1. Research project objectives/Hypothesis

Bilateral congenital limb malformations (CLMs) are clinically and genetically heterogeneous group of developmental anomalies, predominantly caused by genetic factors. Despite recent progress of the knowledge on human genetics and constant improvement of genetic diagnostic methods, the molecular cause of CLMs remains unidentified in about 40-70% of the cases. For several subtypes of isolated CLMs (f. e. isolated split-hand/foot malformation, radial ray aplasia, ulnar ray hypoplasia) genetic origin is unknown in the majority of cases. Importantly, most of the studies successful in deciphering genetic basis of isolated CLMs published within the past decade were performed by means of array CGH and identified causative copy-number variations (CNVs), usually located in the non-coding part of the genome. Such mutations disturb the regulatory landscape of a developmental gene or gene cluster resulting in their misexpression and abnormal limb morphogenesis. Other studies performed with the use of whole-exome sequencing (WES) showed limited diagnostic yield of this approach, substantiating the hypothesis that genetic lesions responsible for CLMs are rather located in the non-coding DNA.

Genome-wide interaction studies by chromosome conformation capture-based approaches (f. e. Hi-C or 4C) revealed that mammalian genome is partitioned into topologically associated domains (TADs). Disruption of TADs including boundaries can change the long-range regulatory landscape of a locus leading to misregulation of developmental genes and consequently to various pathological phenotypes. For these reasons, we are going to study a cohort of unrelated patients affected by bilateral CLMs, in whom a comprehensive molecular screening failed to reveal any known pathogenic changes. The project is aimed at identification of novel, previously unreported genetic variants contributing to the development of CLMs in human individuals. We are going to apply high-resolution array comparative genomic hybridization (array CGH) combined with bioinformatic analysis and functional studies in order to:

[A] identify novel disease-causing CNVs associated with CLMs in humans,

[B] identify novel regulatory elements responsible for the formation of limbs,

[C] gain insight into the molecular mechanisms of normal and abnormal embryonic limb development.

2. Research project methodology

A cohort of about 150 unrelated patients presenting with CLMs (both familial and sporadic cases) will be recruited for the study. Order of genetic screening will depend on the patient's phenotype and the patients will be first tested for the mutations in probably causative genes. The first tier screening will include Sanger sequencing and copy-number analysis using MLPA or quantitative PCR (qPCR) of the relevant, probably causative genes or regions. Since small submicroscopic CNVs (deletions/duplications) account for 10-15% of CLMs, whole genome array CGH (with a minimal resolution of 1M) will be performed in all undiagnosed patients as the final step of genetic analysis. We are going to apply array CGH to study about 100 unrelated patients and qPCR to test the parents. Newly identified potentially pathogenic CNVs will be subject to bioinformatic analyses in order to check if they affect TAD boundaries, change the regulatory landscape of the locus, and give rise to gene misregulation due to so called "enhancer adoption". In case of probably pathogenic CNVs, breakpoints sequencing followed by functional studies and an extensive screening of patients manifesting similar relevant phenotypes will be undertaken. To further prove the association of putative regulatory mutations with the phenotype, we will apply circularized chromosome conformation capture (4C) method, which allows for identifying differences in long-range intrachromosomal interactions in patients' derived cell lines (skin fibroblasts, LCLs) in reference to healthy controls. Finally, we will recapitulate the CNVs in a mouse model using CRISPR-Cas9 approach.

3. Research project impact

Identification of novel genetic factors (either coding or non-coding mutations) responsible for embryonic limb formation will represent an important contribution to the field of clinical genetics, developmental biology, and developmental genetics. The results will extend the knowledge on human congenital malformation phenotypes, as well as provide an insight into the processes of gene regulation, and limb morphogenesis in humans and other vertebrates. Moreover, thanks to the results of this project, CLMs patients and their families will receive better medical care and more reliable genetic counseling. The results are expected to give rise to novel, more complete genetic tests (encompassing also non-coding parts of the genome), hence improving the status of genetic diagnostics and counseling, which can be fully trustworthy only when based on appropriate genetic testing. By providing Polish patients/families affected by CLMs with a comprehensive molecular genetic diagnostics, we will be able to achieve a high rate of diagnostic success. Consequently, we will be able to offer prenatal and pre-implantation diagnosis to all interested families. To conclude, better delineation of the genetic etiology of CLMs (apart from the marked input to basic science) will undoubtedly contribute to the improvement of medical care, enabling better diagnostics, prognosis, treatment, and counseling in this highly heterogeneous group of developmental abnormalities.