Dual-Color High-Resolution Fiber-FISH Analysis on Lethal White Syndrome Carriers in Sheep

UNIVERSITÀ DEGLI STUDI DI TORINO

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Abstract

Molecular defects occurring in the endothelin receptor type B (*EDNRB*) gene are known to be associated with pigmentary anomalies and intestinal aganglionosis in humans, rodents and horses. We have carried out a cytogenetic investigation in two ewes heterozygous for the deletion of the *EDNRB* gene and in two more females as control. The RBA-banded karyotypes of all four ewes were. *EDNRB* gene specific probes were produced by PCR and cloning. The application of the R-banding and Propidium Iodide-staining Florescent In Situ Hybridization (RBPI-FISH) allowed the mapping of the gene to OAR 10q22 and confirmed the heterozygous status of the ewes investigated for the *EDNRB* gene deletion. For the fine estimation of the gene length in sheep and for the correct sizing of the chromosomal gap a dual-color FISH has been applied to high resolution DNA fibers in combination with digital imaging microscopy. The comparison of the DNA fiber bar-codes indicated a chromosomal deletion larger than the only *EDNRB* gene. The length of the gene, not known for sheep until now, was estimated to be ~21kb, whereas the micro-chromosomal deletion was ~100kb. *EDNRB* is located in a chromosomal region previously shown to be a fragile site. The applied method allowed to locate the potential breakpoints thus permitting further interesting prospective of investigation also in the field of the fragile sites in sheep.

Key words: *EDNRB*; Lethal White Syndrome; Sheep; Fluorescence In Situ Hybridization; Chromatin fibers; Breakpoints.

Introduction

Migration, proliferation and differentiation of neural crest cells are fundamental stages for the arrangement of a functional enteric nervous system (ENS). Their breakdown results in an intestinal region characterized by the absence of enteric neurons along varying lengths of

the colon (aganglionic zone). As enteric neurons are indispensable for gut motility, the aganglionic region remains tonically constricted, hampering the movement of faecal material (Wallace and Anderson 2011). In humans, this condition is also known as Hirschsprung's disease (HD), a particular type of the Waardenburg syndrome (WS) characterized by the association of pigmentation abnormalities and aganglionic megacolon (Omenn and McKusick 1979, Shah et al. 1981, Badner and Chakravarti 1990, Bonnet et al. 1996) which occurs in approximately 1:5000 live births (Amiel et al. 2008).

Humans are not the only mammals to suffer from aganglionosis. Lethal White Foal Syndrome (LWFS) of Paint Horses is an autosomally inherited condition of newborn foals born to parents of the overo lineage (Trommershausen-Smith 1977, Schneider and Leipold 1978). The foals are totally or almost totally white and are affected by intestinal aganglionosis (Hultgren 1982, Vonderfecht et al. 1983, McCabe et al. 1990).

The molecular event responsible for the LWS in American (Santschi et al. 1998, Matallinos et al. 1998) and Australian (Yang et al. 1998) Paint horses is a dinucleotide exchange in the endothelin type-B receptor gene (*EDNRB*).

The *EDNRB* gene is known to be involved in the developmental regulation of neural crest cells that become enteric ganglia and melanocytes. It was mapped to chromosome 13q22 in human (Arai et al. 1993), chromosome12q22 in cattle (Schläpfer et al. 1997), and to chromosome 10q22 in sheep (Iannuzzi et al. 2001).

In mice, mutations in the *EDNRB* gene are responsible for disorders associated with the white coat spotting and intestinal aganglionosis (Hosoda et al. 1994, Ceccherini et al. 1995).

A hypopigmented phenotype and signs of intestinal obstruction similar to aganglionosis reminiscent of the lethal white foal syndrome occurred also in sheep. The deletion of the entire *EDNRB* gene in sheep was shown to be the cause of the observed phenotype (Lühken et al. 2012, Pauciullo et al. 2012a). However, the lack of a published

DNA sequence within that region of the sheep chromosome 10 allowed the authors only to estimate the gap as comparison to the homologous bovine sequence. Hence, further investigation is necessary to clarify the length of the deletion.

