, neboli Langerova mesomelická dysplázie (MIM 249700), je dosti vzácné onemocnění a v podstatě se jedná o extrémnější formu Léri-Weillova syndromu vznikající jako důsledek homozygotní deficience genu *SHOX*. Pacienti s Langerovým syndromem vykazují extrémně malý vzrůst a závažné dysmorfie kostí končetin, často dochází k hypoplázii nebo aplázii kosti loketní a lýtkové (Hintz, 2002).

Idiopatický malý vzrůst (MIM 300582) je charakterizován signifikantně malým vzrůstem (< -2 SDS), trvale nízkým tempem růstu a nepřítomností jiného postižení, které by mohlo být příčinou. Patologie genu *SHOX* se ukazuje jako původce přibližně 10 % tohoto onemocnění, což ji činí (s frekvencí 1:300 v celkové populaci) nejčastější monogenní příčinou malého vzrůstu (Marchini et al., 2016).

Významnou roli hraje heterozygotní deficience genu *SHOX* pravděpodobně i v rámci klinického profilu Turnerova syndromu, jehož příčinou je gonosomální monosomie (45,X0). Více než 90 % žen s Turnerovým syndromem je abnormálně malého vzrůstu a právě haploinsuficience genu *SHOX* by mohla být příčinou této složky fenotypu (Hintz, 2002). Naopak gonosomální aneuploidie, při nichž dochází ke zvýšení počtu gonosomů (47,XXX; 47XXY; 47,XYY; 48,XXXX; atd.), se projevují zvýšenou postavou, což také bývá přičítáno změně v počtu alel genu *SHOX* (Binder et al. 2001).

Předkládaná ***publikace II*** (Hirschfeldova et al., 2012, P-II) shrnuje výsledky výzkumu provedeného na 98 jedincích s diagnostikovanou Léri-Weillovou dyschondrosteosou nebo idiopatickým malým vzrůstem. Publikace popisuje mutace nalezené v genu *SHOX* a jeho regulačních oblastech. Dále předkládá rozbor fenotypových projevů pacientů a diskutuje jejich molekulárně genetické pozadí, zejména kryptické přestavby, jejichž klinický význam je nejasný (tzv. delece pod MLPA sondou L05101; různé duplikace).

Předkládaná ***publikace III*** (Solc et al., 2014, P-III) uvádí výsledky menší studie zaměřené konkrétně na tzv. deleci pod MLPA sondou L05101. Po porovnání frekvence jejího výskytu mezi pacienty s idiopatickým malým vzrůstem a zdravými jedinci vyhodnocuje deleci jako klinicky nevýznamnou součást přirozené variability. Studii doplňuje haplotypová analýza sekvenčních polymorfismů nalezených u pacientů (Hirschfeldova et al., 2012, P-II), ovšem bez statisticky významného výsledku.

Předkládaná ***publikace IV*** (Hirschfeldova et Solc, 2017, P-IV) představuje komparativní analýzu duplikací detekovaných v souboru 250 zdravých jedinců v porovnání s rozsahem a umístěním duplikací u pacientů z předchozích studií autorů, jež označila duplikace s vysokým patogenním potenciálem. Výsledky byly podpořeny metaanalýzou publikovaných duplikací ve sledované oblasti.

2.4 Kryptické přestavby chromosomálního regionu 8q24

Poslední oblastí vědeckého zájmu, jíž se zabývá tato práce, jsou strukturní aberace spojené s chromosomálním regionem 8q24, konkrétně s genem *TRPS1*, a mutace v genu *TRPS1*. V klinické rovině jsou tyto mutace asociované s tzv. trichorhinofalangeálním syndromem.

Trichorhinofalangeální syndrom (trichorhinophalangeal syndrome; TRPS) je velmi vzácné genetické onemocnění s typickými kraniofaciálními a skeletálními malformacemi. V současné době se rozlišují na klinické i molekulárně genetické úrovni tři typy označované jako TRPS I (OMIM #190350), TRPS II (OMIM #150230) a TRPS III (OMIM #190351).

Podtyp TRPS I představuje základní, nejčastější formu TRPS. Jeho charakteristickými klinickými znaky jsou krátké, řídké, jemné, pomalu rostoucí vlasy, hruškovitý tvar nosu, dlouhé philtrum, laterálně řídnoucí obočí, kuželovitě tvarované epifýzy především středních článků prstů rukou i nohou, mírně zkrácené metakarpální a metatarsální kosti, brachydaktylie (převážně způsobena brachymesofalangií), mírná postnatální růstová retardace a zpožděný kostní věk (Ludecke et al. 2001). Dále byly u některých pacientů zjištěny též vybočení prstů v proximálních interfalangeálních kloubech (Corsini et al. 2014), malé a nepravidelně tvarované femorální epifýzy (Vaccaro et al. 2005) a problémy s vývinem druhé dentice a opožděný zubní věk (Machuca et al. 1997). Příčinami TRPS I na molekulární úrovni mohou být mikrodelece, nebo bodová mutace v genu *TRPS1* (Ludecke et al. 2001; Solc et al., 2017, P-V).

TRPS II (jinak též Langer-Giedionův syndrom (LGS)) vzniká v důsledku heterozygotní mikrodelece zahrnující gen *TRPS1* a společně s ním také distálněji lokalizovaný gen *EXT1*; jedná se tedy o typický syndrom přilehlých genů (Ludecke et al. 1995). Delece postihuje přinejmenším všechny geny od *TRPS1* až po *EXT1*, tj. více než 1 Mb (Shanske et al. 2008), ale může být i rozsáhlejší – proximálně může zasahovat až do oblasti 8q22.2 a distálně až do oblasti 8q24.2 (Hilton et al. 2001; Hou et al. 1995; Ludecke et al. 1999; Solc et al., 2017, P-V). Klinické projevy tohoto syndromu v sobě spojují klasické symptomy TRPS I s mnohočetnými dědičnými exostózami typu I s původem v mutacích genu *EXT1* (hereditary multiple exostoses; OMIM #133700), a u většiny pacientů se projevuje též mírná až střední mentální retardace. Exostózy mohou být velmi variabilní co do počtu i velikosti (Tsang et al. 2014). Bylo popsáno, že pacienti s TRPS II mají v

průměru menší vzrůst, nežli pacienti s TRPS I či se samotnými izolovanými exostózami, pravděpodobně z důvodu kumulativního efektu, protože oba geny ovlivňují růst a vývoj skeletu. Pacienti s menší delecí vykazují i mírnější stupeň mentální retardace, ve srovnání s pacienty s rozsáhlejší delecí (Ludecke et al. 1991). Kandidátním genem pro mentální retardaci by u TRPS II mohl být proximálně se nacházející gen *CSMD3*, který je hojně exprimován ve fetálním i adultním mozku. Zdá se, že tento gen je neporušen u pacientů bez mentální retrdace, avšak minimálně u části pacientů s TRPS II trpících mentální retardací je deletován. Je tedy pravděpodobné, že haploinsuficience genu *CSMD3* by mohla být zodpovědna za mentální retardaci u pacientů s TRPS II (Ludecke et al. 1991; Riedl et al. 2004). U některých pacientů s TRPS II byly popsány i další symptomy, např. kožní defekty, laxity a hypermobilita kloubů, zvýšená predispozice ke kostním frakturám, mikrocefalie, problémy s příjmem potravy v dětství, u novorozenců hypotonie, opožděný psychomotorický vývoj s pozdním nástupem řeči apod. (Vaccaro et al. 2005). Zaznamenána byla i větší náchylnost k urogenitálním a respiračním infekcím (Michalek et al. 2009). Přítomnost či absence těchto a dalších znaků může být spojena s rozsahem delece u jednotlivých pacientů, tedy projevuje se zde i nadále jev popisovaný jako syndrom přilehlých genů (Fantauzzo and Christiano 2012; Hou et al. 1995; Pereza et al. 2012; Shanske et al. 2008).

Podtyp TRPS III, nejvzácnější ze spektra TRPS, se liší od TRPS II normální inteligencí a nepřítomností exostóz a od TRPS I podstatně výraznější růstovou retardací, brachydaktylií způsobenou zkrácením metakarpálních kostí a článků prstů a častým vybočením kostí v proximálních interfalangeálních kloubech (Niikawa and Kamei 1986). TRPS III tedy svou fenotypovou závažností představuje z klinického hlediska extrémní projev TRPS I. Je to dáno skutečností, že TRPS III vzniká v důsledku specifického typu mutací v genu *TRPS1*. Konkrétně se přitom jedná o *missense* mutace v exonu 6, jehož převážná část kóduje DNA-vazebný GATA motiv zinkového prstu (Hilton et al. 2002; Kobayashi et al. 2002; Ludecke et al. 2001; Maas et al. 2015).

Je ovšem nutno podotknout, že fenotypové projevy TRPS vykazují vysokou míru variability. Fenotyp se liší v závislosti na věku a pohlaví, a to jak u nepříbuzných pacientů nesoucích identickou mutaci, tak u pacientů příbuzných (Ludecke et al. 2001) a dokonce i u monozygotních dvojčat (Naselli et al. 1998).

Gen *TRPS1* (GeneID #7227) byl identifikován na dlouhém raménku chromosomu 8, v oblasti 8q24.1. Tvoří jej sedm exonů a zahrnuje přibližně 260,5 kb genomické DNA,

přičemž exony 2-7 jsou kódující (Momeni et al. 2000). Je exprimován ve více než 50 různých adultních i fetálních tkáních (Chang et al. 2002; Kunath et al. 2002; Malik et al. 2001; Momeni et al. 2000).

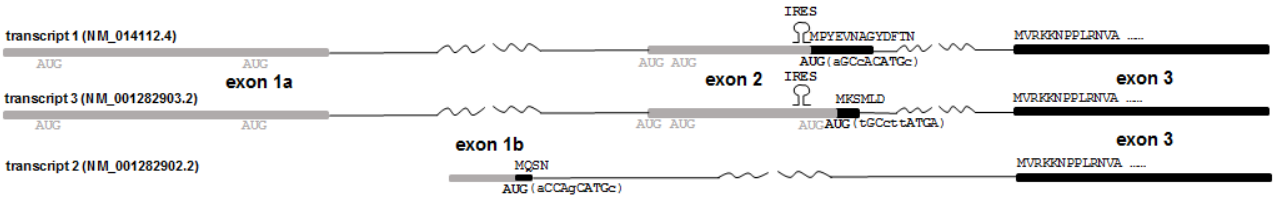
Gen *TRPS1* zasahují delece, jež mohou být různě velké. Popsány byly delece o velikosti jednoho, dvou či několika málo párů bazí, měnící čtecí rámec (tzv. „frameshift" mutace) (Nan et al. 2013), ale stejně tak i delece zahrnující značnou část dlouhého raménka chromosomu 8 (Hilton et al. 2002). Dále byly mnohokrát popsány i bodové *nonsense* či *missense* mutace (Ito et al. 2013), sestřihové mutace (Ludecke et al. 2001), paracentrické inverze zahrnující region 8q24.11 (Sasaki et al. 1997), inzerce (Vaccaro et al. 2005) či složitější přestavby – např. balancované translokace (Hou et al. 1995; Nardmann et al. 1997). S ohledem na publikovaná data lze říci, že přibližně polovina pacientů s TRPS I je nositeli mutace zděděné, zatímco u druhé poloviny došlo ke sporadické mutaci *de novo* (Chen et al. 2011; Corsini et al. 2014; Hilton et al. 2002; Ludecke et al. 2001).

Gen *TRPS1* kóduje protein TRPS1 skládající se z 1281 aminokyselin fungující jako jaderný transkripční faktor a řadící se do nové třídy transkripčních faktorů „multi-type zinc-finger". Protein TRPS1 vykazuje v jádře nehomogenní distribuci a je lokalizován v rozsáhlých avšak málo početných jaderných tělískách (Kaiser et al. 2003). Vedle toho existují náznaky, že by protein mohl vykonávat další minoritní funkci mimo jádro, která však zatím není dobře definována (Fantauzzo et al. 2008).

Ve struktuře tohoto proteinu se objevuje zcela unikátní kombinace několika rozdílných typů motivů zinkového prstu včetně GATA-type a IKAROS-like. Sekvence proteinu také obsahuje dva pravděpodobné lokalizační jaderné signály. Jedná se o jediný známý transkripční faktor, který ve své struktuře spojuje devět motivů zinkových prstů čtyř odlišných typů (Momeni et al. 2000). Tři z motivů zinkového prstu jsou typu C2H2 a sdílejí konsenzuální sekvenci C2X14H2. Další tři motivy zinkového prstu sdílejí konsenzuální sekvenci C2X12H2. Sedmý motiv zinkového prstu je typu C2C2 (CXNCX17CNXC) a vykazuje podobnosti s více než 160 proteinovými sekvencemi, zejména členů rodiny DNA-vazebných transkripčních faktorů GATA, což poukazuje na možné zahrnutí v regulaci genové exprese. Zbývající dva zinkové prsty typu C2H2 se nalézají na C-konci a vykazují vysokou míru podobnosti s téměř třemi sty dalšími motivy zinkového prstu u proteinů mnoha různých organismů. Nejvyšší stupeň podobnosti byl přitom nalezen u dvou charakteristických zinkových prstů z rodiny IKAROS (Chang et al. 2002; Malik et al. 2001).

Protein TRPS1 působí jako represor transkripce genů regulačně závislých na dalších transkripčních faktorech typu GATA. Pro tuto aktivitu je přitom nezbytná přítomnost C-terminální IKAROS-like domény (Malik et al. 2001), jejímž prostřednictvím se TRPS1 váže s kofaktory a aktivuje se (Ludecke et al. 2001; Sun et al. 1996). Aktivita TRPS1 jako transkripčního faktoru je rovněž kontrolována posttranslačními modifikacemi. V případě TRPS1 byla potvrzena SUMOylace na C-konci proteinu (navázání malých proteinů, tzv. small ubiquitin-like modifier). Jedná se pravděpodobně o klíčový mechanismus regulace aktivity tohoto transkripčního faktoru (Kaiser et al. 2007).

Mutace genu *TRPS1* mohou vést k tvorbě zkráceného proteinu postrádajícího C-terminální IKAROS-like doménu, který ztrácí schopnost se aktivovat. Předpokládá se, že proteiny s *missense* mutací jsou schopné dosáhnout jádra a formovat dimery či multimery skrze své IKAROS-like domény, nicméně mají nejspíše sníženou afinitu k DNA. Pokud ale *missense* mutace postihuje přímo GATA doménu, vzniklý protein se není schopen vázat na DNA a regulovat transkripci; takto mutované proteiny jsou tedy transkripčně neaktivní (Momeni et al. 2000).



Obrázek 7

Schéma 5'UTR a oblastí translačních startů v různých transkriptech genu TRPS1. – převzato ze Solc et al., 2017, P-V

Regulace exprese genu *TRPS1* není dosud objasněna. Jako klíčový transkripční faktor podílející se na řadě procesů, mimo jiné i na regulaci buněčného cyklu, lze předpokládat jeho přísnou kontrolu. Podle známých transkriptů byla rekonstruována organizace genu *TRPS1*. Není překvapením přítomnost alternativního sestřihu na 5´ konci genu, který vytváří různě dlouhé a různě strukturované 5´UTR sekvence a který vyžaduje přítomnost alternativních promotorů. Rovněž rozsáhlá 3´UTR oblast má jistě významnou

regulační úlohu (viz obrázek 7). Lze předpokládat, že se v okolí genu *TRPS1* budou nacházet i další *cis* regulační elementy. Řízení celého procesu transkripce a translace a odlišná biologická funkce odlišných transkriptů zůstává dosud neobjasněna (Solc et al., 2017, P-V).

Předkládaná ***publikace V*** (Solc et al., 2017, P-V) shrnuje výzkum provedený na souboru devíti nepříbuzných jedinců s trichorhinofalangeálním syndromem různého typu. Zahrnuje analýzu jejich fenotypu a odhaluje pravděpodobné kauzální příčiny vzniku syndromu na úrovni DNA. Zvolená metodika umožnila vedle kódujících oblastí genu analýzovat i rozsáhlé 5‘ a 3‘ netranslatované oblastí genu *TRPS1* a detekovat zde řadu polymorfismů. Získané poznatky jsou uvedeny do kontextu dosavadních publikovaných studií, zvýšená pozornost je věnována také regulaci exprese genu *TRPS1*.

2.5 Perspektivy výzkumu

Výzkum kryptických přestaveb lidských chromosomů se i do budoucna jeví jako velmi perspektivní a stojí před řadou zajímavých otázek. Oproti svým počátkům, kdy se pozornost soustředila zejména na klinicky významné přestavby s přímým patologickým dopadem na svého nositele, současný výzkum se vedle toho výrazněji zaměřuje: zaprvé na CNV, jež jsou součástí přirozené variability lidského genomu a nemají klinický význam (včetně těch, jež mohou představovat evoluční stopu); a zadruhé na přestavby, jež mohou nějakým způsobem hrát roli při vzniku komplexních, polygenně podmíněných a multifaktoriálních onemocnění.

Pakliže se zaměříme konkrétně na témata, jimž se věnuje tato práce, pak výše uvedené hraje významnou roli při studiu souvislostí mezi kryptickými přestavbami a mentální retardací (či v širším pohledu patologickým kognitivním a intelektuálním fungováním nervové soustavy). Výzkum, k němuž se snaží přispět i tato práce, zdaleka není u svého konce – množství kryptických přestaveb, jež jsou kauzálně spojeny se vznikem syndromů zahrnujících ve fenotypovém projevu mentální retardace (ale i těch bez tohoto symptomu), se neustále zvyšuje. Ovšem stále častěji se jedná nikoli o dobře definované, opakující se přestavby a lokusy, nýbrž o unikátní aberace vedoucí ke vzniku vzácných syndromů. Tato situace staví před nové výzvy jak lékaře, tak vědce, neboť jim neumožňuje opřít se při léčbě pacienta, potažmo při interpretaci biologického významu aberace, o bohatou evidenci a s ní již spojenou praxi, a vede je tak k hledání nových medicínských i vědecko-metodologických přístupů. Situaci při detekci vzácných kauzálních aberací navíc komplikuje výše uvedený fakt, že se zvyšující se schopností zmapovat genom člověka roste množství popsaných a u každého člověka též detekovaných CNV (např. Gallasso et al., 2010). Ulehčení naopak představují obsáhlé databáze (např. DECIPHER), které v poslední době umožňují spolehlivěji identifikovat strukturní polymorfismy a o něž se lze opřít při vyhodnocování významu nalezených přestaveb.

