ARTICLE

Genetic analysis of congenital hemimelia in buffaloes from Southern Italy

Simona Tafuri, Luigi M. Pavone*, Dionea Santoro, Sara Albarella, Silviana Rea, Valeria De Pasquale, Rossella Della Morte, Vincenzo Peretti

.

All Res. J. Biol., **2013**, 4, 2-6

All your results are good results!

THE ALL RESULTS JOURNALS

Issue 1, Vol 4, 2013, 2-6

Genetic analysis of congenital hemimelia in buffaloes from Southern Italy

Simona Tafuri[a], Luigi M. Pavone[b,*], Dionea Santoro[b], Sara Albarella[a], Silviana Rea[b], Valeria De Pasquale[b], Rossella Della Morte[a], Vincenzo Peretti[a]

*[a]Department of Veterinary Medicine and Animal Productions, University of Naples Federico II, Via F. Delpino 1, 80137 Naples, Italy; [b]Department of Molecular Medicine and Medical Biotechnology, University of Naples Federico II, Via S. Pansini 5, 80131 Naples, Italy; *Corresponding author: luigimichele.pavone@unina.it*

Abstract: Hemimelia is a common congenital limb abnormality found in water buffaloes from Southern Italy. In humans, such a defect has been associated with mutations in WNT7A and ESCO2 genes. These two candidate genes were analyzed by polymerase chain reaction in the genomic DNA extracted from the blood of buffaloes, and cows for control. No differences in WNT7A and ESCO2 sequences between affected and healthy buffaloes were identified. However, comparing sequences of control cows and buffaloes, WNT7A showed simple species polymorphisms, and ESCO2 showed seven base-pair substitutions. These results demonstrate that limb malformations in buffaloes are not related to congenital defects in WNT7A gene. Interestingly, our findings highlight for the first time differences in the sequences of WNT7A and ESCO2 genes between buffaloes and cows.

Keywords: Buffaloes, Cows, Hemimelia, ESCO2, WNT7A.

Hemimelia is a congenital malformation characterized by the absence of a limb portion. It is anatomically classified into two types: transversal hemimelia, characterized by complete absence of distal portion of the limb, and paraxial hemimelia characterized by aplasia of either radius or ulna, or tibia and fibula. In humans, these malformations are sporadic or very rare, with an incidence of approximately 1 per 1 million of live births. Some genetic mutations that cause limb deficiencies are associated with an autosomal dominant inheritance; other genetic causes include an autosomal recessive inheritance and chromosomal aberrations. However, different teratogenic agents and drugs have been also related to hemimelia[1]. Many case reports of hemimelia in cattle, sheep, dog, cat and goat have been reported[2-4]. Recent studies highlighted the major molecular components that coordinate limb outgrowth along three axes: fibroblast growth factors (FGFs) control the proximodistal axis, the sonic hedgehog (SHH) the anteroposterior axis, while the dorsoventral axis is regulated by bone morphogenetic proteins (BMPs), engrailed-1 (EN1), and wingless-type member 7A protein (WNT7A)[5]. The activities of these genes are mutually dependent. The WNT7A gene encodes a secreted signalling molecule that plays several roles in vertebrate development, and it is expressed in limbs, central nervous system, and urogenital tract. Functional studies have demonstrated that WNT7A is needed for normal patterning of the limb buds[6]. Homozyguos missense mutations in WNT7A cause two distinct limb-malformation disorders in humans: the Fuhrmann syndrome and the Al-Awadi/Raas-Rothschild phocomelia syndrome[7]. However, the WNT7A G204S mutation results to be associated with both Al-Awadi-Raas Rothschild syndrome and Fuhrmann syndrome phenotypes[8]. A phenotype similar to Fuhrmann syndrome was detected in WNT7A knockout mice[9].

Furthermore, the autosomal recessive human disorder Roberts syndrome, characterized by craniofacial anomalies, tetraphocomelia, and loss of cohesion at heterochromatic regions of centromeres and Y chromosome, has been associated to mutations in ESCO2 gene[10]. This gene encodes a protein belonging to the highly conserved Eco1/Ctf7 family of acetyl-transferases, and it is involved in the regulation of sister chromatid cohesion[11]. In humans, most ESCO2 mutations cause premature stop codons that may result in truncated proteins or mRNA instability due to nonsense-mediated mRNA decay[12].