High resolution fiber FISH is a method which allows the direct visualization of DNA sequences along the chromatin fibers released from interphase nuclei (Fidlerova et al. 1994) and it's often used for resolving size gapping problems (Florijn et al. 1995). Recently, this technique was also used to visualize allele-specific gene copy number variation and orientation by using specific probes (Perry et al. 2007).

The aim of this study was to provide further cytogenetic information on the ovine *EDNRB* gene by using fluorescence in situ hybridization (FISH) with a set of specific probes in order to determine the size of the chromosomal deletion and to estimate the length of the gene in the sheep.

Material and Methods

Animals

Five completely white-coated lambs with blue eyes were born in a small group of Cameroon sheep, where only one ram was used for several consecutive years and mated to his relatives. All white coated lambs died shortly after birth. Cytogenetic investigations were carried out on two ewes, both previously found to be heterozygous carriers of a gene deletion (*EDNRB* +/-) (Lühken *et al.* 2012). Two normal ewes (*EDNRB* +/+) belonging to the same flock were used as controls.

Karyotyping of the samples

Peripheral blood cell cultures from the investigated animals were treated for conventional and late-incorporation of BrdU (15 μg/ml) to obtain R-banding preparations. Hoechst 33258 (30 μg/ml) was simultaneously added to BrdU 6 h before harvesting to

enhance the R-banding patterns. The sheep were karyotyped according to standard methods (Iannuzzi and Di Berardino 2008) for RBA-banding techniques. Chromosome identification followed the R-banded standard ideogram according to the latest international nomenclature for domestic bovids chromosomes (ISCNDB 2000). The conventional and R-banding preparations were further used for FISH analysis.

Chromatin fibers preparation

Chromatin fibers (Figure 1a) were prepared according to the method described by Fidlerova et al. (1994) with minor modifications. In brief, lymphocyte cells spread on the slides were quickly transferred to a coplin jar containing 1X PBS for 70 sec and gently stretched three times with the edge of a coverslip by using 100 µl of 0.1N NaOH : 100% ethanol (5:2). After sodium hydroxide treatment, two drops of the classical methanol-acetic acid (3:1) fixative were applied to the slides to improve the reproducibility of the release technique. Slides were dried at room temperature and dehydrated sequentially at room temperature for 2 minutes each with 70% and 95% ethanol.

Probes preparation

The *EDNRB* gene probes were prepared by PCR amplification of 3 DNA fragments spread over the gene itself and one DNA fragment directly upstream the endothelin receptor type B gene (primers are provided in Table 1). The PCR reaction mix (50 µl) comprised: 100 ng of genomic DNA, 1X GeneAmp PCR Buffer II (Applied Biosystems, Germany), 2 mM $MgCl_2$, 2 pmol of each primer, dNTPs each at 200 µM, 2.5 U of AmpliTaq® DNA Polymerase (Applied Biosystems, Germany). PCR was performed under the following conditions: 95°C for 4 min, 35 cycles at 95°C for 1 min, 60 °C for 45 s, 72°C for 2 min, with the final extension at 72°C for 10 min. All the amplified fragments were analyzed by

electrophoresis on 1.5% agarose gels in 0.5X TBE buffer and stained with ethidium bromide (Figure 1b).

PCR products were purified by Invisorb® Fragment Cleanup kit (Invitek, Germany) and cloned using the pGEM®-T Easy Vector System (Promega, USA). White recombinant clones were randomly chosen and screened by PCR using standard vector primers M13. Recombinant clones underwent plasmid purification by PureYield™ Plasmid Midiprep System (Promega, USA) and then sequencing reaction using the BigDye® Terminator Sequencing kit (Applied Biosystems, Germany). Sequencing was accomplished using an ABI 3130 Genetic Analyzer (Applied BioSystems, Germany). Approximately 1.5 µg of each purified plasmid was labeled with biotin-16-dUTP by standard nick translation (Roche, Germany) and then used for FISH analysis.