Vedle toho se začínají odhalovat i souvislosti mezi konkrétními přestavbami a heterogenními patologickými projevy, jako je např. epilepsie (např. Mullen et al., 2013). Ovšem zřejmě nejambicioznějším je v současnosti výzkum snažící se objasnit spojení kryptických přestaveb se vznikem a podobou poruch autistického spektra. Vznik této multifaktoriální patologie s vysoce polygenním genetickým pozadím byl v rámci mnoha studií asociován s bodovými mutacemi, strukturními aberacemi i epigenetickými mutacemi

mnoha genů. Aktuální výzkumy se snaží o nový pohled, kdy se nehledá spojení konkrétní mutace ani přesné role, kterou hrají jednotlivé geny, nýbrž přechází se na komplexnější pohled na jiných úrovních, nežli je genom, a to na úrovni transkriptomu, proteomu a interaktomu. Zde je již (zatím ovšem pouze v hrubých rysech a s požadavkem na další studie a posilování evidence) možné identifikovat určité signální a regulační dráhy, jejichž narušení (skrze bodovou, strukturní nebo epigenetickou mutaci kteréhokoli z velkého množství zapojených genů) je pravděpodobně spojeno se vznikem poruch autistického spektra (pro podrobné zpracování viz např. Krum et al., 2014).

Perspektivy výzkumu cíleného pouze na konkrétní chromosomální regiony, resp. oblasti asociované s konkrétními geny, jejž představují i další dvě témata této práce, se z povahy věci samozřejmě liší. Jak v případě genu *TRPS1* a trichorhinofalangeálního syndromu, tak v případě genu *SHOX* a s ním asociovaných onemocnění pokračují výzkumy zabývající se konkrétními patologiemy genů a spojující je s konkrétními klinickými důsledky a perspektivami. Vedle patologií kódujících sekvencí, vedoucích k absenci proteinového produktu či jeho defektům, se pozornost zaměřuje i na patologie regulace genové exprese. Zde jsou jak v případě genu *TRPS1*, tak genu *SHOX* intenzivně zkoumány nekódující sekvence exonů (5'UTR a 3'UTR) a také promotorové sekvence. V případě genu *SHOX* je ovšem stále větší důraz kladen i na výzkum regulačních, enhancerových sekvencí v okolí genu. Moderní metodologické přístupy umožňují v současné době analýzu právě i nekódujících sekvencí, jejichž výzkum byl dříve často kvůli jejich délce a nízké komplexnosti opomíjen. V případě genů jako jsou transkripční faktory *TRPS1* a *SHOX*, kde se předpokládá velmi přísná mnohaúrovňová regulace exprese genu, by měla analýza regulačních oblastí přejít do rutinní praxe. Překážkou zatím zůstává nedostatečně standardizovaný a zjednodušený systém testování a interpretace funkčního významu nalezených variant. Nicméně vzhledem k bouřlivému rozvoji tohoto odvětví se dá předpokládat, že i tato překážka bude brzy odstraněna. Výzkum duplikací v pseudoautosomální oblasti odhaluje komplikace spojené s jejich interpretací, které mají obecnější charakter. Důležitou roli hraje uspořádání duplikované oblasti, zda jsou primárně narušeny kódující či regulační sekvence, ale i možnost kompetice duplikovaných regulačních elementů o transkripční faktory, jejichž množství může být omezené, případně zda nové prostorové uspořádání chromosomální domény nenarušuje spávnou interakci mezi faktory v různých *cis* regulačních elementech (Hirschfeldova et Solc (2017, P-IV)). Výzkum genu *SHOX*, s ohledem na množství jeho známých regulačních elementů, které

jsou i relativně dobře charakterizované, a na častý výskyt drobných přestaveb, může sloužit jako vzor pro výzkum dalších podobných genů.

Předmětem výzkumu zůstává v případě obou genů i funkce kódovaných proteinů. V případě TRPS1 nejsou všechny jeho funkce ještě zdaleka zmapované. V případě SHOX se jedná spíše o detailnější výzkum, např. v rámci jeho aktivity v prehypertrofických chondrocytech je třeba upřesnit, která z jeho funkcí se v kterém kroku procesu uplatňuje, jak jsou tyto odlišné funkce koordinovány apod. Vedle toho je třeba věnovat pozornost tomu, jakou úlohu hraje SHOX v dalších tkáních. Zejména by protein SHOX podle některých náznaků mohl hrát roli při vývoji nervové tkáně (specificky se to týká nově popsaných izoforem zahrnujících exony 7-1, 7-2 a 7-3). V poslední době se dokonce objevují i teorie, že právě i patologie SHOX by mohla přispívat ke vzniku poruch autistického spektra (viz např. Tropeano et al., 2016).

Souhrnem lze říci, že kryptické přestavby lidských chromosomů představují výzkumnou oblast, která skrývá mnohá tajemství a jejíž studium nám může přinést řadu poznatků o přirozené strukturní variabilitě lidského genomu a jeho evoluci, fyziologickém i patologickém fungování genů a proteinů, regulaci genové exprese i o složitém genetickém pozadí polygenních a multifaktoriálních onemocnění. A čím více se o kryptických přestavbách lidských chromosomů dozvídáme, tím očividnější je, že ještě nesrovnatelně více toho zůstává před našimi zraky ukryto.

2.6 Použité zkratky

3'UTR ... 3' untranslated region (3' netranslatovaný region)

5'UTR ... 5' untranslated region (5' netranslatovaný region)

aCGH ... array CGH

C ... C konec proteinu

CGH ... comparative genomic hybridization (komparativní genomová hybridizace)

CKII ... casein kinase II (casein kináza II)

CNE ... conservative noncoding element

CNV ... copy number variations

CSMD3 ... CUB And Sushi Multiple Domains 3 (lidský gen)

DNA ... deoxyribonucleic acid (deoxyribonukleová kyselina)

Ex ... exon

EXT1 ... exostosin 1 (lidský gen)

FGFR3 ... fibroblast growth factor receptor 3 (lidský protein)

FISH ... fluorescent *in situ* hybridization (fluorescenční *in situ* hybridizace)

FoSTeS ... fork stalling and template switching

HB ... homeobox (homeobox)

HD ... homeodomain (homeodoména)

LCR ... low-copy repeats

MAPH ... multilocus amplifiable probe hybridization

m-FISH ... multicolour FISH (mnohobarevná FISH)

MLPA ... multiplex ligation-dependent probe amlification

N ... N konec proteinu

NAHR ... non-allelic homologous recombination (nealelická homologní rekombinace)

NHEJ ... non-homologous end joining (nehomologní spojování konců)

NMD … nonsense-mediated mRNA decay (degradace transkriptů s předčasným terminačním kodónem)

NLS ... nuclear localization signal (jaderný lokalizační signál)

OAR ... otp, aristaless and rax (transaktivační doména)

P ... místo fosforylace

PAR1 ... pseudoautosomal region 1 (pseudoautosomální region 1)

PCR ... polymerase chain reaction (polymerázová řetězová reakce)

qPCR ... quantitative PCR (kvantitativní PCR)

RUST … regulative unproductive splicing and translation (tvorba neproduktivních transkriptů jako mechanismus regulace exprese genu)

SHOX ... short stature homeobox-containing gene (lidský gen)

SHOX ... short stature homeobox-containing gene (lidský protein)

SHOX2 ... short stature homeobox-containing gene 2 (lidský gen)

Shox2... short stature homeobox-containing gene 2 (myší gen)

SKY ... spectral karyotyping (spektrální karyotypizace)

SOX ... SRY-related HMG-box (rodina genů)

SUMO … small ubiquitin-like modifier (signální protein)

TAR … telomere associated repetition

Tel … telomera

TRPS ... trichirhinophalangeal syndrome (trichorhinofalangeální syndrom)

TRPS1 ... transcriptional repressor GATA binding 1 (lidský gen)

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3. Výstupy výzkumu

3.1 Publikace v impaktovaných časopisech

Publikace I

Hirschfeldova K, Baxova A, Kebrdlova V, **Solc R**, Mihalova R, Lnenicka P, Vesela K, Stekrova J (2011) *Cryptic chromosomal rearrangements in children with idiopathic mental retardation in the Czech population*. Genetic Testing and Molecular Biomarkers, 15(9), 607-611

Impact factor (2011) = 1,110

Journal ranking (2011) = Q4 (Genetics et Heredity)

Citační ohlas (ke dni 17.10.2017)
dle WOS 1x citováno; dle SCOPUS 1x citováno; dle ResearchGate 3x citováno

Shrnutí obsahu

Publikace shrnuje a diskutuje výsledky výzkumu provedeného na souboru jedinců s diagnostikovanou idiopatickou mentální retardací. U těchto pacientů byly pomocí metody MLPA vyhledány kryptické přestavby, jež mohly být kauzální příčinou onemocnění. Pozitivní nálezy byly popsány a diskutovány.

Podíl předkladatele

Předkladatel se na publikačním výstupu podílel zejména teoretickou prací, a to obecnou rešerší problematiky (teoretický úvod článku) a cílenou konfrontací pozitivních nálezů s publikovanými případy a studiemi (součást diskuse).

Publikace II

Hirschfeldova K, **Solc R**, Baxova A, Zapletalova J, Kebrdlova V, Gaillyova R, Prasilova S, Soukalova J, Mihalova R, Lnenicka P, Florianova M, Stekrova J (2012) SHOX *gene defects and selected dysmorphic signs in patients of idiopathic short stature and Léri-Weill dyschondrosteosis*. Gene, 491(2), 123-127

Impact factor (2012) = 2,196

Journal ranking (2012) = Q3 (Genetics et Heredity)

Citační ohlas (ke dni 17.10.2017)
dle WOS 21x citováno; dle SCOPUS 23x citováno; dle ResearchGate 25x citováno

Shrnutí obsahu

Publikace shrnuje a diskutuje výsledky výzkumu provedeného na souboru jedinců s diagnostikovaným Léri-Weillovým syndromem (dyschondrosteózou), či izolovaným malým vzrůstem (idiopatickým). U těchto pacientů byly pomocí metody MLPA vyhledány kryptické přestavby, jež mohly být kauzální příčinou onemocnění, a dále byla provedena mutační analýza exonů genu *SHOX* za využití Sangerova sekvenování. Pozitivní nálezy byly popsány a diskutovány. Vedle kauzálních aberací byly zachyceny též takové, jejichž vztah k patologii byl nejasný (rozlišné duplikace, delece pod sondou L05101 (MLPA kit P018)).

Podíl předkladatele

Předkladatel se podílel na provedení laboratorních analýz (sběr dat) a dále prováděl teoretickou práci, a to obecnou rešerši problematiky (teoretický úvod článku) a cílenou konfrontaci pozitivních nálezů s publikovanými případy a studiemi (součást diskuse).

Publikace III

Solc R, Hirschfeldova K, Kebrdlova V, Baxova A (2014) *Analysis of common* SHOX *gene sequence variants, and ~4.9kb PAR1 deletion in ISS patients*. Journal of Genetics, 93(2), 505-508

Impact factor (2014) = 1,093

Journal ranking (2014) = Q4 (Genetics et Heredity)

Citační ohlas (ke dni 17.10.2017)
dle WOS 1x citováno; dle SCOPUS 1x citováno; dle ResearchGate 1x citováno

Shrnutí obsahu

Publikace shrnuje a diskutuje výsledky dílčí studie mapující výskyt delece pod sondou L05101 (MLPA kit P018) a porovnávající jej s výskytem v souboru pacientů s izolovaným malým vzrůstem, přičemž je delece vyhodnocena jako populační polymorfismus bez klinického významu. Tuto studii doplňuje konstatace výsledků haplotypové analýzy bodových polymorfismů, jež ovšem nepřináší žádný pozitivní výsledek.

Podíl předkladatele

Předkladatel je autorem designu studie týkající se delece pod sondou L05101 (MLPA kit P018), provedl veškeré laboratorní práce s ní spojené a je autorem manuskriptu (s výjimkou části týkající se haplotypové analýzy). Na haplotypové analýze se nepodílel.

Publikace IV

Hirschfeldova K, **Solc R** (2017) *Comparison of* SHOX *and associated elements duplications distribution between patients (Lëri-Weill dyschondrosteosis/idiopathic short stature) and population sample*. Gene, 627, 164-168

Impact factor (2016) = 2,415

Journal ranking (2016) = Q3 (Genetics et Heredity)

Citační ohlas (ke dni 17.10.2017)
dle databází dosud necitováno

Shrnutí obsahu

Publikace charkaterizuje duplikace v regionu genu *SHOX* nalezené v souboru 250 zdravých jedinců. V rámci následující metaanalýzy jsou porovnávány duplikace popsané v této a předchozích publikacích spoluautorů a další duplikace nalezené u zdravých či pozitivně diagnostikovaných osob publikované v literatuře. Na jejím základě jsou vyvozeny závěry ohledně patogeničnosti duplikací a ohledně funkčního významu oblastí sekvencí v regionu genu *SHOX*.

Podíl předkladatele

Předkladatel je autorem celkové koncepce výzkumu, organizoval vytvoření souboru dárců, provedl veškeré laboratorní analýzy a spolupodílel se na psaní manuskriptu. Metaanalitickou část práce rozpracovali oba spoluautoři samostatně ve více variantách a následně ve spolupráci dokončili, přičemž finální podoba vychází z varianty navržené předkladatelovou školitelkou a spoluautorkou.

Publikace V

Solc R, Klugerova M, Vcelak J, Baxova A, Kuklik M, Vseticka J, Beharka R, Hirschfeldova K (2017) *Mutation analysis of* TRPS1 *gene including core promoter, 5´UTR, and 3´UTR regulatory sequences with insight into their organization*. Clinica Chimica Acta, 464(1), 30-36

Impact factor (2016) = 2,873

Journal ranking (2016) = Q2 (Medical Laboratory Technology)

Citační ohlas (ke dni 17.10.2017)
dle databází dosud necitováno

Shrnutí obsahu

Publikace shrnuje a diskutuje výsledky výzkumu provedeného na souboru jedinců s diagnostikovaným trochorhinofalangeálním syndromem. U těchto pacientů byly pomocí metody MLPA vyhledány kryptické přestavby, jež mohy být kauzální příčinou onemocnění, a dále byla provedena mutační analýza kódujících i nekódujících částí exonů genu *TRPS1* za využití sekvenování nové generace a kontrolního Sangerova sekvenování. Pozitivní nálezy byly popsány a diskutovány.

Podíl předkladatele

Předkladatel je autorem celkové koncepce výzkumu, provedl značnou část laboratorních analýz, spolu s lékaři analyzoval klinický profil pacientů a napsal významnou část manuskriptu.

3.2 Příspěvky na mezinárodních odborných konferencích

1) **Solc R**, Hirschfeldova K: Comparison of the distribution of duplicated regions associated with *SHOX* gene between LWD/ISS patients and population sample. European human genetic conference (ESHG), May 2017, Copenhagen; poster

2) **Solc R**, Hirschfeldova K, Klugerova M, Vcelak J, Baxova A, Kuklik M, Vseticka J: *TRPS1* gene defects in patients with trichorhinophalangeal syndrome. European human genetic conference (ESHG), May 2016, Barcelona; poster

3) Mihalova R, Hirschfeldova K, Baxova A, Kebrdlova V, Lnenicka P, **Solc R**, Stekrova J, Vesela K: Detection efficiency of FISH vs. MLPA method in microdeletion syndromes patients. European human genetic conference (ESHG), May 28.-31. 2011, Amsterdam RAI, Netherlands; poster, abstrakt: European Journal of Human Genetics, 2011,Vol.19,Suppl.2,p.145

4) Hirschfeldova K, Kebrdlova V, **Solc R**, Florianova M, Lnenicka P, Stekrova J, Mihalova R, Baxova A: *SHOX* gene polymorphic variants and their association with isolated short stature. European human genetics conference (ESHG), May 28.-31. 2011, Amsterdam, Netherlands; poster, abstrakt: European Journal of Human Genetics, 2011,Vol.19,Suppl.2,p.120-121

5) Hirschfeldova K, Kebrdlova V, Mihalova R, Stekrova J, Vesela K, Lnenicka P, Florianova M, **Solc R**, Baxova A: MLPA analysis in children with mental retardation. European human genetics conference (ESHG) , June 12.-15. 2010, Gothenburg, Sweden; poster, abstrakt: European Journal of Human Genetics, 2010, Vol.18, Suppl.1, p.121.