In livestock, several congenital malformations such as amelia, polymelia, ectromelia and hemimelia have been associated with genomic instability[4,13]. In this study, we screened WNT7A and ESCO2 genes in water buffaloes affected by limb defects in order to check whether mutations in these genes could be associated with their hemimelia phenotype. Bovine and human WNT7A genes differ for one additional exon present in the human genome, and bovine ESCO2 includes 10 exons whereas human *ESCO2* comprises 11 exons spanning 30.3 kb, with the start codon in exon 2 and the stop codon in exon 11. We analyzed twenty-six Mediterranean Italian buffaloes from one day to six month old, 13 of which were affected by hemimelia (Figure 1), and 13 were healthy. Thirteen healthy cows were also studied controls.

Figure 1. Italian Mediterranean buffalo calf with left hind limb amputated off proximal epiphysis metatarsus.

The clinical and radiological patterns observed in the malformed animals are reported in Table 1. In their malformed limbs, all the animals showed more or less developed outlines of claws.

Materials and Methods

Genomic DNA was extracted from peripheral blood samples (1 ml) of all the animals using a PureLink genomic DNA mini kit (Invitrogen, Milan, Italy) according to manufacturer's instructions. Using genomic DNA templates, polymerase chain reaction (PCR) was performed to amplify the three exons of the bovine WNT7A gene and exon 2 of bovine ESCO2 gene. This ESCO2 exon was selected because of its high susceptibility to mutations in humans. Primers were selected from bovine WNT7A and ESCO2 gene sequences (Ensembl Genome Browser) using the Primer3 Input 0.4.0 program, because buffalo genome has not been sequenced yet. PCR mix contained in a final volume of 25 μl: 60 ng of genomic DNA, 1 μM primer, 1.5 mM $MgCl_2$, 1 U Taq polymerase (Eppendorf, Milan, Italy), and 0.2 mM dNTPs. PCR to amplify WNT7A exons was performed using a Gene Amp Mj Mini (BioRad Laboratories, Rome, Italy) as it follows: 1X (94°C for 4 min) and 38X (94°C for 45 s, 58.2°C (exon 3) and 63°C (exon 1 and 2) for 30 s, 72°C for 1 min). PCR to amplify ESCO2 exon 2 from genomic DNA was performed as it follows: after an initial 5 min denaturation step at 95°C, 35 amplification cycles (94°C for 40 s, 60°C for 30 s, 72°C for 1 min) were carried out followed by a 10 min incubation at 72°C. The oligonucleotide primer sequences, and PCR product size for each exon are reported in Table 2.

Table 1. Clinical and radiological patterns observed in malformed animals.

1 female	Hind limbs amputated, the right amputated off the second tarsus bones and the left amputated off the proximal epiphysis metatarsus, and the right thoracic limb hypoplasic
2 females 1 male	Left hind limb amputated off the proximal epiphysis metatarsus
1 female	Left hind limb amputated off the third tarsus bones
1 female 1 male	Left hind limb amputated off the tibia
1 female	Left hind limb amputated off the distal epiphysis metatarsus
1 male	Left hind limb amputated off the first phalanx
1 male	Right hind limb amputated off the proximal epiphysis metatarsus
1 female	Left hind limb amputated off the proximal epiphysis tibia
2 males	Right hind limb amputated off the proximal epiphysis tibia

Table 2. Primers for amplification and sequencing of WNT7A exons and ESCO2 exon 2.

	Forward primer	Reverse primer	bp
WNT7A Exon 1	5'-GTCTGCAGGCTGTGCCCCGC-3'	5'-CCACTTTGAGCTCCTTGCCG-3'	298
WNT7A Exon 2	5'-GGAGCCGGGAGGCCGCCTTC-3'	5'-CTTCCGGCCTGCCTCATTAT-3'	272
WNT7A Exon 3	5'-ATCCTGGAGGAAAACATGAA-3'	5'-TCACTTGCACGTGTAGACCT-3	480
ESCO2 Exon2part1	5'-ATCAATGGACTGTTTCCTTT-3'	5'-GGCTTAGAACTCGAGGAGCA-3	579
ESCO2 Exon2part2	5'-TGCAAGGAAAACCAGTCTGC-3'	5'-TTAGAAGCTATGAATTTCCA-3	504

Results and Discussion

The PCR products were separated by electrophoresis to verify the expected length of amplified fragments. Figure 2 shows the PCR products of the three WNT7A exons and ESCO2 exon 2 from cows, healthy and malformed buffaloes. No length differences were observed between the amplified products from cows, healthy and malformed buffaloes. PCR products were purified and sequenced, and the sequence of each exon was analyzed using CodonCode Aligner software. PCR amplifications and sequencing were performed in triplicate.