6) Baxova A, Kebrdlova V, **Solc R**, Lnenicka P, Florianova M, Stekrova J, Mihalova R, Vesela K, Hirschfeldova K: PAR1 deletion/duplication in patients with dyschondrosteosis or idiopathic short stature. European human genetics conference (ESHG) , June 12.-15. 2010, Gothenburg, Sweden; poster, abstrakt: European Journal of Human Genetics, 2010,Vol.18,Suppl.1,p.342

3.3 Příspěvky na domácích odborných konferencích

1) **Šolc R**, Klugerová M, Včelák J, Baxová A, Kuklík M, Všetička J, Beharka R, Hirschfeldová K: Komplexní molekulárně genetická analýza genu *TRPS1* u pacientů s trichorhinofalangeálním syndromem. 20. celostátní konference DNA diagnostiky, listopad 2016, Dolní Morava; přednáška

2) **Šolc R**, Klugerová M, Hirschfeldová K, Baxová A, Kuklík M: Molekulárně genetická analýza u pacientů s trichorhinofalangeálním syndromem. 19. celostátní konference DNA diagnostiky, listopad 2015, České Budějovice; přednáška

3) **Šolc R**, Klugerová M, Hirschfeldová K, Baxová A, Kuklík M: Strukturní aberace chromozomové oblasti 8q24 a mutace v kódujících oblastech genu *TRPS1* u pacientů s trichorhinofalangeálním syndromem 48. cytogenetická konferenc SLG ČLS, září 2015, Brno; poster, abstrakt ve sborníku

4) **Šolc R**, Hirschfeldová K, Kebrdlová V, Baxová A: Role ~4,9 kb delece v regulačních oblastecch genu *SHOX* v regionu PAR1: příčina malého vzrůstu, anebo populační polymorfismus? Konference GSGM, 24.-26. září 2014, Průhonice; poster, abstrakt ve sborníku: str. 92

5) Hirschfeldová K, Kebrdlová V, Mihalová R, Štekrová J, Lněnička P, **Šolc R**, Vranová V, Baxová A: Komplexní genetická analýza u dětí s mentální retardací a opožděným PMR vývojem. 15. celostátní konference DNA diagnostiky, 24.-25. listpadu 2011, Praha; poster

6) Hirschfeldová K, Baxová A, Kebrdlová V, **Šolc R**, Lněnička P, Gaillyová R, Mihalová R, Veselá K, Štekrová J: Komplexní analýza *SHOX* genu u pacientů s dyschondrosteózou a idiopatickým malým vzrůstem. 14. celostátní konference DNA diagnostiky, 25.-26. listopadu 2010, Brno; poster, abstrakt ve sborníku: str.55

4. Předkládané publikace

Publikace I 43
Hirschfeldova K, Baxova A, Kebrdlova V, **Solc R**, Mihalova R, Lnenicka P, Vesela K, Stekrova J (2011) *Cryptic chromosomal rearrangements in children with idiopathic mental retardation in the Czech population*. Genetic Testing and Molecular Biomarkers, 15(9), 607-611

Publikace II 48
Hirschfeldova K, **Solc R**, Baxova A, Zapletalova J, Kebrdlova V, Gaillyova R, Prasilova S, Soukalova J, Mihalova R, Lnenicka P, Florianova M, Stekrova J (2012) SHOX *gene defects and selected dysmorphic signs in patients of idiopathic short stature and Léri-Weill dyschondrosteosis*. Gene, 491(2), 123-127

Publikace III 53
Solc R, Hirschfeldova K, Kebrdlova V, Baxova A (2014) *Analysis of common* SHOX *gene sequence variants, and ~4.9kb PAR1 deletion in ISS patients*. Journal of Genetics, 93(2), 505-508

Publikace IV 57
Hirschfeldova K, **Solc R** (2017) *Comparison of* SHOX *and associated elements duplications distribution between patients (Lėri-Weill dyschondrosteosis/idiopathic short stature) and population sample*. Gene, 627, 164-168

Publikace V 62
Solc R, Klugerova M, Vcelak J, Baxova A, Kuklik M, Vseticka J, Beharka R, Hirschfeldova K (2017) *Mutation analysis of* TRPS1 *gene including core promoter, 5´UTR, and 3´UTR regulatory sequences with insight into their organization*. Clinica Chimica Acta, 464(1), 30-36

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Cryptic Chromosomal Rearrangements in Children with Idiopathic Mental Retardation in the Czech Population

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Aims: The aim of our study was to scan for cryptic rearrangements using the multiplex ligation probe amplification method in a cohort of 64 probands with mental retardation or developmental delays in combination with at least one of the following symptoms: hypotonia after birth, congenital anomalies, or face dysmorphisms; but without a positive cytogenetic finding. The study contributes to the knowledge of microdeletion syndromes and helps disclose their natural phenotypic variability. *Results:* In total, 10 positives (16%) were detected, particularly 3 duplications (Xpter-p22.32; 17p11.2; 22q11) and 6 different deletions (1p36; 7q11.23; 10p15; 15q11-q13; 17p11.2; 17p13.3), 1 of these in 2 probands. Besides the well-characterized syndromes, less-often described rearrangements with ambiguous phenotype associations were also detected. *Conclusions:* Some rearrangements, particularly duplications, are associated with vague phenotypes; and their frequency could be underestimated.

Introduction

Mental retardation may occur as either isolated or associated with several malformations or dysmorphias. The underlying cause of mental retardation remains unknown in up to 80% of all patients (Rauch *et al.*, 2006). Chromosomal aberrations are the most commonly known cause of mental retardation. Standard cytogenetic banding techniques hold resolution limits of 5–8 Mb; and are, thus, not powerful enough to detect chromosomal rearrangements below 5 Mb in size (Salman *et al.*, 2004). It is for this reason these types are referred to as "cryptic" rearrangements.

Cryptic chromosomal rearrangements have been detected on all human chromosomes (Ballif *et al.*, 2007) and may arise all over the genome; however, some chromosomal areas are more prone to them than are others. Microdeletions/duplications mostly arise as the result of frequent unequal crossover events, between sites sharing more than a 97% homology (the so-called, low copy segments, LCRs) (Shaffer *et al.*, 2007). Such high-homology sites evolved during genome evolution by chromosomal segment duplication (Conrad and Antonarakis, 2007). Subtelomeric regions are more frequently the subject of microdeletion than are the other parts of the chromosomes (Mefford and Trask, 2002). There are DNA sequences adjacent and centromeric to the $(T_2AG_3)_n$ sequence; these are the so-called telomere associated repeats, made up of blocks of repetitive DNA (Flint *et al.*, 1997). These sequences are shared among many different subsets of telomeres and evolved as a result of nonhomologous chromosome recombination (Shaffer and Lupski, 2000).

Over the last several years, the employment of modern cytogenetic and molecular genetic techniques has enabled progressive diagnosis of distinctive cryptic rearrangements as the underlying causes of idiopathic mental retardation (Rooms *et al.*, 2006). However, due to the extensive phenotypic variation, as well as mutually overlapping phenotypes, the correct syndrome diagnosis is often difficult. Thus, our study contributes to the level of knowledge of these microdeletion syndromes and helps further disclose their natural phenotypic variability.

Materials and Methods

Subjects

Overall, 64 probands were included in the study. Study subjects were recruited from the Department of Medical Genetics (1st Faculty of Medicine and General Teaching Hospital, Charles University in Prague) in close cooperation with both the Department of Neurology and the Department of Pediatrics and Adolescent Medicine, which are the specialist consultancy locations for these matters within the entire Czech Republic.

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Patients' selection criteria were the presence of either mild-to-severe mental retardation or developmental delay in combination with at least one of the following symptoms: hypotonia after birth, congenital anomalies, and face dysmorphisms; but without a positive cytogenetic finding during standard cytogenetic karyotyping. The introduction of a patient into the project was the sole decision of the clinical geneticist. All patients who were introduced (or their official legal representatives) signed an informed consent form for the taking of blood, DNA analysis, and agreement to submitting to the study. If required, an informed consent form for blood taking and DNA analysis was signed with the proband's relative, as well.

DNA analysis

The EDTA blood was stored at 4°C and then processed within 48 h after venisection. Genomic DNA was isolated from the peripheral blood using QIAGEN spin columns on a QIAcube (QIAGEN, GmbH). Both DNA purity and concentration was measured on a NanoPhotometer (IMPLEN GmbH). DNA concentration was adjusted to 30–80 ng/μL before further analysis. The detection of microdeletion rearrangements was carried out by using the multiplex ligation probe amplification (MLPA) method (MRC-Holland), which enables simultaneous analysis of tens of genomic regions (Schouten *et al.*, 2002).

Each proband was analyzed by use of MLPA kit P245 to detect the 21 most common microdeletion syndromes. Additional microdeletion syndromes were analyzed using MLPA kit P297. The subtelomeric regions were analyzed with the use of the MLPA kit P036. Additional MLPA kits (P018, P064, P147, P250, and ME028) were used for further deletion/duplication verification and specification; and if used are mentioned in the results section of a particular case. The MLPA reaction was run with 50–150 ng of DNA, according to the manufacturer's instructions. The MLPA analysis was carried out by fragmentation analysis conducted on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). Visual examination of the peak patterns and peak areas normalization was done for each sample according to the instructions of the manufacturer for each particular MLPA kit.

Results

A total of 64 probands were screened for cryptic deletion/duplication using the MLPA method. The patients' ages ranged from 2 months to 15 years, and the male-to-female ratio was 1.4:1 (37:27). The most frequent age at diagnosis was between 1 and 4 years (34 probands). Mental retardation could only be assessed in those patients who were at least 3 years old ($n = 38$).

Overall, 10 positives (16%) were detected; specifically 3 duplications and 7 deletions using the MLPA kits P245, P036, and P297 (Table 1).

In the case of the 1p36 deletion syndrome, only the TNFRSF4 probe of MLPA kit P245 was involved. The GNB1 probe and the GABRD probe were both outside of the deleted region. Additional deletion specification (P147) detected two separate deletions. One deletion, <1 Mb, spanned from the TNFRS18 probe to the DVL1 probe; the second and largest (about 4 Mb) deletion covered the chromosomal area from the AJAP1 probe to the SLC45A1 probe. The nondeleted region between the two deletions is about 3 Mb. The 3-year-old boy involved was 95 cm tall and 17 kg in weight. Considerable characteristic craniofacial dysmorphisms were detected. This boy suffered from mild mental retardation, hypotonia, and epilepsy. No auditory findings were detected. Both phenotypically healthy parents were analyzed using the MLPA kit P147 to exclude the presence of a 3 Mb duplication, which can mask the proband's nondeleted area. No such duplication was detected in either parent.

In the case of Williams syndrome (7q11.23), Smith-Magenis syndrome (17p11.2), and Miller-Dieker syndrome (17p13.3), all probes from the MLPA kit P245 were deleted. Both probands with Williams syndrome display the deletion of all appropriate probes from the P064 MLPA kit. The deletion extends >1 Mb and encompasses the ELN and LIMK1 genes, among others. In a 3-year-old boy, the Williams syndrome was combined with the typical osteogenesis imperfecta (OI). This proband possessed the characteristic facial features, developmental delay, short stature, as well as pulmonary artery and aortic stenosis. The second proband with Williams syndrome was a 7-month-old girl. She was found to be having intrauterine growth retardation, characteristic facial features,

Table 1. Summary of All Rearrangements Found and the Multiplex Ligation Probe Amplification Kits Used for Their Detection and Specification

	MLPA kit				
Proband no.	*P245*	*P297*	*P036*	*Aberration*	*Additional MLPA kits*
MD4	+	–	+	1p36 deletion	P064; P147
MD6	–	–	+	Xpter-p22.32 (SHOX) duplication	P018
MD20	+	–	–	7q11.23 deletion	P064
MD21	+	–	–	22q11 duplication	P250
MD22	+	–	–	17p13.3 deletion	P064
MD27	+	–	–	17p11.2 duplication	P064
MD39	+	–	–	15q11.2-q1.2 deletion	ME028
MD48	+	–	–	17p11.2 deletion	P064
MD53	+	–	–	10p15 (GATA3) deletion	P250
MD63	+	–	–	7q11.23 deletion	P064

+ indicates a positive analysis; – indicates a negative analysis.
MLPA, multiplex ligation probe amplification.

and developmental delay; but no cardiac developmental defects were detected. A proband with Smith-Magenis syndrome displayed the deletion of all P064-relevant probes. The deletion extends > 2.5 Mb. The 6-year-old boy displayed hypotonia after birth, craniofacial dysmorphism, microcephaly, and mild mental retardation. Additionally, sleep disturbances and auto-aggressive behaviors were detected. In the case with Miller-Dieker syndrome, three of seven MLPA P064 probes were deleted. The deletion encompasses the METT10D and LIS1 genes, and its range is below 1.5 Mb. The girl proband was hospitalized at 2 months because of repeated apnoeic pauses and seizures. She was further found to be having developmental delay, microcephaly, extension hypertonia, and severe congenital brain defects (lyssencephaly type I; agyria).

In the case with Prader-Willi syndrome (PWS), all of the MLPA kit P245 probes were deleted. Further analysis (ME028) displayed the paternal origin of the deleted fragment. The 2-month-old boy suffered from severe hypotonia, cryptorchidism, characteristic facial dysmorphism, and developmental delay.

In one case, the 10p deletion of one MLPA P245 probe was detected. The deleted probe targeted the GATA3 gene exon 1 and corresponds to the DiGeorge 2 region (10p12-15). The MLPA kit P250 revealed the GATA3 probe deletion targeting exon 4. The 9-year-old girl displays intrauterine growth retardation, developmental delay, microcephaly, short stature, auditory defects, nephropathy, and syndactyly (incomplete simple syndactyly between the third and fourth finger of the upper limbs and complete simple syndactyly between the second and third toes).

Three various duplications were detected. In one case, the P245 analysis showed a duplication of all three 17p11.2 probes, corresponding to the Smith-Magenis region. The duplication was verified using the P064 MLPA kit; and all relevant 17p11 probes were duplicated. The 18-year-old girl suffered from moderate mental retardation, language impairment, short stature, a gait defect, facial dysmorphism (narrow face, prominent nose, short philtrum, pointed chin), and a cataract. A 22q11 duplication was detected in a 10-year-old girl with mild mental retardation, learning disability, disharmonic emotional development, mild craniofacial dysmorphism, and slight stature. All MLPA P425 probes were duplicated. The MLPA kit P250 was used for additional specification; showing that the duplication covers the region 22q11 A-D, and spans > 2.5 Mb. The same duplication was found in the proband's mother.

Surprisingly, in one case with mild mental retardation, the interstitial duplication in the PAR1 region (including the SHOX gene) was detected using the P036 MLPA kit, and then the P018 kit for additional specification. The 3-year-old girl displayed hypotonia, macrocephaly, facial dysmorphism, and short stature. The same duplication was also found in the proband's father.

Discussion

We used the MLPA method to search for cryptic deletions/duplications in a cohort of 64 probands. The high methodological effectiveness (16%) in our study sample was established by stringent introduction of the probands by clinical geneticists with abundant experience in the fields of mental retardation and dysmorphias in children. The detection rate of molecular karyotyping could be around 20% in such a cohort of patients (Gijsbers *et al.*, 2009). Besides the well-characterized syndromes, less-often described rearrangements with ambiguous phenotype associations were also detected.

Monosomy 1p36 (MIM 607872) is the most common terminal deletion syndrome, with an estimated prevalence of 1 in 5000 (Shapira *et al.*, 1997; Shaffer and Lupski, 2000). In our case, two distinct interstitial deletions were detected: a small deletion, < 1–1.5 Mb distant from the p-telomere; as well as a large deletion, 4 to 8(9) Mb distant from the p-telomere. Such complex rearrangements have also been described in other studies (Heilstedt *et al.*, 2003). In our proband, a number of characteristic craniofacial symptoms are present; however, the common auditory findings were not detected (Shapira *et al.*, 1997; Heilstedt *et al.*, 2003). It seems that the critical auditory region is outside of the deleted area. Windpassinger *et al.* (2002) suggested that the *GABRD* gene (gene ID: 2563) may be a candidate for the neurodevelopmental and neuropsychiatric anomalies seen in this syndrome. Surprisingly, the *GABRD* gene was not affected in our proband.

In two cases, the deletion of 7q11.23 was detected as associated with Williams syndrome (WBS; MIM 194050). The estimated WBS frequency is between 1 in 7500 and 1 in 20,000 (Greenberg, 1989; Stromme *et al.*, 2002). Three large region-specific segmental duplications are responsible for recurrent chromosomal rearrangements in 7q11.23 locus (Valero *et al.*, 2000). In both our cases, the deletion is > 1 Mb long, and it encompasses both the *ELN* (gene ID: 2006) and *LIMK1* genes (gene ID: 3984). Consequently, we assume the most common 1.55 Mb deletion exhibited in most patients (95%) (Bayés *et al.*, 2003). Mutations/deletions of the *ELN* gene are responsible for vascular and connective tissue abnormalities (Ewart *et al.*, 1993). In one proband, the *ELN* deletion is not associated with congenital cardiac defects; however, considering her age at diagnosis, some less-severe heart abnormalities such as heart murmurs could later emerge. In the proband with OI, the condition was inherited from the mother (OI type III; MIM 259420), and the 7q11.23 deletion seems to be a coincidental event.

Two probands with a 17p11.2 rearrangement were detected. The overall phenotype, including sleep defects and self-injury in our proband with the 17p11.2 deletion, is in accordance with the diagnosis of Smith-Magenis syndrome (MIM 182290). The phenotype of the proband with the 17p11.2 duplication fulfills the characteristics for Potocki-Lupski syndrome (MIM 610883). The cataract condition was inherited from the mother. Both syndromes are the result of a nonallelic homologous recombination between region-specific LCRs (Chen *et al.*, 1997; Potocki *et al.*, 2000). In both our cases with Smith-Magenis syndrome and site duplication, the rearrangement area is > 2.5 Mb long. The most frequent recurrent deletion/duplication is ~3.7 Mb long (Juyal *et al.*, 1996; Chen *et al.*, 1997).

The 17p13.3 deletion is associated with the Miller-Dieker syndrome (MIM 247200). A deletion or mutation in the *LIS1* gene only (gene ID: 601545) appears to cause the isolated lissencephaly (ILS; MIM 607432) (Cardoso *et al.*, 2003). The common prevalence of Miller-Dieker syndrome and ILS is 1 in 40,000 live births (Fleck *et al.*, 2000). In our proband, the deletion is < 1.5 Mb in range (and no facial dysmorphisms were detected); so, ILS seems to be the correct diagnosis.

PWS (MIM 176270) is a neurogenetic disorder usually caused by chromosomal deletion on chromosome 15q11-q13, by uniparental disomy or by imprinting defects. The chromosomal alterations result in an aberrant expression profile of gene loci that are subject to imprinting. The clinical features, as well as methylation status of CpG islands in the *SNRPN* gene (gene ID: 6638), in our proband are in compliance with a diagnosis of PWS. The estimated frequency of PWS is ~1 in 10,000 (Steffenburg *et al.*, 1996; Cassidy, 1997). Carrozzo *et al.* (1997) suggested recombination or an intrachromosomal loop as the mechanisms that underlie the interstitial *de novo* deletions at 15q11-q13 locus.

The 22q11 duplication is a reciprocal rearrangement to the deletion mainly associated with DiGeorge syndrome (MIM 188400) with birth prevalence of at least 1 in 4000 (Devriendt *et al.*, 1998). The high frequency of 22q11 copy number changes is due to the presence of several copies of a repeat sequence (LCR22). The extent of the 22q11 rearrangement is variable, although 87% extends from the first (LCR22-A) until the fourth (LCR22-D) repeat (Shaikh *et al.*, 2000). Unlike deletion, the site duplication is associated with many variable and ambiguous phenotypes (Courtens *et al.*, 2008). In our case, the duplication was detected to the same extent in the proband's mother (who displays neither pathological features nor behaviors). Similarly, most individuals in whom the 22q11.2 duplication was diagnosed and with obvious clinical features have inherited the duplication from a parent with a normal or near-normal phenotype (Courtens *et al.*, 2008). It could be a susceptible genotype for mental retardation without full penetrance; or simply an ascertainment bias, where the duplication represents population variability with no direct effect on the phenotype.

The DiGeorge 2 critical region (MIM 601362) is located on 10p13-14. It seems that the more telomeric (10p14-10pter) deletion is responsible for the HDR syndrome phenotype (MIM 146255) (Van Esch *et al.*, 2000). Haploinsufficiency of the *GATA3* gene (gene ID: 2625) is only associated with the HDR syndrome (MIM 146255). *GATA3* is essential in the embryonic development of the parathyroids, auditory system, and kidneys (Van Esch *et al.*, 2000). Our proband displays hearing defects and nephropathy; however, no hypoparathyroidism. However, symptoms of the HDR syndrome are variably expressed between and within families (Muroya *et al.*, 2001). It seems that the proband is simultaneously a carrier of the syndactyly type 1 (MIM 185900).

In one of our probands, the interstitial duplication of the *SHOX* gene (gene ID: 6473) (PAR1 region; Xp) was detected. Deletions of the *SHOX* gene are well documented (Bertorelli *et al.*, 2007). In contrast, *SHOX* duplication is rare; only a few cases have thus far been described, and the associated phenotype is highly variable (Thomas *et al.*, 2009). Recently, *SHOX* duplication was associated with type I Mayer-Rokitansky-Kuster-Hauser syndrome (MIM 277000) (Miozzo *et al.*, 2010). In our proband, we found a short stature without complying with the increased *SHOX* gene copy number; additionally, the overall phenotype does not satisfy the criteria for the Mayer-Rokitansky-Kuster-Hauser syndrome. *SHOX* duplications are likely to be under-ascertained, and more cases need to be well characterized, to accurately determine their phenotypic consequences.

The MLPA method is sufficient to reasonably analyze a number of susceptible chromosomal regions at the same time. It is particularly beneficial for probands with vague phenotypes. However, the MLPA method is not able to detect reciprocal rearrangements. Our data support the assumption that the majority of the symptoms may be the result of a small deletion within critical regions. Consequently, it is essential to precisely evaluate the extent of the rearrangement, to enable the determination of such syndrome critical regions and to assess symptom penetrance and variable expressivity. Besides the role of genes in the involved interval, there are multiple factors such as regulatory sequences, epigenetic mechanisms, parental origin, and nucleotide variations in the nonaffected allele that may also be important.

Acknowledgment

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Disclosure Statement

No competing financial interests exist.

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SHOX gene defects and selected dysmorphic signs in patients of idiopathic short stature and Léri–Weill dyschondrosteosis

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ABSTRACT

The aim of the study was to analyze frequency of *SHOX* gene defects and selected dysmorphic signs in patients of both idiopathic short stature (ISS) and Léri–Weill dyschondrosteosis (LWD), all derived from the Czech population.

Overall, 98 subjects were analyzed in the study. Inclusion criteria were the presence of short stature (−2.0 SD), in combination with at least one of the selected dysmorphic signs for the ISS+group; and the presence of Madelung deformity, without positive karyotyping for the LWD+group. Each proband was analyzed by use of P018 MLPA kit, which covers *SHOX* and its regulatory sequences. Additionally, mutational analysis was done of the coding portions of the *SHOX*.

Both extent and breakpoint localizations in the deletions/duplications found were quite variable. Some PAR1 rearrangements were detected, without obvious phenotypic association. In the ISS+group, MLPA analysis detected four PAR1 deletions associated with a *SHOX* gene defect, PAR1 duplication with an ambiguous effect, and two *SHOX* mutations (13.7%). In the LWD+group, MLPA analysis detected nine deletions in PAR1 region, with a deleterious effect on *SHOX*, first reported case of isolated *SHOX* enhancer duplication, and *SHOX* mutation (68.8%). In both ISS+and LWD+groups were positivity associated with a disproportionately short stature; in the ISS+group, in combination with muscular hypertrophy.

It seems that small PAR1 rearrangements might be quite frequent in the population. Our study suggests disproportionateness, especially in combination with muscular hypertrophy, as relevant indicators of ISS to be the effect of *SHOX* defect.

1. Introduction

The *SHOX* (Short Stature Homeobox-Containing Gene; Gene ID: 6473) is located in the main pseudoautosomal region (PAR1) and is grouped among the so-called homeobox genes, which are distinguished by a homeodomain. Like all genes in the pseudoautosomal region, it escapes X inactivation and shows a "pseudo-autosomal" inheritance pattern (Rao et al., 1997). Mutations or deletions/duplications affecting the production of the *SHOX* are associated with: Turner syndrome; Léri–Weill dyschondrosteosis (LWD; MIM ID: 127300); its homozygote form, Langer mesomelic dysplasia (MIM ID: 249700); as well as with idiopathic short stature (ISS; MIM ID: 300582) (Chen et al., 2009; Ellison et al., 1996; Sabherwal et al., 2007). In these syndromes, *SHOX* defects are implicated in inaccurate bone development and longitudinal body growth.

The clinical symptoms of the *SHOX* deficiency are highly variable, and the phenotype can be markedly different, even among affected

Abbreviations: A, adenine; c, cDNA level; C, cytosine; CEN, centromeric; CNE, conserved non-coding DNA element; DMSO, dimethylsulfoxide; fhGH, recombinant human growth hormone; G, guanine; ISS, idiopathic short stature; Kb, kilobase(s) or 1000 bp; L, leucine; LWD, Léri–Weill dyschondrosteosis; Mb, megabase(s) or 1000 000 bp; MLPA, multiplex ligand-dependent probe amplification; N, asparagine; OAR, otp, aristaless, and rax domain; p, protein level; P, proband; PAR1, main pseudoautosomal region; R, arginine; S, serine; *SHOX*, short stature homeobox-containing gene; SD, standard deviation; T, thymine; TEL, telomeric.

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members of the same family (Grigelioniene et al., 2001). The symptoms are usually more pronounced in women, which is the effect of estrogen (Fukami et al., 2004; Ogata et al., 2001).

The variability of the *SHOX* pathology contains both deletions/duplications (mainly cryptic) and point mutations (Chen et al., 2009; Sabherwal et al., 2007). Size of deletions does not correlate with the intensity of the clinical phenotype (Schiller et al., 2000). Deletions can include both the *SHOX* and its regulating regions (Benito-Sanz et al., 2005; Fukami et al., 2006). Partial *SHOX* duplications appeared to have a more deleterious effect on skeletal dysplasia and duplication height gain than complete *SHOX* duplications (Benito-Sanz et al., 2011b). However, the effect of duplications and the associated phenotype is highly variable (Hirschfeldova et al., 2011; Thomas et al., 2009). One exon which is mostly affected by causal point mutations is exon 3 (Marchini et al., 2007), coding the greater part of the homeodomain. The management of short stature in ISS and LWD patients, caused by SHOX-related haploinsufficiency includes treatment with recombinant human growth hormone (rhGH), in order to improve the final adult height (Blum et al., 2007). Early diagnosis is thus highly advantageous.

The aim of the study was to analyze the frequency of *SHOX* defects, as well as selected dysmorphic signs in patients of both ISS and LWD (all derived from the Czech population, and in order to improve its diagnosis within the Czech Republic).

2. Materials and methods

2.1. Subjects

The study's participants were screened for deletions or mutations in the *SHOX*, and its known regulatory sequences. Overall, 98 subjects were analyzed in the study. To follow the indication criteria for extended *SHOX* gene analysis in our laboratory practice the ISS+ and LWD+ study samples were designed. The ISS+ study sample consists of 51 unrelated probands and 11 relatives; the LWD+ study sample included 16 probands and 20 relatives. Study subjects were recruited from the Department of Medical Genetics of the 1st Faculty of Medicine and the General Teaching Hospital, Charles University in Prague, in close cooperation with the Department of Medical Genetics of the University Hospital in Brno as well as the Department of Paediatrics of the University Hospital in Olomouc. All of the patients that were introduced into the study (or their legal representatives) signed an informed consent form for the taking of blood, DNA analysis, plus an agreement to submit to the study. If required, an informed consent form for blood taking and DNA analysis was signed with the proband's relative, as well.

The probands' selection criteria for the ISS+ study sample were the presence of short stature (−2.0 SD), in combination with at least one of following dysmorphic signs: disproportionate stature, cubitus valgus, short forearm, bowing of forearm, muscular hypertrophy, or dislocation of ulna (at elbow). The recommended dysmorphic signs were obtained from the study by Rappold et al. (2007) that provides quantitative clinical guidelines for testing of the *SHOX* gene.

No GH deficiency or resistance, as well as no known growth-influencing medications, were detected in the selected probands. For the LWD+ study sample, the probands' inclusion criteria were the presence of at least unilateral Madelung deformity, but without a positive cytogenetic finding during standard cytogenetic karyotyping.

2.2. DNA analysis

The EDTA blood was stored at 4 °C, and then processed within 48 h after venesection. The genomic DNA was isolated from the peripheral blood using QIAGEN spin columns on a QIAcube (QIAGEN, GmbH, Germany). Detection of the PAR1 rearrangements was carried out by use of the multiplex ligand-dependent probe amplification (MLPA) molecular genetic method (MRC-Holland, the Netherlands) (Schouten et al., 2002).

Each proband was analyzed by use of a MLPA kit P018, which covers the *SHOX*, its regulatory sequences, and the adjacent X-specific region. The MLPA reaction was run with 50–150 ng of DNA, according to the manufacturer's instructions. The MLPA analysis was carried out by fragmentation analysis, conducted on an ABI PRISM 310 Genetic Analyser (Applied Biosystems, CA, USA). At first, using the raw data, a visual examination of the peak patterns was performed for each sample, as well as between each sample; additionally, negative controls were also run. The peak areas were normalized, according to the instructions of the manufacturer. In order to obtain the final results, the normalized probe ratios of each sample were divided by the normalized probe ratios of the negative control(s) run. A personally constructed Microsoft Excel table was used for the entry of all of these calculations.

Additionally, DNA sequencing was done of the coding portions of the *SHOX* exons 2, 3, 4, 5, 6a, and 6b. We used primers by Huber et al. (2001), with minor modifications to amplify exons 2 through 5. The following primers were used to amplify exons 6a and 6b: 5′ taggggagaagaggcacgtt 3′ as a forward, and 5′tcctcaggcctcttgcag 3′ as a reversed primer for 6a amplification; and 5′ttcaccgtgttagccaggaa 3′ as a forward, and 5′ggatcacctgaggtcaggagtt 3′ as a reversed primer for 6b amplification. The 25 µl reaction mixture contained: 50–120 ng of genomic DNA, 1.5 mM $MgCl_2$, 0.2 µM of each primer, 0.1 mM of each dNTP, and 0.5 unit of *Taq* DNA Polymerase (recombinant) in the manufacturer's provided $(NH_4)_2SO_4$ buffer (Fermentas, Vilnius, Lithuania). The amplification conditions were: 95 °C for 2 min as a denaturation step, followed by 40 cycles of 1 min at 94 °C, 30 s at 68 °C (exon 3, exons 4–5); or 61 °C (exon 6a and 6b), and 30 s at 72 °C, ending with 3 min at 70 °C. Exon 2 was amplified at adjusted conditions of 95 °C for 2 min as a denaturation step, followed by 10 cycles of 1 min at 94 °C, 45 s at 72 °C, followed by 30 cycles of 1 min at 94 °C, 45 s at 69 °C, ending with 3 min at 70 °C. The sequencing reaction was performed according to the manufacturer's instructions (we added DMSO), using the BigDye® Terminator v1.1 Cycle Sequencing Kits (Applied Biosystems, Foster City, CA, USA), and run on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

3. Results

All probands were screened for copy number variation in the PAR1 region, including the *SHOX*, and its regulatory sequences, using the MLPA kit P018. The largest deletion is in the range of around ~6 Mb (P61); the smallest deletion has less than 0.5 Mb (P24, P25). Duplications in the PAR1 region range of ~130 kb (P7), ~500 kb (P10), and 200–600 kb (P67). Both the extent and telomeric (TEL) or centromeric (CEN) breakpoint localization in the deletions/duplications found were quite variable (Fig. 1). The *SHOX* mutation analysis involved the coding portions of the *SHOX* exons 2, 3, 4, 5, 6a, and 6b.

3.1. The ISS + group

The ISS+ probands included 31 women and 20 men, ranging in age from 5 to 29. The primary inclusive criteria were growth retardation, diagnosed as a height below −2.0 SD. In 64% of the patients, the short stature was familial. One proband was adoptive. The listing and frequencies of selected dysmorphic signs can be found in Table 1.

The MLPA analysis detected four PAR1 deletions associated with the *SHOX* defect (7.8%); three *SHOX* deletions, and one enhancer CNE9 deletion (CNE; conserved non-coding DNA element). The extent of the deletion is outlined in Fig. 1. In a boy proband (P3), the familial short stature (−2.0 SD) was associated with muscular

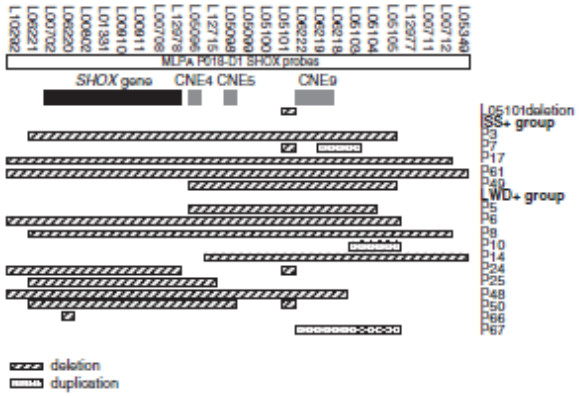

Fig. 1. The MLPA probes deleted/duplicated in particular probands (P) from the ISS+ and the LWD+ groups are indicated. Three known downstream *SHOX* enhancers are shown (CNE4, CNE5, CNE9) (Sabherwal et al., 2007). The L05101 deletion is a common deletion without any evidential effect on phenotype. Detailed characterization of the MLPA probe mix is available on MRC Holland websites (www.mlpa.com).

hypertrophy and a disproportionate stature. The deletion was inherited from the proband's father (168 cm; −2.0 SD). In a girl proband (P17), the idiopathic short stature (−2.0 SD) was associated with a short forearm, bowing of the forearm, the appearance of muscular hypertrophy, and disproportionateness. The proband's parents, with normal stature, were not analyzed. In another girl proband (P49), familial short stature (−2.5 SD) was associated with a short forearm, muscular hypertrophy, and disproportionateness. The proband's relatives, with proportion short stature, were negative for the deletion. Surprisingly, there was only one positive proband of the seven potential, coming from families with an incidence of the Madelung deformity. The boy proband (P61) has a short stature (−2.5 SD) associated with a short forearm, muscular hypertrophy and disproportionateness. Besides the causal deletions, a small deletion covering only a single probe was detected in six probands (11.8%). The deletion localization is outlined in Fig. 1 as a L05101 deletion. The same deletion was observed among relatives with normal heights, as well. One proband (P7) had the L05101 deletion in combination with a small duplication at the PAR1 region (Fig. 1). It is not clear whether the *SHOX* regulatory sequences (enhancer CNE9) are impaired by this duplication. The P7 proband displays disproportionate stature, short forearm, and cubitus valgus. The *SHOX* mutation analysis detected two distinct mutations in exon 6a (P1; P11), plus a single common polymorphism in exon 6b (Table 2); the rare G allele frequency in the ISS probands were 0.457±0.051. The mutation p.S275N of exon 6a localizes to the conserved C-terminal amino acid portion known as the OAR/aristaless domain. Proband P1 displays a short stature, the appearance of muscular hypertrophy, and disproportionateness. The p.R280L mutation of exon 6a in proband P11 is associated with a

Table 1
Frequency of monitored dysmorphic signs in *SHOX* defect positive and negative probands from the ISS+ and the LWD+ groups.

Dysmorphic sign	ISS+ group		LWD+ group	
	Negative	Positive	Negative	Positive
	n=45 (%)	n=6 (%)	n=5 (%)	n=11 (%)
Disproportionate stature	28 (62.2)	6 (100.0)	4 (80.0)	9 (81.8)
Cubitus valgus	19 (42.2)	1 (16.7)	3 (60.0)	5 (45.5)
Short forearm	32 (71.1)	4 (66.7)	4 (80.0)	11 (100.0)
Bowing of forearm	10 (22.2)	1 (16.7)	4 (80.0)	8 (72.7)
Muscular hypertrophy	23 (51.1)	5 (83.3)	4 (80.0)	9 (81.8)
Dislocation of ulna (at elbow)	0	0	0	0

Table 2
Summary of all detected *SHOX* gene variants in the ISS+ and the LWD+ group found in coding part of exon 2-6a/6b.