Table 3 summarizes the nucleotide differences between WNT7A sequences of cows, healthy and malformed buffaloes. The base-pair substitutions between cows and healthy buffaloes encode for the same amino acid, thus suggesting the occurrence of polymorphisms. No mutations were observed in WNT7A coding sequences of malformed buffaloes compared to healthy animals

Table 4 reports the specie specific nucleotide differences observed between ESCO2 exon 2 sequences of cows and healthy buffaloes.

In three cases, the base-pair substitutions encode for the same amino acid, whereas, in five cases, the base-pair substitutions encode for amino acids with similar chemical properties, and, in two cases, for amino acids with different chemical properties.

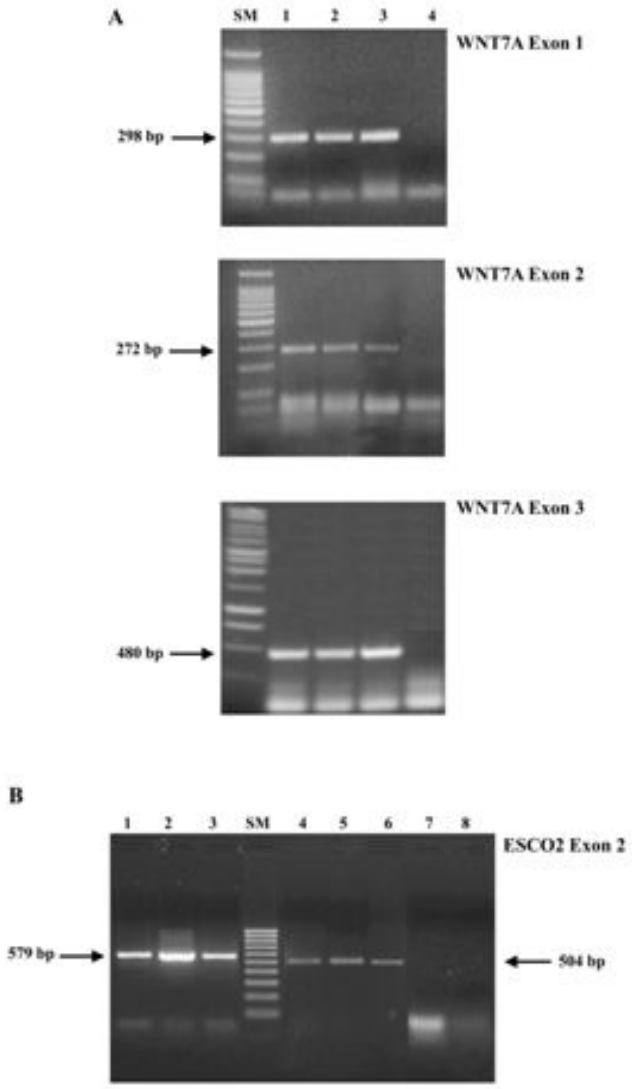

Figure 2. PCR products of WNT7A exons and ESCO2 exon 2. A. PCR product of WNT7A exons from extracted DNA of control cow (lane 1), healthy buffaloes (lane 2) and malformed buffaloes (lane 3). Lane 4: negative control, SM: DNA ladder. B. PCR product of ESCO2 exon 2 part 1 and exon 2 part 2 from extracted DNA of control cows (lanes 1 and 4), healthy buffaloes (lanes 2 and 5) and malformed buffaloes (lanes 3 and 6). Lanes 7 and 8: negative controls, SM: DNA ladder. Arrows indicate the size of PCR products.

Figure 3 shows the sequences of ESCO2 exon 2 that give rise to different amino acids between cows and healthy buffaloes. No differences were observed in the ESCO2 exon 2 sequences between healthy and malformed buffaloes (Table 4).

Table 3. WNT7A bp differences between control cows, healthy and malformed buffaloes.

Control cow		Healthy buffaloes		Malformed buffaloes	
bp	**aa**	**bp**	**aa**	**bp**	**aa**
48 g	L 16	48 a	L 16	48 a	L 16
273 t	T 91	273 c/t	T 91	273 c/t	T 91
372 c	T 124	372 g/c	T 124	372 g	T 124
393 c	C 131	393 t	C 131	393 t	C 131
474 c	Y 158	474 t/c	Y 158	474 t	Y 158
519 a	K 173	519 g	K 173	519 g	K 173
609 c	H 203	609 t	H 203	609 t	H 203
633 g	T 211	633 c	T 211	633 c	T 211
684 c	L 228	684 t	L 228	684 t	L 228
798 t	T 266	798 c	T 266	798 c	T 266
969 g	Q 323	969 a	Q 323	969 a	Q 323

The bp numbers are referred to *bos taurus* WNT7A cDNA (ENSBTAT00000002188)

Table 4. ESCO2 bp and aa differences between control cows, healthy and malformed buffaloes.