Variant	Protein	Exon	Group	Status	SHOX database ID[a]
c.361 C>T	p.R121C	3	LWD+	Mutation	–
c.824 G>A	p.S275N	6a	ISS+	Mutation	–
c.839 G>T	p.R280L	6a	ISS+	Mutation	–
c.63 C>T	p.G21G	2	LWD+	polymorphism	SHOX_00068
c.657 A>G	p.P219P	6b	ISS+, LWD+	polymorphism	SHOX_00135

[a] SHOX® http://www.hd-lovd.uni-hd.de/ (Flanagan et al., 2002; Fokkema et al., 2005).

short stature, a short forearm, and disproportionateness. In both probands' relatives, the mutation was associated with disproportionate short statures, not with a proportional short stature. The frequency of the co-occurrence of muscular hypertrophy and disproportionate stature in the ISS+ group is 40.4%; however, it was present in all but one of the positive probands.

3.2. The LWD + group

The LWD+ probands included 14 women and 2 men, ranging from 8 to 43 years of age. The inclusive criteria were the presence of the Madelung deformity accompanied in all but three probands (−1.0 SD) with short stature (−2.0 SD). The list and frequencies of the monitored dysmorphic signs are summarized in Table 1. There was no obvious difference in the incidence of monitored dysmorphic signs between the *SHOX* defect positive and negative probands. The MLPA analysis detected nine deletions in the PAR1 region, with a deleterious effect on the *SHOX*. In seven probands, the deletion covers the *SHOX* coding sequences (in one proband P66 only the *SHOX* exon 2 was involved); in two probands, only the *SHOX* downstream enhancer CNE9 was involved. In proband P67 duplication outside the *SHOX* coding region was detected. The duplication covers CNE9 enhancer sequences and proband P67 displays Madelung deformity associated with proportional short stature (−2.5 SD), short forearm, cubitus valgus, and muscular hypertrophy. The extent of the deletions/duplication is outlined in Fig. 1. The L05101 deletion was detected in two probands (12.5%); in both probands, it was in combination with the *SHOX* deletion. The *SHOX* mutation analysis discloses a mutation in exon 3 (P10), and two polymorphisms (exon 2 and 6b). All of the detected *SHOX* variants are shown in Table 2. The girl proband (P10) had inherited the exon 3 mutation, as well as a small PAR1 duplication (Fig. 1) from her mother diagnosed for LWD, and the exon 2 polymorphism from a phenotypically normal father. The rare allele frequencies of the polymorphic sites in the LWD+ probands were: frequency of the c.63 T allele 0.077±0.052; and the c.657 G allele frequency 0.423±0.097.

In 20 of the probands' relatives the *SHOX* defect was associated with both disproportionate short stature (especially in men), or the Madelung deformity. However, proportional short stature was present in the probands' relatives, without any related *SHOX* defect being found.

4. Discussion

The study was performed in order to determine the frequency of *SHOX* defects in both the ISS+ and LWD+ groups, derived from within the Czech population. The selected dysmorphic signs were monitored at the same time, in order to improve the diagnosis, particularly in the ISS+ group. Both the extent and the telomeric or centromeric breakpoint localization in the deletions/duplications found were highly heterogeneous, and no hotspot was observed. This is in contrast to studies by both Schneider

et al. (2005) and Zinn et al. (2006); but in compliance with the findings of Benito-Sanz et al. (2006). The PAR1 region exhibits one of the highest recombination rates throughout the entire human genome due to the high frequency of repeat elements. Duplications and the common L05101 deletion, with no obvious phenotypic association, indicate a relatively frequent occurrence of PAR1 rearrangements in the general population.

The ISS covers a diverse cohort of patients of short statures; therefore only those who disclosed at least one of the recommended dysmorphic signs were inclosed (ISS+ group). The most frequent dysmorphic sign was a short forearm; however, all but one positive patient were characterized by the co-occurrence of muscular hypertrophy and a disproportionate stature. The L05101 deletion corresponds to the ~4.9 kb deletion described by Benito-Sanz et al. (2011b); and it is considered as a non-pathogenic copy number variant (Benito-Sanz et al., 2011a). Overall, there were five PAR1 rearrangements; one with an ambiguous effect on the phenotype, as well as two point *SHOX* mutations. The effect of the duplication in proband P7 could be pathogenic due to a direct CNE9 enhancer involvement or a positional effect. The higher detection rate (13.7%), compared to some previous studies is caused by the study inclusion criteria (Jorge et al., 2007; Rappold et al., 2002).

The most frequent dysmorphic sign in the LWD+ group was a short forearm, followed by a disproportionate stature. It seems that true shortening of the total length of radius is the primary feature of Madelung deformity caused by *SHOX* gene haploinsufficiency. Origin of Madelung deformity in negative probands may be a result of different processes. No obvious difference in the incidence of the monitored dysmorphic signs between the positive and negative LWD+ patients can be explained by the existence of additional undetected *SHOX* defects or locus(es), with minor effects. The duplication in proband P10 is combined with *SHOX* exon 3 mutation in both, proband and her mother. The effect of duplication is thus hard to assess. The duplication does not encompass CNE9 enhancer sequences but it may include a novel enhancer or act due to a positional effect. The only duplication notably related to *SHOX* expression was detected in proband P67. It is the first case described to have isolated duplication of *SHOX* enhancer sequences. Our data thus contributes to studies trying to determine the potential phenotypic consequences of *SHOX* duplications (Thomas et al., 2009). Our detection rate of the *SHOX* defects in the LWD+ group (68.8%) is consistent with the multi-ethnic study by Chen et al. (2009). The greater portion of women in the LWD+ group, when compared with men; as well as the association of a disproportionate short stature without the Madelung deformity with haploinsufficiency of the *SHOX* in probands' male relatives, is in compliance with the estrogen effect (Ogata et al., 2001). The relatively high frequency of proportional short stature in families with LWD+, without associated *SHOX* haploinsufficiency, could be the result of asorting matings. As with most mammals, humans tend to mate with like individuals, particularly with visible or noticeable traits. We are not able to unambiguously consider the role of a rare polymorphic variant c.63 C>T. The rare T allele was detected in two patients in the LWD+ group; however, in none within the ISS+ group. In the family of proband P10, the variant was not segregating with the phenotype. We analyzed its frequency in the population sample of 96 unrelated individuals, derived from the Czech population. The population frequency was 0.016±0.009, and is comparable to previously reported frequencies (Flanagan et al., 2002). Its high frequency in the LWD+ group could be a result of small sample size or of stratification.

In conclusion, small PAR1 rearrangements, without obvious phenotype association, could be quite frequent in the population. Our study suggests disproportionateness, especially in combination with muscular hypertrophy, as relevant indicators of ISS to be the effect of the *SHOX* defect.

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RESEARCH NOTE

Analysis of common *SHOX* gene sequence variants and ~4.9-kb PAR1 deletion in ISS patients

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[Solc R., Hirschfeldova K., Kebrdlova V. and Baxova A. 2014 Analysis of common *SHOX* gene sequence variants and ~4.9-kb PAR1 deletion in ISS patients. *J. Genet.* **93**, 505–508]

Introduction

Defects of the *SHOX* gene (short stature homeobox-containing gene), localized in the pseudoautosomal region 1 (PAR1) have been associated with Léri–Weill dyschondrosteosis (LWD; MIM ID: 127300) (heterozygous microdeletion or causal point mutation), Langer syndrome (MIM ID: 249700) (homozygous defect), and idiopathic short stature (ISS; MIM ID: 300589). The same pathological phenotype can be caused by an aberration in flanking regulatory sequences of *SHOX* gene (f.e. Flanagan *et al*. 2002). Defects of the *SHOX* gene or its regulatory sequences are the origin of LWD in about 70% of patients (Kosho *et al*. 1999; Ogata *et al*. 2002; Hirschfeldova *et al*. 2012) and in about 3% of ISS patients (f.e. Rappold *et al*. 2002). What we do not know is the role of common *SHOX* gene sequence variants and a small common ~4.9-kb deletion (MLPA kit P018 SHOX, probe L05101) ~200 kb downstream of *SHOX* gene near its known regulatory sequences (f.e. Benito-Sanz *et al*. 2006; Hirschfeldova *et al*. 2012). Common variants could be responsible for common phenotypes such as ISS. A common variant can act as a predisposition and should be more frequent in patients but their presence in controls is not excluded. Deletion L05101 is quite common in ISS and LWD groups but was also detected in healthy people (Chen *et al*. 2009).

Materials and methods

The population group was 84 healthy individuals (51 women and 33 men). We used quantitative real-time PCR with a TaqMan probe specific for L05101 deletion (Custom TaqMan, Applied Biosystems, Prague, Czech Republic) for population sample analysis. We considered difference in threshold cycle number C_t between the locus examined by the L05101 probe and the control locus of the *GADPH* gene in one reaction. For L05101 locus we used forward primer 5′-CGGGAAATCGTAACCACTGTCA-3′, reverse primer 5′-GGAATTGGAGAATGCGGTTTGTTAA-3′ and FAM-labelled TaqMan probe 5′-CTGAGAGACCCAAATTG-3′. For the *GADPH* locus we used a probe that targeted exonic sequences (kindly provided by Ales Horinek). We expected that if C_t(L05101)–C_t(GADPH) = n go for people without deletion in L05101, than for people with heterozygous deletion go C_t(L05101)–C_t(GADPH) = $n + 1$. The frequency of L05101 deletion in the ISS group was obtained in well-characterized ISS group from our previous study as a part of MLPA analysis (Hirschfeldova *et al*. 2012). Only *SHOX*-defect-negative ISS patients were included ($N = 45$) for this purpose.

Direct sequencing of *SHOX* gene exon 1 and a noncoding part of exon 2 was conducted in the ISS group (primers on request). Exon one analysis was designed to cover adjacent 5′ end sequences. Analyses of coding part of exon 2 and of exons 3, 4, 5, 6a and 6b were conducted as a part of our previous study. In population sample only exons where polymorpic variants were detected in the ISS group were analysed (exons 1, 2, 6b).

Each polymorphic site was tested for Hardy–Weinberg equilibrium (HWE). To analyse an indirect effect of polymorphic variants we estimated common haplotypes. The linkage disequilibrium measurement between each pair of polymorphic sites (significance level = 0.05) and haplotype frequencies estimation based on a Gibbs sampling strategy were done using

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Keywords. *SHOX*; deletion; PAR1; idiopathic short stature; polymorphism.

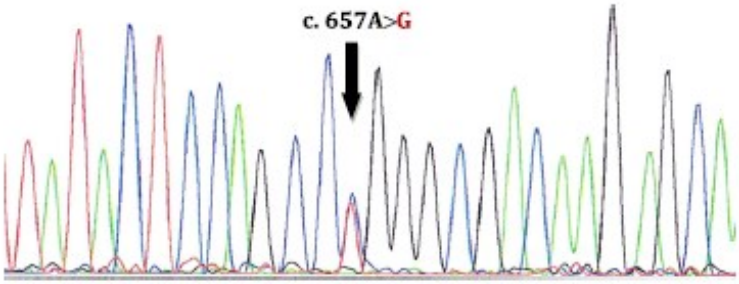

Figure 1. Polymorphic variant detected in exon 6b of *SHOX* gene (antisense strand).

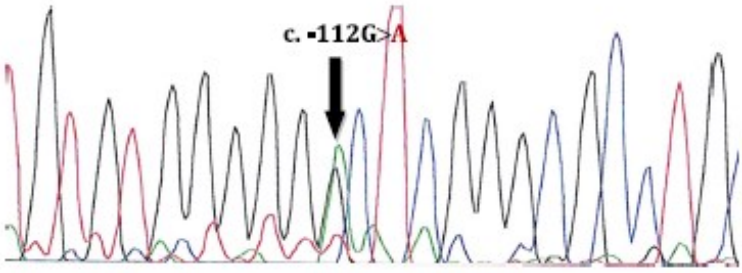

Figure 2. Polymorphic variant detected in exon 2 of *SHOX* gene (untranslated part).

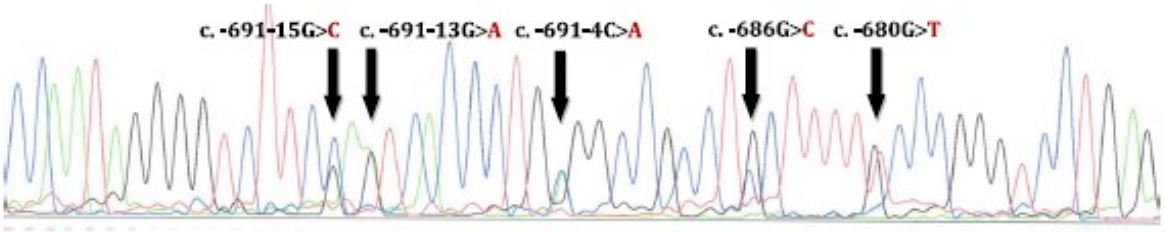

Figure 3. Polymorphic variants detected in the 5′ end and exon 1 of *SHOX* gene.

Arlequin software ver. 3.1. (Excoffier *et al.* 2005). Rare allele and haplotype frequencies were compared by chi-squared test (STATISTICA ver. 9.1) (StatSoft 2010).

Results

In population sample L05101 deletion was found in 11 individuals (nine women and two men) (13.1%). In ISS group we detected the L05101 deletion in six probands (13.3%). It reflects the L05101 deletion frequency is not significantly different between patiens with ISS and population sample ($P = 0.05$). No significant difference of the L05101 deletion frequnency was detected when comparing men and women in both ISS patients and in the population group.

Overall, 10 polymorphic variants were detected in the ISS group and population sample using the direct sequencing of the *SHOX* gene and adjacent 5′ regulatory sequences

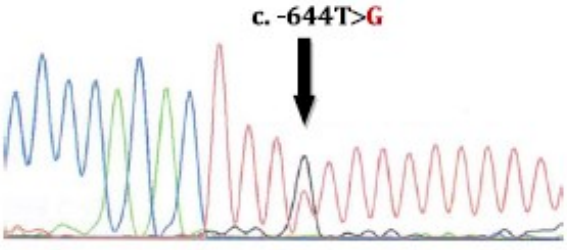

Figure 4. Polymorphic variants detected in exon 1 of *SHOX* gene.

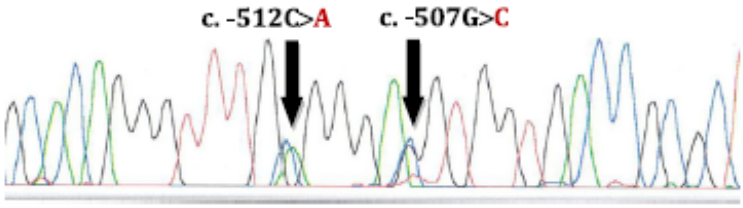

Figure 5. Polymorphic variants detected in exon 1 of *SHOX* gene.

Table 1. Frequency and localization of common *SHOX* gene polymorphic sites in the ISS group and population sample. Sequence variants are described according to HGVS recommendations based on the coding DNA reference sequence (NM_006883.2) (den Dunnen and Antonarakis 2000).

Variant	Localization		Frequency ± SD		SHOX	
			ISS group	Population	Database ID[1]	refSNP
c. −691 -15G>C	5′ end	Promoter	0.244 ± 0.047	0.253 ± 0.032	–	–
c. −691 -13G>A	5′ end	Promoter	0.244 ± 0.047	0.253 ± 0.032	–	–
c. −691 -4C>A	5′ end	Promoter	0.268 ± 0.049	0.242 ± 0.031	–	–
c. −686 G>C	Exon 1	Promoter	0.244 ± 0.047	0.242 ± 0.031	SHOX_00163	rs28475683
c. −680 G>T	Exon 1	Promoter	0.256 ± 0.048	0.258 ± 0.032	SHOX_00216	rs3813940
c. −644 T>G	Exon 1	Promoter	0.354 ± 0.053	0.300 ± 0.033	–	–
c. −512 C>A	Exon 1	Promoter	0.025 ± 0.017	0.042 ± 0.015	SHOX_00365	rs113313554
c. −507 G>C	Exon 1	Promoter	0.325 ± 0.052	0.363 ± 0.035	SHOX_00366	rs111549748
c. −112 G>A	Exon 2	Promoter	0.110 ± 0.035	0.135 ± 0.025	–	–
c. 657 A>G	Exon 6b	p.Pro219Pro	0.452 ± 0.054	0.422 ± 0.036	SHOX_00135	Flanagan *et al.* (2002)

[1]SHOX @ http://www.hd-lovd.uni-hd.de/.

(figures 1–5) (table 1). Five polymorphisms were already described in the *SHOX* database. The position of polymorphic variants and corresponding rare allele frequencies in both study groups are summarised in table 1. Allele frequencies were in HWE in both ISS group and population sample. There was no statistically significant difference in the rare allele frequencies between the ISS group and population sample. Linkage disequilibrium between each pair of polymorphic sites was analysed. Strong linkage disequilibrium was only preserved among polymorphic sites from 5′ end sequences and exon 1. The c. − 512C > A polymorphism was excluded from further analysis because of low frequency. Nine-pol haplotypes were estimated in both study groups and frequencies were compared. Only haplotypes estimated to have frequency of at least 2% in the population sample were included for association analyses. Overall 14 9-pol haplotypes were analysed. There was no statistically significant difference in haplotype frequencies between the ISS group and the population sample ($P = 0.05$).

No linkage disequilibrium was detected between the *SHOX* gene polymorphic sites and the L05101 deletion.

Conclusion

The frequency of common L05101 deletion (~4,9 kb) is not significantly different between the ISS group and the population sample. Absence of linkage disequilibrium between the *SHOX* gene polymorphic sites and the L05101 deletion is in compliance with high recombination rate in the area (May *et al.* 2002). Our results correspond with conclusions published by Benito-Sanz *et al.* (2011), who found L05101 deletion in 11.5% of patients with ISS and in 12.1% of healthy people. Further, comparing our data and data from Benito-Sanz, we found no significant difference in the L05101 deletion frequency between men and women in ISS patients as well as in the population group. We are convinced that we can confirm the thesis of Benito-Sanz *et al.* (2011) that this small PAR1 deletion represents a nonpathogenic polymorphism.

Common sequence variants were detected in the *SHOX* gene coding sequences including 5′ end region. Especially the 5′ end and exon 1 seem to be quite polymorphic. A strong linkage disequilibrium between corresponding polymorphic sites is comprehensible. Nonsignificant linkage disequilibrium between these sites and more distant *SHOX* gene polymorphic variants seems to be due to a high local recombination rate and the different age. No association was detected for common sequence variants nor single nor 9-pol haplotypes with the ISS phenotype.

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Research paper

Comparison of *SHOX* and associated elements duplications distribution between patients (Léri-Weill dyschondrosteosis/idiopathic short stature) and population sample

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ABSTRACT

The effect of heterozygous duplications of *SHOX* and associated elements on Léri-Weill dyschondrosteosis (LWD) and idiopathic short stature (ISS) development is less distinct when compared to reciprocal deletions.

The aim of our study was to compare frequency and distribution of duplications within *SHOX* and associated elements between population sample and LWD (ISS) patients.

A preliminary analysis conducted on Czech population sample of 250 individuals compared to our previously reported sample of 352 ISS/LWD Czech patients indicated that rather than the difference in frequency of duplications it is the difference in their distribution. Particularly, there was an increased frequency of duplications residing to the CNE-9 enhancer in our LWD/ISS sample. To see whether the obtained data are consistent across published studies we made a literature survey to get published cases with *SHOX* or associated elements duplication and formed the merged LWD, the merged ISS, and the merged population samples. Relative frequency of particular region duplication in each of those merged samples were calculated. There was a significant difference in the relative frequency of CNE-9 enhancer duplications (11 *vs.* 3) and complete *SHOX* (exon1-6b) duplications (4 *vs.* 24) (p-value 0.0139 and p-value 0.000014, respectively) between the merged LWD sample and the merged population sample.

We thus propose that partial *SHOX* duplications and small duplications encompassing CNE-9 enhancer could be highly penetrant alleles associated with ISS and LWD development.

1. Introduction

The major pseudoautosomal region (PAR1) located at the terminus of the p arm of both sex chromosomes is the site of at least one obligatory recombination in male meiosis. Such recombination is essential for a proper disjunction of gonosomes in heterogametic sex. PAR1 is rich in GC-content and repetitive sequences. It makes PAR1 to have an exceptionally high male crossover rate, which is 17-fold higher than the genome-wide average (Schmitt et al., 1994). All of this results in region highly prone to chromosomal rearrangements. At the population level, there is a rapid breakdown of linkage disequilibrium even between close polymorphic loci (May et al., 2002). From the 24 annotated genes within the PAR1 only *SHOX* (Short stature HOmeoboX; MIM ID 6473) is explicitly associated with pathological phenotype. Heterozygous deletion or aberration of *SHOX* or one of its numerous enhancers have been reported to be responsible for Léri-Weill dyschondrosteosis (LWD; MIM 127300) and for a small portion of Idiopathic Short Stature (ISS; MIM 300582) (Hirschfeldova et al., 2012; Gatta et al., 2014; Chen et al., 2009). Haploinsufficiency of *SHOX* in LWD patients is associated with variable degrees of growth impairment with or without a spectrum of skeletal abnormalities. Homozygous or compound heterozygous *SHOX* defects are responsible for the rare Langer Mesomelic Dysplasia (LMD;

Abbreviations: ASMT, acetylserotonin *O*-methyltransferase; *CNE*, conserved non-coding elements; *CNV*, copy number variant; *DECIPHER*, Database of Chromosomal Imbalance and Phenotype in Humans using Ensembl Resources; *DGV*, Database of Genomic Variants; *ISS*, idiopathic short stature; *LMD*, Langer Mesomelic Dysplasia; *LWD*, Léri-Weill dyschondrosteosis; *MLPA*, multiplex ligation-dependent probe amplification; *MRKH*, Mayer-Rokitansky-Kuster-Hauser syndrome; *PAR1*, major pseudoautosomal region; *SD*, standard deviation; *SHOX*, short stature homeobox gene

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MIM 249700) characterized with more severe phenotype (Zinn et al., 2002). Despite the phenotype variability within afflicted family members the role of PAR1 deletions encompassing the *SHOX* or associated regulatory elements is unambiguous. However, effect of reciprocal duplications is less distinct. There are several phenotypes put into context with three copies of the *SHOX*: tall stature, as well as ISS and LWD, neurodevelopmental disorders, or Mayer-Rokitansky-Kuster-Hauser syndrome type I (MRKH type I) (Gervasini et al., 2010; van Duyvenvoorde et al., 2014; Tropeano et al., 2016; Benito-Sanz et al., 2011; del Rey et al., 2010; Ottesen et al., 2010). Analysis of LWD and ISS patients revealed that duplications encompassing *SHOX* and associated elements are with them less frequent compared to the reciprocal deletions (Benito-Sanz et al., 2011). The relevant *SHOX* associated elements are conserved non-coding elements (CNE) adjacent to the 3′ end (CNE-3,4,5,7,8,9) and 5′ end (CNE-2,3,5) of the gene. The aim of our study was to determine the population frequency of duplications covering both *SHOX* and associated regulatory elements. Moreover, the distribution of duplicated regions was compared between a group of patients (ISS; LWD) and Czech population sample. To determine whether the data are consistent across published studies a literature-based search for published cases diagnosed with LWD or ISS and with a duplication within *SHOX* or its associated elements were executed.

2. Materials and methods

2.1. Subjects

The study was approved by the local ethical committee and all participants provided informed consent. Population sample of 250 unrelated Czech individuals for preliminary study was obtained from the Department of Anthropology and Human Genetics (Faculty of Science, Charles University in Prague) and consists of 197 females (average height 168.49 cm ± 4.93 SD) and 53 males (average height 182.76 cm ± 5.63 SD). The average male height and female height is 180.30 cm ± 7.42 SD and 167.20 cm ± 7.11 SD, respectively, in the Czech Republic. Frequency of duplications and their extent detected in the LWD/ISS sample of 352 unrelated Czech individuals from routine diagnostic practice is described in detail elsewhere (retrospective group of patients from the Institute of Biology and Medical Genetics, General University Hospital and The First Faculty of Medicine of Charles University in Prague) (Hirschfeldova et al., 2017).

We used the search terms "*SHOX* duplications" and "*SHOX* rearrangement" in PubMed database at the National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov) to collect published cases diagnosed with LWD or ISS and with detected duplication encompassing *SHOX* or associated elements. There are several studies in which population sample was analyzed for *SHOX* or associated elements duplication as well. We have combined obtained data with our data to form the merged LWD, the merged ISS, and the merged population samples. The merged LWD cohort includes 31 cases (Benito-Sanz et al., 2011; Hirschfeldova et al., 2017; Bunyan et al., 2016; Auger et al., 2016) the merged ISS cohort includes 29 cases,(Benito-Sanz et al., 2011; Hirschfeldova et al., 2017; Bunyan et al., 2016; Fukami et al., 2015; Bunyan et al., 2013; Mitka et al., 2016; Caliebe et al., 2012; Valetto et al., 2016), and the merged population sample consists of 36 individuals (Tropeano et al., 2016; Fukami et al., 2015; Thomas et al., 2009).

2.2. Methods

The genomic DNA was isolated from the peripheral blood using QIAGEN spin columns on QIAcube (QIAGEN, GmbH, Germany). Czech population sample of 250 unrelated individuals was screened for PAR1 duplication using the multiplex ligation-dependent probe amplification (MLPA), MLPA kit P018-G1 SHOX probemix (MRC-Holland, the Netherlands) according to standard protocol described in detail elsewhere (Hirschfeldova et al., 2017). Second independent MLPA analysis was performed for verification.

As for the merged samples, there were various methods used for duplication detection in different studies. So, we converted the referred duplication extent to appropriate MLPA probes duplication (MLPA kit P018-G1) for each sample included.

Relative frequency of each probe duplication was calculated for our preliminary Czech population sample and our LWD/ISS sample as well as for the literature-based merged LWD, merged ISS, and merged population samples. In the preliminary study the relative frequency of probe duplication was compared between our Czech population sample and our LWD/ISS sample. As for the literature-based merged samples, relative frequency of probe duplication was plotted and compared between the patients (the merged LWD or the merged ISS samples) and the merged population sample. The Fisher exact test ($p = 0.05$) was used for statistical significance testing.

The following publicly accessible databases of human genomic variants were inspected for *SHOX* or associated elements duplication: DECIPHER (Database of Chromosomal Imbalance and Phenotype in Humans using Ensembl Resources) (https://decipher.sanger.ac.uk) and DGV (Database of Genomic Variants) (http://dgv.tcag.ca/). Only duplications with at least one boundary within the critical PAR1 chromosomal region (hg19; chrX: 398,000–980,000) were analyzed. For reasons of brevity we use the term "*SHOX* area" in the following text. The term "*SHOX* area" is characterized as a chromosomal region where the *SHOX* gene and associated regulatory elements are situated (hg19; chrX: 398,000–980,000).

3. Results

During the preliminary study, overall ten duplications in heterozygous state were detected within the PAR1 region in our Czech population sample of 250 individuals using the MLPA probe mix (Fig.1). Five of them (2.0%) carry a known copy number variant (CNV) within *ASMT* that is not part of the "*SHOX* area" and thus was excluded from further evaluation. Four duplications (1.6%) cover *SHOX* or associated regulatory elements: single complete *SHOX* duplication, single partial *SHOX* duplication (not interfering with transcript SHOXa), single duplication of all upstream regulatory elements (CNE-2,3,5), and a complete duplication of downstream regulatory elements (CNE-3,4,5,7,9). In one individual (0.4%) a duplication of chrX:970,000 area (hg19) was detected. There was not a significant difference in duplication frequency between our LWD/ISS sample and our Czech population sample (1.6 *vs.* 2.3; *p*-value 0.7691). However, there is a tendency to higher incidence of CNE-9 area duplication in our LWD/ISS sample compared to our Czech population sample (6 *vs.* 1).

We have created a literature-based merged LWD, merged ISS, and merged population samples to analyze whether tendency to increased frequency of CNE-9 area duplication among LWD/ISS patients is consistent across published studies. Relative frequency of each probe duplication was calculated for the merged LWD, the merged ISS, and the merged population sample and plotted in the graph (Fig. 2.). The amount of duplications affecting only downstream regulatory elements and involving CNE-9 enhancer were 11 (35.5%) in the merged LWD sample, 8 (27.6%) in the merged ISS sample, and 3 (8.3%) in the merged population sample (significant difference in the frequency between the merged LWD sample and the merged population sample *p*-value 0.0139). Moreover, it seems that in the merged LWD sample duplications have a smaller extent compared to the merged population sample. Therefore, we have compared the frequency of complete *SHOX* duplications (exon 1–6b) between the merged LWD (4 individuals) and the merged population sample (24 individuals) and detected a statistically significant difference in complete *SHOX* duplication frequency (4 *vs.* 24; *p*-value 0.000014).

The marked peak (L20176 probe) at the 3′ end of the "*SHOX* area" in the merged ISS sample duplication distribution is a result of recurrent

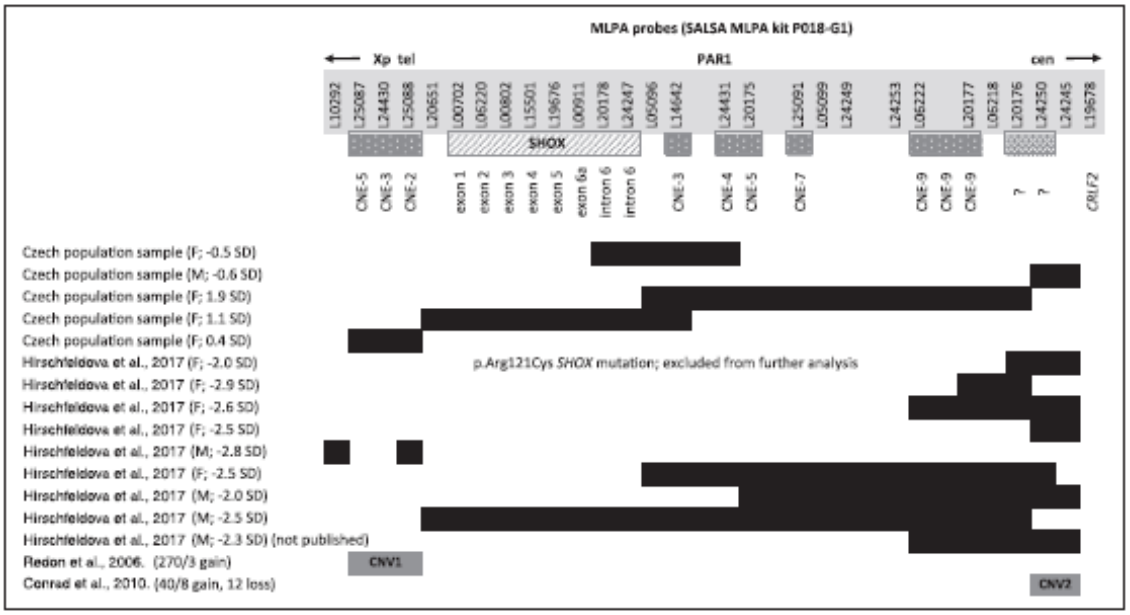

Fig. 1. Summary of duplications detected within *SHOX* and associated elements in our Czech population sample of 250 individuals (with corresponding height SD score). Chromosomal region of *ASMT* probe duplication is not included. Duplications detected in the LWD/ISS cohort of 352 Czech individuals obtained from routine diagnostic practice (with corresponding height SD score) (Hirschfeldova et al., 2017). A single male ISS patient with duplication and corresponding height SD score detected in routine diagnostic practice and not included in our previous study. Common duplications derived from the DGV database are depicted in grey colour as CNV1 and CNV2 with appropriate study group frequency indicated in parenthesis. Localization of *SHOX* associated elements CNEs (conserved non-coding elements) acting as enhancers is demonstrated. The region of putative regulatory element (hg19; chrX:970,000) is marked with question marks. (F – female; M – male).

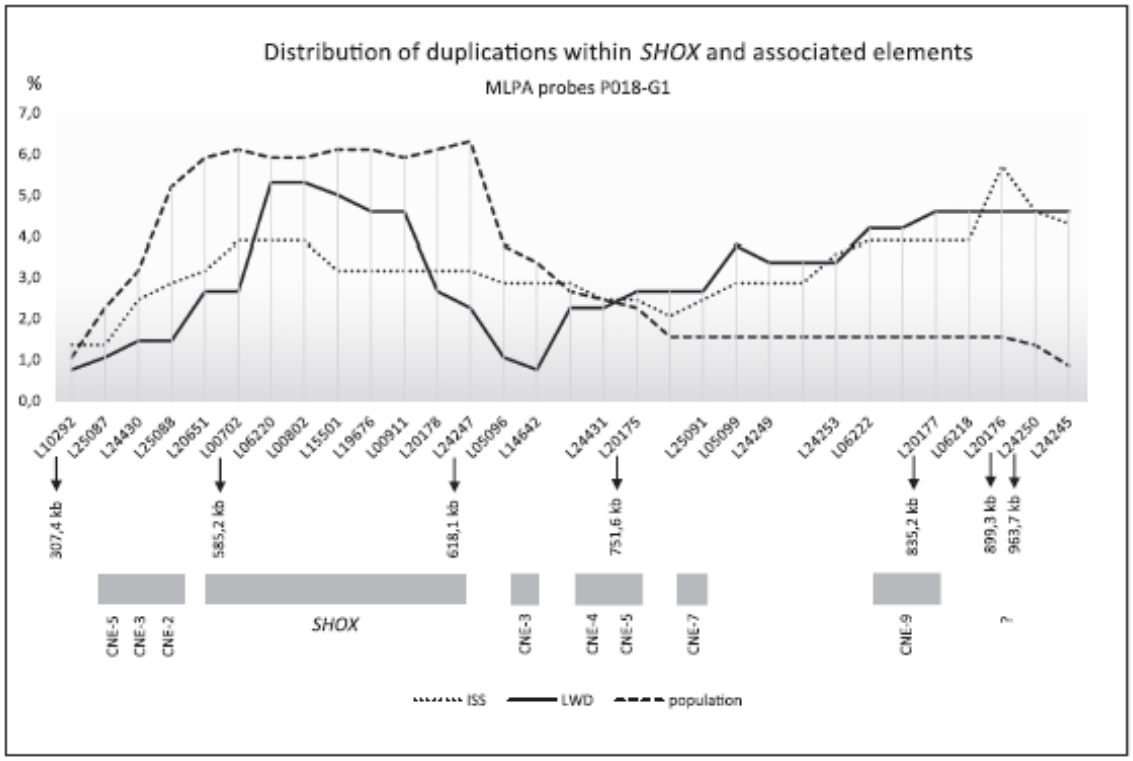

Fig. 2. Distribution of duplicated probes (MLPA kit P018-G1) in the merged LWD, the merged ISS, and the merged population samples expressed as a relative frequency of each probe duplication (%). Localization of *SHOX*, and associated CNEs acting as enhancers are demonstrated. The region of putative regulatory element (hg19; chrX:970,000) is marked with question mark. Genomic coordinates in hg19 on X chromosome for selected probes are indicated.