Control cow		Healthy buffaloes		Malformed buffaloes	
bp	**aa**	**bp**	**aa**	**bp**	**aa**
209 g	R 70	209 a	K 70	209 a	K 70
262 g	A 88	262 t	S 88	262 t	S 88
272 t	V 91	272 c	A 91	272 c	A 91
313 t	L 105	313 c	L 105	313 c	L 105
498 g	V 166	498 a	V 166	498 a	V 166
566 a	Y 189	566 c	S 189	566 c	S 189
571 g	A 191	571 a	T 191	571 a	T 191
626 t	V 209	626 c	A 209	626 c	A 209
673 t	S 225	673 a	T 225	673 a	T 225
831 c	N 277	831 t	N 277	831 t	N 277

The bp numbers are referred to *bos taurus* ESCO2 exon 2+exon 1 cDNA (ENSBTAT00000008606). In red highlighted species polymorphisms, in black highlighted conservative mutations and in green highlighted mutations for amino acids with different properties.

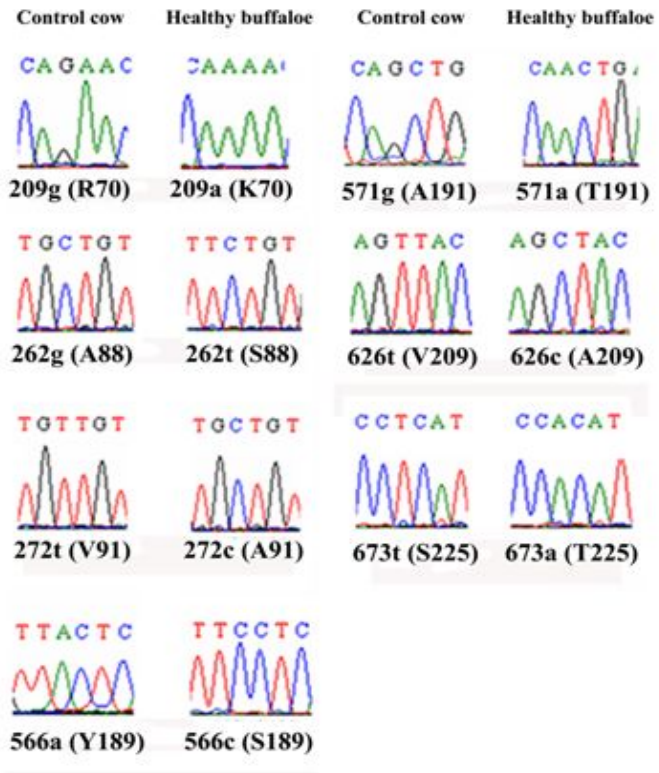

Figure 3. Sequence analysis of the ESCO 2 exon 2 shows differences in the coding sequence between control cow and healthy buffaloes.

In recent years, an increasing number of calves born in Southern Italy shows limb defects, and in particular, transversal hemimelia[4]. Genomic instability has been demonstrated in these animals as proved by the high rates of structural chromosomal aberrations and increased sister chromatid exchanges detected in affected calves[4,13-14]. Due to the economic and social impact of such a problem, molecular genetic studies, which allow identifying the genes responsible for these congenital defects, will help to find adequate strategies for the prevention of the disease. Here, we investigated for the first time the WNT7A and ESCO 2 genes that are the main candidate genes involved in human severe limb pathologies such as Fuhrmann syndrome and Roberts syndrome. Our results do not show genetic alterations in the WNT7A exons and ESCO2 exon 2 coding sequences of malformed buffaloes, although further studies on ESCO2 gene are needed to rule out its involvement in the pathogenesis of these congenital malformations. These findings suggest that the pathogenesis of hemimelia in buffaloes from Southern Italy could be probably related to genetic alterations in other genes involved in embryonic limb development. Interestingly, our findings highlight for the first time differences in the sequences of WNT7A and ESCO2 genes between buffaloes and cows.

Acknowledgments

This work was supported by a grant "Analisi genetica dell'emimelia congenita nel bufalo" from Regione Campania, Italy. We thank Dr. E. Cirillo for administrative help.