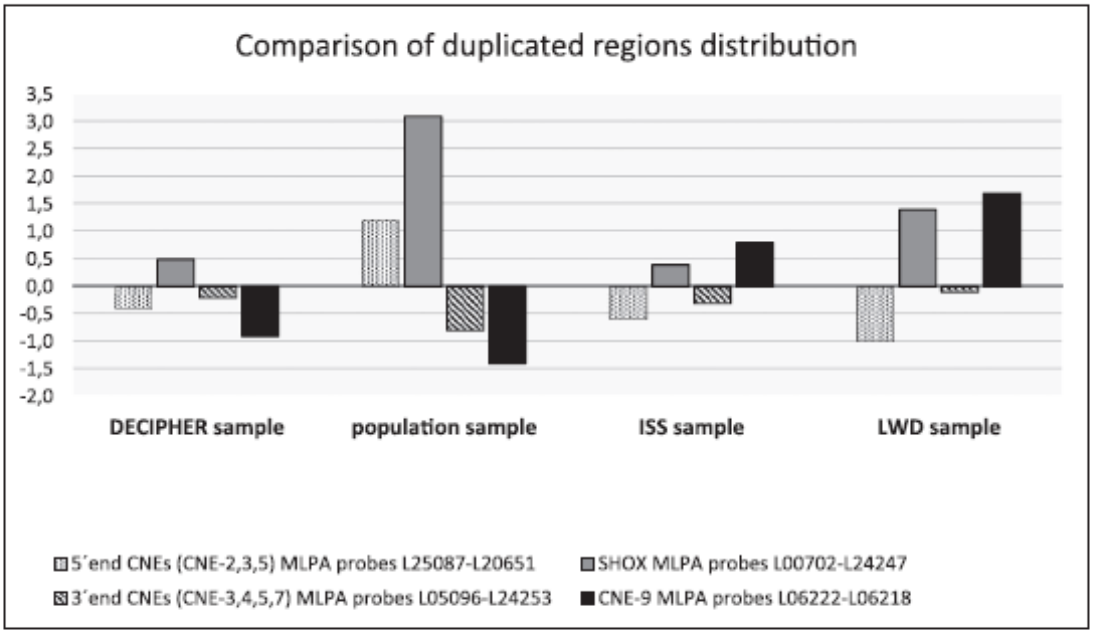

Fig. 3. Comparison of duplicated regions distribution among the DECIPHER sample, the merged population sample, the merged ISS sample, and the merged LWD sample expressed as the difference from the appropriate median of the relative probe duplication frequency. The critical chromosomal region of PAR1 (hg19; chrX:398,000–980,000) was divided into four areas corresponding to upstream *SHOX* regulatory elements, *SHOX* exons, downstream *SHOX* regulatory elements except the CNE-9 which forms a separate area. Particular MLPA P018-G1 probes for each selected area are indicated.

325 kb duplication (probes L20176-L24245) from study by Bunyan et al. (2016). and corresponds to the CNV2 polymorphism described below.

Overall 75 duplications with at least one boundary within the "*SHOX* area" were obtained from DECIPHER database. The most frequent associated phenotype was surprisingly intellectual disability and global developmental delay. We have converted the referred duplication extent to appropriate MLPA probes (MLPA kit P018-G1) and calculated the relative frequency of each probe duplication. Distribution of duplicated probes in DECIPHER sample was rather uniform and resembles that in the merged population sample (Fig.3).

The overview of "*SHOX* area" duplications found in DGV (Database of Genomic Variants) is summarized in Fig.1. Two recurrent PAR1 duplications representing structural variation in healthy control samples are recognized as the CNV1 (esv2758854; 218.7 kb), and the CNV2 (esv26183; 144.2 kb). Both of them were detected in our population sample (0.40%), the CNV2 was present in one case from our LWD/ISS sample as well (0.28%).

4. Discussion

The nature of PAR1 is predisposing to frequent rearrangement occurrence. There must be a strong evolutionary pressure to maintain genes tolerating gains or losses. It could be the reason why *SHOX* is the only gene from PAR1 explicitly associated with known pathological phenotype. Recurrent partial duplication of *ASMT* was in some studies associated with autism spectrum disorders (Cai et al., 2008). Such duplication had frequency of 2.0% in our population sample which is comparable to its frequency of 2.3% in our cohort of 352 LWD/ISS patients and to its frequency in population sample from another study (Cai et al., 2008). To our knowledge none of the individuals that carry the *ASMT* duplication and was detected in our laboratory suffer from autism spectrum disorders.

In spite of a substantial phenotype variability, there is an unambiguous pathological significance of *SHOX* deletions. Pathological significance of reciprocal duplications is much less clear. Previously, it was postulated that three copies of *SHOX* are predisposing to tall stature. However, it is not so straight forward as number of regulatory sequences are essential for proper *SHOX* expression (Chen et al., 2009; Fukami et al., 2006; Rosin et al., 2013). It seems that only duplication of the whole critical chromosomal region (hg19; chrX:398,000–980,000) could have a positive effect on height. However, such duplications are often part of more complex gonosomes rearrangements and are thus associated with gonadal dysgenesis and low estrogen levels, another factor increasing the final height. It may overlap the true effect of extra *SHOX* copy.

It was shown that duplications within PAR1 are principally organized tandemly (Bunyan et al., 2016). Hence, partial duplications of the critical chromosomal region (hg19; chrX: 398,000–980,000) are a mixture of duplications with highly variable pathogenic potential. It seems, that pathogenic potential of a specific duplication is determined by a probability of disrupting the right level of SHOX production. It is consistent with our data, that revealed partial *SHOX* duplications and small duplications encompassing CNE-9 regulatory elements are more abundant in LWD (ISS) sample compared to population sample. The CNE-9 enhancer seems to be the key regulatory element of *SHOX* expression in growth plate environment (Chen et al., 2009; Fukami et al., 2006). Small tandem duplication of CNE-9 may execute its negative effect on *SHOX* expression in several ways. It could decrease an effective communication between the CNE-9 enhancer and *SHOX* promoter. Such interaction requires DNA bending or other strand rearrangement which are generally more responsive to shortening then to extension. We propose that small duplications involving CNE-9 enhancer result in two tandemly organized CNE-9 enhancers what can directly disrupt CNE-9 full function or can compete for transcription factors binding and *SHOX* promoter interaction. It can subsequently decrease effective transcription of *SHOX* in *cis*. It is consistent with the finding that less conserved sequences flanking CNE-9 are necessary to fully execute the limb enhancer activity (Rosin et al., 2013). There are number of indicators, such as long untranslated regions and number of upstream and downstream regulatory elements, that *SHOX* expression is under tight control (Durand et al., 2011; Blaschke et al., 2003). It seems that its

decreased expression from one allele could be sufficient for the development of disease phenotype. Phenotype penetrance and expressivity are then modulated by genetic background and non-genetic factors.

Two common duplications labeled as CNV1 and CNV2 were detected within the critical chromosomal region (hg19; chrX: 398,000–980,000) in DGV (Conrad et al., 2010; Redon et al., 2006). These two duplications together with two tiny deletions of 47.5 kb (esv2661336; hg19: chrX: 780 549-828 092) and of 4.1 kb (esv28534; hg19: chrX: 821 704-825 830) represent a common PAR1 population variability (Conrad et al., 2010; Genomes Project et al., 2012). However, the CNV2 is encompassing the putative regulatory element (hg19: chrX:970,000) and both loss and gain alleles occur in the population (Bunyan et al., 2014). It is likely that allele frequencies are population specific (Bunyan et al., 2016). It has been suggested, that loss alleles could be associated with ISS and LWD development in the autosomal recessive manner while gain alleles seem to be without any phenotypic effect (Bunyan et al., 2016; Bunyan et al., 2014; Tsuchiya et al., 2014). Its real functional significance has yet to be confirmed by functional analysis.

5. Conclusions

Effect of both deletions, and duplications encompassing *SHOX* or its associated regulatory elements on final height is less or more modified by a genetic background. We propose that partial *SHOX* duplications and small duplications residing CNE-9 enhancer are highly penetrant alleles associated with ISS and LWD development.

Conflict of interest

No competing financial interests exist.

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Mutation analysis of *TRPS1* gene including core promoter, 5′UTR, and 3′ UTR regulatory sequences with insight into their organization

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ABSTRACT

The TRPS1 protein is a potent regulator of proliferation, differentiation, and apoptosis. The *TRPS1* gene aberrations are strongly associated with rare trichorhinophalangeal syndrome (TRPS) development.

We have conducted MLPA analysis to capture deletion within the crucial 8q24.1 chromosomal region in combination with mutation analysis of *TRPS1* gene including core promoter, 5′UTR, and 3′UTR sequences in nine TRPS patients. Low complexity or extent of untranslated regulatory sequences avoided them from analysis in previous studies. Amplicon based next generation sequencing used in our study bridge over these technical limitations. Finally, we have made extended *in silico* analysis of *TRPS1* gene regulatory sequences organization.

Single contiguous deletion and an intragenic deletion intervening several exons were detected. Mutation analysis revealed five *TRPS1* gene aberrations (two structural rearrangements, two nonsense mutations, and one missense substitution) reaching the overall detection rate of 78%. Several polymorphic variants were detected within the analysed regulatory sequences but without proposed pathogenic effect. *In silico* analysis suggested alternative promoter usage and diverse expression effectivity for different *TRPS1* transcripts.

Haploinsufficiency of *TRPS1* gene was responsible for most of the TRPS phenotype. Structure of *TRPS1* gene regulatory sequences is indicative of generally low single allele expression and its tight control.

1. Introduction

Trichorhinophalangeal syndrome (TRPS) is an extremely rare malformation syndrome with typical craniofacial and skeletal abnormalities, which can be well characterized clinically and radiologically. A discriminative phenotype is caused by a mutation or deletion of *TRPS1* gene (GeneID 7227), which is localized on the long arm of chromosome 8 (8q24.1) [1].

The inheritance of TRPS syndrome is autosomal dominant, although more than half of the reported cases are sporadic [2]. TRPS exhibits almost complete penetrance, but variable expressivity [3]. Phenotype can be highly variable depending on age and gender, even among patients carrying identical mutation, affected members of the same family [2], or in monozygotic twins [4].

Characteristic clinical features are sparse hair, pear-shaped nose, long and flat philtrum, thin upper lip, protruding ears, small stature caused by progressive growth retardation, brachydactyly and cone-shaped epiphysis on middle phalanges. Three types of TRPS syndrome are recognized. Trichorhinophalangeal syndrome type I (MIM 190350) and III (MIM 190351) are result of *TRPS1* gene mutation. Studies show that TRPS III is with phenotypic severity at the edge of the TRPSI spectrum and probably represents only the extreme of clinical manifestations of TRPSI [2,3]. There is a specific type of *TRPS1* gene mutation associated with TRPSIII phenotype namely missense mutations affecting exon 6, that encodes the GATA DNA-binding motifs [2].

TRPS II (Langer-Giedion syndrome, LGS; MIM 150230) is caused by deletion of both *TRPS1* gene and distally localized *EXT1* gene (GeneID 2131) [5]. The critical subregion 8q24.11-q24.13 also called "Langer-Giedion syndrome chromosomal region" (LGCR) is responsible for the characteristic phenotype [6], but in some cases it may be more extensive [7]. Patients with TRPS II in addition to general characteristics of TRPS phenotype suffer from multiple exostosis and much higher variability

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in phenotype compared to TRPSI and TRPSIII. There is a correlation between the extent of deletion (beyond the critical LGCR) and the variability of symptoms [7,8].

The *TRPS1* gene encodes a large nuclear transcription factor, which consists of 1281 amino acids and displays a repression activity on GATA regulated genes [9]. Protein TRPS1 combines in its structure nine potential zinc-finger motifs of four different types, including DNA-binding GATA motif and IKAROS-like zinc-finger motif. The two IKAROS-like sequences at the carboxy terminus mediate the transcriptional repressive function [10]. Currently some 59 mutations mostly missense and nonsense or small deletions and insertions within the coding part of *TRPS1* gene are listed in the public HGMD database (Human Gene Mutation Database; www.hgmd.org).

Besides the coding sequences there are extensive 5′UTR and 3′UTR regions which play a considerable regulatory role. The scattered low complexity areas or their extent avoided them from previous mutation analyses. We decided to implement these regions as well as core promoter sequences in the mutation analysis of *TRPS1* gene. We further summarized information regarding these *TRPS1* gene regulatory sequences and reveal some interesting data.

2. Materials and methods

2.1. Subjects

Study subjects were recruited from the Department of Medical Genetics of the 1st Faculty of Medicine and the General University Hospital, Charles University in Prague, and from the Genetic Department Olsanska, 1st and 3rd Faculty of Medicine, Charles University in Prague with the contribution of Genetika Ostrava s.r.o., and the Department of Medical Genetics, University Hospital Brno. Contributing physicians have abundant experiences with genetic consultation and syndromology and the introduction of a patient to the study was their sole decision and was done based on proper previous investigation. All patients introduced to the study (or their legal representatives) signed an informed consent form for blood withdrawal and DNA analysis. Control sample was obtained from the Department of Anthropology and Human Genetics (Faculty of Science, Charles University in Prague) and consists of 100 individuals with height within the normal and expected range based on the population data and the particular parents' height. The research was prospectively reviewed and approved by a duly constituted ethics committee.

2.2. DNA analysis

The EDTA blood was stored at 4 °C, and then processed within 48 h after venesection. The genomic DNA was isolated from the peripheral blood using QIAGEN spin columns on QIAcube (QIAGEN, GmbH, Germany). Detection of the 8q24 rearrangement covering the *TRPS1* gene was carried out using the MLPA (multiplex ligand-dependent probe amplification) method (MRC-Holland, the Netherlands) [11]. Each proband was analysed by use of a MLPA kit P228-B1, which covers the relevant 8q24 region, mainly *TRPS1* and *EXT1* genes. The MLPA reaction was run with 50–150 ng of DNA, according to the manufacturer's instructions and fragmentation analysis was conducted on a 3130xl Genetic Analyzer (Applied Biosystems, CA, USA). Both visual and computational evaluation was performed for each sample against the run negative control. The peak areas were normalized according to the instructions of the manufacturer. A personally constructed Microsoft Excel table was used for the entry of all of these calculations.

Additionally, all exons (1a, 1b–7) including exon/intron boundaries, 5′UTR and 3′UTR sequences and core promoter sequences were amplified. Primers were designed using the NCBI primer-blast (Primer3 algorithm). Primers and amplification conditions are available upon request. Exon 7 is more than 6000 bp long and required long range PCR polymerase mixture Long PCR Enzyme Mix (Fermentas, Vilnius, Lithuania). DNA libraries were prepared with Nextera XT DNA kit according to the standard protocol (Illumina, Inc., San Diego, USA). Libraries were quantified by qPCR using KAPA Library Quantification kit (Kapa Biosystems, Inc. Wilmington, USA) and the LightCycler 480 Real-Time PCR System (Roche, Basel, Switzerland). Paired-end sequencing was performed on MiSeq desktop sequencer using MiSeq v2 reagents (500 cycles) and 2 × 250 pb reads (Illumina, Inc., San Diego, USA).

Mutations or rare variants were confirmed and specified using direct Sanger sequencing of relevant fragments with adjusted set of primers. The sequencing reaction was performed according to the manufacturer's instructions, using the BigDye® Terminator v3.1 Cycle Sequencing Kits (Applied Biosystems, Foster City, CA, USA), and run on a 3130xl Genetic Analyzer (Applied Biosystems). Frequency estimation of the novel variant from the 3′UTR region in the control sample was done by restriction analysis of amplified specific fragment (Sau3AI enzyme; Fermentas).

2.3. Bioinformatic analysis

The next-generation sequencing data were processed using the MiSeq Reporter analysis software v.2.5, (standard PCR Amplicon workflow, variant filter quality cutoff was set to 30) followed by variant annotation with the Illumina Variant Studio Version 2.2.3 (www.illumina.com) in combination with the Integrative Genomics Viewer (IGV) [12].

To estimate functional effect of variants found within the regulatory sequences we checked them for conservation applying ECR Browser [13] and UCSC Blat (https://genome.ucsc.edu/), samely as for transcription factor or miRNA binding site prediction using a combination of TRANSFAC professional 8.1 (www.biobase-international.com), TargetScan Release 7.0 [14], and miRanda [15], as well as for secondary structure stability (*mfold* 3.1) [16] and presence of relevant regulatory elements within the 5′and 3′UTR sequences (RegRNA 2.0) [17].

Moreover, nucleotide sequence comparison was carried out for all three known transcript variants of TRPS1 gene. We checked the 5′UTR sequences for conservation, upstream open reading frames, Kozak sequence compliance, and translation initiation context using the public NCBI websites (http://www.ncbi.nlm.nih.gov/), ECR, and UCSC Browsers and associated databases. Secondary structure of 5′UTR for all transcripts was estimated by *mfold* 3.1 algorithm. Alternative promoter search upstream of the exon 1b was done using the GP miner (gpminer.mbc.nctu.edu.tw) and estimation of regulatory elements was done by RegRNA software and by using 'DNA pattern' tool of the Regulatory Sequence Analysis Tools (http://rsat.ulb.ac.be/rsat/).

3. Results

TRPS syndrome is an extremely rare disorder. We recruited nine probands from the Czech population based on their characteristic phenotypic symptoms. Both common and less frequent features are summarized in Table 1.

3.1. TRPS1 coding sequences aberration

Prior to mutation analysis all probands were screened for deletion within the relevant 8q24 chromosomal region. In proband P5 there was a large deletion encompassing chromosomal region including probes L21674 and L07025 (9.7–12 Mb). There is thus hemizygosity not only for *TRPS1* and *EXT1* genes but also for number of others. It is the only proband with multiple exostosis which led to diagnosis of TRPSII syndrome. In proband P8 a large intragenic deletion was revealed including probes L07409 and L07025 (size between 36 and 250 kb) covering *TRPS1* exons 2–5 and diagnosed with TRPSI syndrome.

In probands with no deletion detected (P1, P2, P3, P4, P6, P7, P9) mutation analysis was conducted employing amplicon based next generation sequencing which enabled us to analyse even the low complexity regions. The *TRPS1* gene aberration were detected in five of these probands reaching the overall detection rate of 78%. For summary of

Table 1
Patients suspected for TRPS syndrome, their pathological features and molecular findings. Variant localization is following the transcript 1 mRNA position (NM_014112.4).

Proband	Age	Sex	TRPS type	Mutation localization (transcript variant 1 NM_014112.4)	Clinical features								Radiological features			TRPS II typical features			Other features
					Long philtrum	Thin upper lip	Pear-shaped nose	Large prominent ears	Sparse hair	Postnatal growth retardation	Clinobrachydactylia	Swelling of proximal interphalangeal joints	Cone-shaped epiphyses of middle phalanges	Spine anomalies (scoliosis, lordosis)	Perthes-like phenotype	Mental retardation	Thickened alea nasi	Multiple exostosis	
P1	10 y	F	I[a]	_	0	+++	+	+	+++	+	+++	−	−	++	−	−	++	−	
P2	22\ y	F	I	*TRPS1*: c.2762G>A (p.R921Q)	−	++	++	−	+	−	++	++	++	−	−	−	−	−	
P3	12 y	M	I[a]	_	++	++	+++	−	−	++	−	−	++	0	−	−	−	−	
P4	38 y	F	I	*TRPS1*: c.2421_2439del19 (p.W807Cfs*13)	+	+	+++	−	+++	−	++	++	+++	−	+	−	−	−	
P5	34 y	M	II	Deletion 8q24 (about 9,7–12 Mb, contains both *TRPS1* and *EXT1*)	++	+	+	+	+	−	+	−	+	+	−	−	+	+++	Osteoporosis, Madelung deformity, cleft palate, positional anomalies of teeth, shorter right limbs
P6	47 y	F	I	*TRPS1*: c.1765A>T (p.K589*)	++	+	+++	+	+++	++	+	+	++	++	−	−	+	−	Arthritic changes of hips, problems with kidneys, hematuria
P7	34 y	F	I	*TRPS1*: c.1940_1947del8ins39 (p.E647Vfs*5)	+	+	++	−	++	+	+++	++	++	−	+	−	−	−	
P7a	6 y	F	I	*TRPS1*: c.1940_1947del8ins39 (p.E647Vfs*5)	++	++	+	−	0	0	0	0	0	−	−	−	−	−	
P8	36 y	F	I	Deletion 8q24 (about 36–250 kb, contains *TRPS1* exons 2–5)	+	+	+++	++	+++	+	++	++	++	+++	−	−	++	−	Not closed canalis sacralis, uterus septus
P9	3 y	F	I	*TRPS1*: c.2371G>T (p.E791*)	++	++	++	++	++	++	++	0	++	−	−	−	++	−	

[+++] very marked presence of the feature; [++] presence of the feature; [+] tenuous presence of the feature; [−] absence of the feature; [0] presence/absence of the feature is not known/detectable.
[a] Both probands (P1 and P3) miss some typical features of TRPS syndrome. Particularly, in proband P1 there are no cone-shaped epiphyses of middle phalanges and the proband P3 misses sparse hair and clinobrachydactylia. It could reflect an alternative gene mutation associated with overlapping phenotype [27].

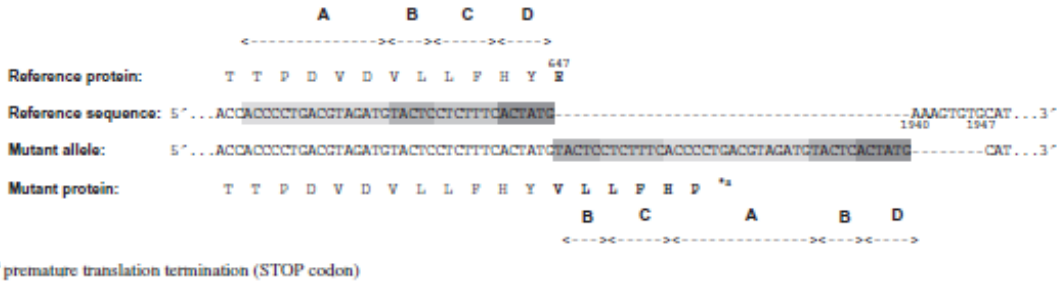
detected mutations and associated phenotypes see Table 1. Two small structural aberrations (P4 and P7) and two nonsense mutations (P6 and P9), seem to be the novel mutations. The missense substitution (P2) residing at the exon 6 is the CM010486 (rs121908435) mutation from the HGMD database [2] and impacts the seventh zinc-finger motif with confirmed GATA binding capacity [9]. Impaired GATA binding domain was in previous studies associated with the more severe TRPSIII phenotype due to dominant negative effect. However, it is not the case in our proband who even lack the postnatal growth retardation.

Small heterozygous deletion of 19 nucleotides (c.2421_2439del19) within the exon 5 was detected in proband P4. It is a frameshift mutation resulting in a premature stop codon formation 13 amino acids downstream. Even in case it escapes the nonsense mediated decay (NMD) machinery degradation the nascent protein is not able to entry the nucleus as it lacks the nuclear localization signal [10]. It is consistent with the TRPSI phenotype in P4. The second structural rearrangement of *TRPS1* gene found in the proband P7 resides the exon 4 and is somewhat more complex (Fig. 1). The microindel mutation (c.1940_1947del8ins39) could be result of an error-prone processing of DNA repair mechanisms. It is evident that the duplicated part evolved in two steps with initial duplication of B-C-D motif followed by duplication of A-B motif and its successive insertion between C and D motifs within the sequence arised before. We found the same rearrangement in proband's daughter (P7a). Despite incomplete clinical data available for proband's daughter, there was an oral comment from the clinical geneticist who was in personal contact with both of the family members confirming very close phenotype pattern of mother and daughter. The shift in reading frame results in the premature termination of translation within the next five amino acids and the nascent protein will be most likely directed to nonsense mediated degradation. The same predestination is expected for truncated protein products of two nonsense mutations from exon 4 and exon 5 detected in proband P6 and P9, respectively. All these truncated proteins do not possess functionally essential GATA and IKAROS-like binding domains as well as nuclear localization signal.

3.2. *TRPS1* regulatory sequences analysis

Sequence analysis of 3′UTR region revealed several polymorphic variants (Table 2). In proband P1 with no proved mutation within the coding part of *TRPS1* gene we detected a novel single base substitution absent in others. The wild type allele of the 3′UTR area is found in a conserved area particularly in primates. It does not produce or alternate any miRNA binding site. The stem-loop secondary structure is predicted to arise from the nearby poly A sequence, but we do not assume that the substitution will have a considerable impact. ChIP-seq experiments performed by the ENCODE project (data available at UCSC websites) identified CTCF binding site within this region. But the CTCF core binding sequence is laying more than hundred nucleotides upstream. The restriction analysis of 100 individuals from the control sample detected four heterozygous carriers reaching the frequency of 0.02. The long 3′ UTR (5528 bp) harbours five nuclear polyadenylation elements (AAUAAA), eight cytoplasmic polyadenylation elements (CPE) (UUUUAU) and a single K box (cTGTGATa) that mediates negative post-transcriptional regulation. Moreover, sixteen AU-rich elements (AREs) of class I (AUUUA) were found in the 3′UTR area. None of these elements as well as predicted miRNA binding sites are effected by polymorphic variants detected in our sample.

We were interested in how the 5′UTR sequences of three known alternative *TRPS1* transcripts are organized. On the Fig. 2 we can see that alternative transcripts 1 and 3 sharing exon 1 (refer as 1a) and arising due to the 3′ alternative splicing of exon 2. The open reading frame from the translation initiation AUG of transcript 1 is terminated after 22 aminoacids due to the alternative splicing in transcript 3. So the first appropriate downstream AUG for restoring the open reading frame is used in transcript 3. On the other hand, the transcript 2 is made up of an alternative exon 1 (refer as 1b) which is spliced directly to exon 3 common to all transcripts. There are several potential upstream open reading frames within the exon 1a and 2 the longest coding for 43 aminoacids. Long 5′UTR sequences with many upstream open reading frames are components of genes whose protein products translation efficacy requires temporary and spatially fine tuning. The expression regulation of such proteins is thus equally under control of both transcription and translation. Exon 1b represents much shorter 5′UTR including only single translation initiation AUG. The amount of TRPS1 protein production from the transcript 2 is more governed by transcription rate with relatively stable translation efficiency. It seems that transcripts 1 (or 3) and 2 play a very distinctive role and are utilised based on the current availability of transcription factors and other regulatory molecules reflecting the actual cell needs. As the codons immediately following the translation start site in all three transcript variants differ there is a unique N-terminus peptide sequence for each nascent peptide. Starting with exon 3 a common peptide sequence evolved. All short distinct N-terminal amino acid sequences are highly conserved in evolution. Moreover, we have analysed the vicinity of each AUG within the 5′UTR and detected a low compliance with Kozak consensus sequence (43–71%) even around the AUGs of open reading frames (Fig. 2). The most striking was the absence of -3A/G (except of transcript 2) and +4G nucleotide rule in sequences surrounding the AUGs of all three open reading frames [18]. It seems that *TRPS1* gene translation rate is generally low. One of the cotranslation modification is the cleavage of terminal methionine what enables further amino-terminal modifications. The probability of cleavage is mostly dependent on the second (penultimate) amino acid residue [19]. The probability of terminal methionine removal is 85%, 40% and 0% for transcripts 1, 2, and 3, respectively [20]. Different amount of terminal methionine cleavage could reflect diverse posttranscription protein modification that involve subcellular protein relocalization, apoptosis or signal transduction [21]. Estimation of secondary structure assembly was measured by the lowest Gibbs free energy of possible folds. In transcripts 1 and 3 even the most stable folding achieves free energy of only $\Delta G = -143$ kcal/mol. However, there was an individual stem loop within the exon 1 included in several predicted foldings with notably low free Gibbs energy of



[a] premature translation termination (STOP codon)

Fig. 1. Heterozygous microindel mutation in exon 4 of *TRPS1* gene (c.1940_1947del8ins39; p.E647Vfs*5), shift of the reading frame and stop codon position. Reference sequence is following the transcript 1 mRNA nucleotide and amino acid position (NM_014112.4).

Table 2
The sequence variants detected in *TRPS1* gene regulatory sequences (3′UTR and 5′UTR). Variant localization is following the transcript 1 mRNA position (NM_014112.4).

Variant	rs	Minor allele (EUR)[a]	Minor allele fr. (EUR)[a]	Status	Localization
c.-300_-295delCTCTCT	rs10546472	–	–	Polymorphism	5′UTR
c.-78G>A	rs2721939	G	0.39	Polymorphism	5′UTR
c.-9dupT	rs35329862	dupT	0.42	Polymorphism	5′UTR
c.*391T>C	rs800899	T	0.49	Polymorphism	3′UTR
c.*1157G>A	rs145622918	A	0.26	Polymorphism	3′UTR
c.*1980_*1981insG	rs11401905	T-	0.13	Polymorphism	3′UTR
c.*4114T>G	–	–	–	Mutation	3′UTR
c.*4315T>G	rs800897	T	0.49	Polymorphism	3′UTR

[a] EUR indicates minor allele and its frequency in individuals of European origin.

ΔG = −32.2 kcal/mol. Moreover, the most stable folding has designed a Y shape stem-loop feature close to the start AUG which reminds to the IRES (Internal Ribosome Entry Site) cellular element. The presence of IRES at transcripts 1 and 3 was estimated by RegRNA software as well. The most stable folding for transcript 2 has a free energy of ΔG = −43 kcal/mol what is in compliance with the short 5′UTR sequence. Overall three polymorphic variants were detected in the 5′ UTR sequences of transcripts 1 and 3 (Table 2). Two of them reside in the area of putative IRES element and the combination of -9dupT and -78A alleles in cis phase abolish the recognition of putative IRES element. There was only the proband P1 in which both relevant alleles (−9dupT and -78A) were in homozygous state. No variation was detected within the exon 1b and its upstream neighbouring sequences.

Alternative splicing of exon 1 used to be connected with alternative promoter. We have done a basic search for promoter elements within the upstream sequences adjacent to exon 1b. No TATA-box but Inr element consensus sequence (TTAATTC) was detected to start at the nucleotide + 4. Surprisingly, there is a TATA box (degenerate TATA-532) [22] consensus sequence (AATTAAAA) some 367 bp upstream from the transcription start site of exon 1b, associated with marked CpG island, several GC boxes and three BRE (B recognition element) elements consensus motifs. It probably represents a proximal promoter regulatory element that could help to assemble and stabilize pre-initiation complex at the core promoter sequences. The primary core promoter upstream of exon 1a is a TATA-less and Inr-less promoter with pronounced CpG island immediately adjacent to the transcription start site containing several GC boxes, and a single BRE element. Different promoter organization of transcript 1 (3) compared to transcript 2 may reflect their distinct role in biological processes. There was no variability detected within both of these suggested promoters in our group of patients. The ENCODE project have identified binding of number of transcription factors associated with regulation of chromatin and promoter activity, cell cycle regulation, differentiation or apoptosis as well as with modulation of bone formation and carcinogenesis. Some of them are predicted to bind to both suggested promoters, others being promoter specific, but their characterization is beyond the scope of this communication.

4. Discussion

The rare TRPS syndrome has a distinctive phenotype and a strong association with *TRPS1* gene aberration. Detection rate of 78% in our sample is in accordance with the large study by Lüdecke et al., [2]. There was a clear phenotype correlation between the mutation and phenotype except for proband P2, in whom mutation of the GATA-binding domain in exon 6 is not associated with more severe TRPSIII syndrome. It is a recurrent mutation previously detected in a Japanese family associated with TRPSIII syndrome and in three patients from the Lüdecke study with ambiguous phenotype association (one TRPSIII family and two individuals without or mild growth retardation and diverse phenotypes) [2,23]. So it seems that the impact of R908Q (in our sample referred as R921Q) mutation is modulated by genetic background in TRPSII phenotype development.

Large deletion encompassing at least ten genes in proband P5 represents a typical contiguous gene syndrome. Final phenotype reflects joint hemizygosity of involved genes. Except *TRPS1* gene, several other genes controlling cell proliferation are deleted (*RAD21*, *NOV*, *MTBP*) and may contribute to developmental defects during embryogenesis of affected tissues. Absence of one copy of *EXT1* and *TNFRSF11B* genes is related to multiple exostosis and osteoporosis, respectively [24,25]. Defect of *TRPS1* gene is generally associated with osteopenia [3]. *TNFRSF11B* gene is coding for osteoprotegrin (OPG), that act as an osteoclastogenesis inhibitor regulating rate of bone resorption and its deletion improves the pro-osteoporosis environment. All *TRPS1* gene abberations altering the reading frame and producing premature stop codon have deleterious effect on functional protein production. The nascent protein lacks nuclear localization signal and thus cannot be transported to the nucleus and is directed to NMD. The second wild type copy is left to produce a functional protein however it is under a tight control of regulatory molecules and the amount of TRPS1 protein is restricted and not sufficient to fully execute its function. The microindel mutation in proband P7 is a rare type of sequence aberration. The origin of microindels still remains an enigma. The Scaringe's research group proposed a model in which microindels are the "scars" of error-prone repair of large, potentially lethal DNA adducts [26].

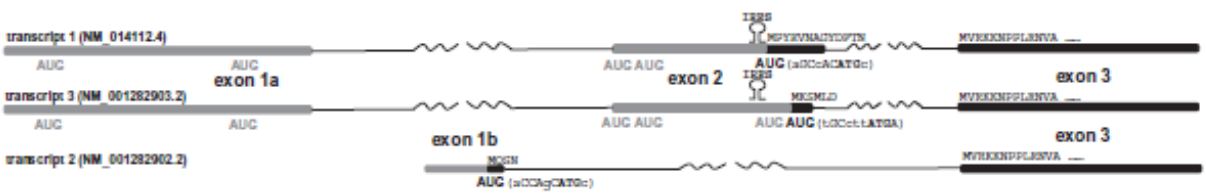


Fig. 2. The scheme of the 5′UTR and translation start site sequence organization in different transcripts of *TRPS1* gene. The putative IRES element and upstream open reading frames are indicated (shadow AUGs). The translation start sites of open reading frames in each transcript are designed in black with adjacent sequences reminiscent to Kozak element in parenthesis. Different amino-terminal peptide sequences are noted having the common amino acid sequence starts with exon 3.

In two patients there was no deletion or mutation within the coding part of *TRPS1* gene detected. However, in proband P1 a novel variant in the 3′UTR sequence was captured. We propose no clear pathogenic effect and population frequency of 2%. The same proband was the only homozygote for two alleles from the 5′UTR that in combination abolish the putative IRES element. Both alleles are frequent in European population but haplotype data are not available. Both probands P1 and P3 miss some typical features of TRPS syndrome. It could reflect both the disturbance of regulatory sequences (active only in certain tissues or developmental stages) and alternative gene mutation associated with overlapping phenotypes [27]. There are various 3′UTR specific regulatory elements demonstrating multilevel post-transcriptional regulation of *TRPS1* gene. Another type of post-transcriptional regulation is represented by length of 5′UTR sequences with upstream open reading frames, IRES element and various effectiveness of translation initiation. Finally, structure of core promoter elements establishes rate and specificity of transcription initiation. The TATA-less promoters predominate in human genome (76%) and in 46% of such promoters the Inr element is absent as well. Overall, genes containing different core promoter elements tend to control different biological processes [28]. We can speculate that primary TRPS1 promoter coupled with transcripts 1 and 3 makes its influence left in basic processes of cell cycle regulation contrary to secondary promoter that could predominate in cell-type specific development of embryonic tissue or growth plate formation.

5. Conclusion

The TRPS1 protein is a significant transcription factor of early embryogenesis and regulator of cell division. As such its transcription and translation efficacy will be controlled at different levels with several upstream and downstream regulatory elements. Alternative promoter usage, long 5′UTR sequence in transcripts 1 and 3 with number of upstream open reading frames, very weak compliance with consensus Kozak element of translation start site, different probability of N-terminus modification for all three transcripts and markedly long 3′UTR these are the main indices for a generally low single allele expression of TRPS1 transcription factor and its very tight control. It is in accordance with the autosomal dominant mode of inheritance of TRPS syndrome. Aberrations in regulatory sequences may affect proper expression on developmental stage, tissue level or as a response to actual cell needs. Moreover, variants within the regulatory sequences may convey their impact not until a homozygotic stage what have not to be leave out of consideration. Novel molecular genetic methods expanding in last decade bridge over technical limitations that exclude the regulatory sequences from previous routine mutation analysis. We think that analysis of regulatory sequences should be a part of regular mutation survey especially in genes where the precise expression regulation is considered.

Conflict of interest

The authors declare no conflict of interest.

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No competing financial interests exist.

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