References

1. Sanders, D.D. and Stephens, T.D. (1991) Review of drug-induced limb defects in mammals. Teratology 44, 335-354.
2. Baum, K.H., Hull, B.L., Weisbrode, S.E. (1985). Radial agenesis and ulnar hypoplasia in two caprine kids. J. Am. Vet. Med. Association *186*, 170-171.
3. Lapointe, J.M., Lachance, S., Steffen, D.J. (2000). Tibial hemimelia, meningocele, and abdominal hernia in Shorthorn cattle. Vet. Pathol. *37*, 508-511.
4. Peretti, V., Ciotola, F., Albarella, S., Restucci, B., Meomartino, L., Ferretti, L., Barbieri, V., Iannuzzi, L. (2008). Increased SCE levels in Mediterranean Italian buffaloes affected by limb malformation (transversal hemimelia). Cytogenet. Genome Res. *120*, 183-187.
5. Niswander, L. (2003). Pattern formation: old models out on a limb. Nat. Rev. Genet. *4*, 138-143.
6. Yang, Y., Niswander, L. (1995) Interaction between the signaling molecules WNT7A and SHH during vertebrate limb development: dorsal signals regulate anteroposterior patterning. Cell 80, 939-947.
7. Woods, C.G., Stricker, S., Seemann, P., Stern, R., Cox, J., Sherridan, E., Roberts, E., Springell, K., Scott, S., Karbani, G., Sharif, S.M., Toomes, C., Bond, J., Kumar, D., Al-Gazali, L., Mundlos, S. (2006). Mutations in WNT7A cause a range of limb malformations, including Fuhrmann syndrome and the Al-Awadi/Raas-Rothschild phocomelia syndrome. Am. J. Hum. Genet. *79*, 402-408.
8. Al-Qattan MM, Shamseldin HE, Alkuraya FS. (2013) The WNT7A G204S mutation is associated with both Al-Awadi-Raas Rothschild syndrome and Fuhrmann syndrome phenotypes. Gene 516, 168-170.
9. Parr, B.A., Avery, E.J., Cygan, J.A., McMahon, A.P. (1998). The classical mouse mutant postaxial hemimelia results from a mutation in the WNT7A gene. Dev. Biol. *202*, 228-234.
10. Vega, H., Waisfisz, Q., Gordillo, M., Sakai, N., Yanagihara, I, Yamada, M., van Gosliga, D., Kayserili, H., Xu, C., Ozono, K., Jabs, E.W., Inui, K., Joenje, H. (2005). Roberts syndrome is caused by mutations in ESCO2, a human homolog of yeast ECO1 that is essential for the establishment of sister chromatid cohesion. Nat. Genet. *37*, 468-470.
11. Gordillo, M., Vega, H., Trainer, A.H., Hou, F., Sakai, N., Luque, R., Kayserili, H., Basaran, S., Skovby, F., Hennekam, R.C., Uzielli, M.L., Schnur, R.E., Manouvrier, S., Chang, S., Blair, E., Hurst, J.A., Forzano, F., Meins, M., Simola, K.O., Raas-Rothschild, A., Schultz, R.A., McDaniel, L.D., Ozono, K., Inui, K., Zou, H., Jabs. E.W. (2008). The molecular mechanism underlying Roberts syndrome involves loss of ESCO2 acetyltransferase activity. Hum. Mol. Genet. *17*, 2172-2180.
12. Vega H, Trainer AH, Gordillo M, Crosier M, Kayserili H, Skovby F, Uzielli ML, Schnur RE, Manouvrier S, Blair E, Hurst JA, Forzano F, Meins M, Simola KO, Raas-Rothschild A, Hennekam RC, Jabs EW. (2010). Phenotypic variability in 49 cases of ESCO2 mutations, including novel missense and codon deletion in the acetyltransferase domain, correlates with ESCO2 expression and establishes the clinical criteria for Roberts syndrome. J. Med. Genet. 47, 30-37.
13. Albarella, S., Ciotola, F., Dario, C., Iannuzzi, L., Barbieri, V., Peretti, V. (2009). Chromosome instability in Mediterranean Italian buffaloes affected by limb malformation (transversal hemimelia). Mutagenesis *24*, 471-474.
14. Di Berardino, D., Iannuzzi, L., Fregola, A., Matassino, D. (1983). Chromosome instability in a calf affected by congenital malformation. Vet. Rec. *112*, 429-432.