NCBI clone finder resource (http://www.ncbi.nlm.nih.gov/clone/) was used to choose a BAC clone. The ovine genomic *EDNRB* sequence available via the sheep chromosome sequence v1.0 (https://isgcdata.agresearch.co.nz) was not annotated and exhibited many gaps. The ambiguous localization of the available BACs in the sheep led us to prefer the bovine clone CH240-51D6, reported in NCBI web site as tested and precisely mapped to chromosome 12q22 in cattle. The clone was purchased from the BAC/PAC collection belonging to Children´s Hospital Oakland Research Institute (CHORI, Oakland, CA).

BAC DNA isolation was carried out according to the alkaline lysis miniprep protocol suggested by CHORI. Before the labeling, the DNA was tested via PCR for the *EDNRB locus* using the following primers: 5´-GAAGATTATTCCTTGATGAGCATTT-3´ (*forward*) and 5´-CAGACTAAGAAAAAGGAATTATGCTCT-3´ (*reverse*) and the same chemical condition aforementioned. The amplified fragment (366 bp) spans over the exon 4 (data not shown).

Approximately 1.5 µg of BAC DNA was combined with 20µl 2.5X random primer (BioPrime aCGH Labeling Module, Invitrogen, Germany) in a total volume of 39µl. Samples

were incubated at 95°C for 5 min and were then placed on ice for 5 min. Next, 5µl 10X dUTP, 1 µl Exo-Klenow Fragment (BioPrime Module) and 5µl (0.6 mM) DIG-11-dUTP (Roche, Germany) were added. Samples were incubated at 37°C for 5 h and then used for *in situ* hybridization.

Fluorescent in situ hybridization (FISH)

R-banding by late BrdU-incorporation and Propidium Iodide-staining FISH (RPBI-FISH) was performed according to Iannuzzi & Di Berardino (2008), whereas fiber-FISH and conventional FISH were performed according to Pauciullo *et al.* (2012b) with minor modifications. Briefly, for each experiment, 500 ng of each labeled DNA probe (BAC probe plus 4 *EDNRB* probes) were mixed together and combined with 5µg of salmon sperm DNA and 10µg of calf thymus DNA. All the mixture was precipitated in ethanol 100%, air-dried and then reconstituted in 7µl hybridization solution (50% formamide in 2X SSC + 10% dextran sulfate), denatured at 75°C for 10 min and incubated at 37°C for 60 min.

The slides were denatured for 3 min in a solution of 70% formamide in 2X SSC (pH 7.0) at 75°C.

The hybridization mixture was applied to the slides, covered with 24 x 24 mm coverslips and incubated in a moist chamber at 37°C over-night. After hybridization, the slides were washed in: 2X SSC at room temperature for 1 min, 0.4X SSC + 0.3% Nonidet P40 (Applichem, Germany) at 73°C for 2 min, 2X SSC at room temperature for 1 min. Slides were then incubated with 75µl 1X hybridization blocking solution (Vector Laboratories, CA, USA) for 30 min at room temperature.

Detection steps were carried out with 1:400 fluorescein isothiocyanate (FITC)-avidin (Vector Laboratories, CA, USA) and 1:200 anti-avidin antibody (Vector Laboratories, CA, USA) for the biotin-labeled probe (green signal), whereas the digoxigenin-labeled probe (red)

was detected using 1:400 rhodamine fluorochrome conjugated to an anti-digoxigenin antibody from sheep (Roche, Germany). Three-step detection and signal amplification were used for RPBI and chromatin fiber slides. Each step was conducted for 45 min at room temperature followed by three washes in 1X PBT for 5 min each at room temperature in a gently shaking.

R-banding slides were mounted with Antifade/Propidium Iodide (3μg/ml), conventional slides were counterstained with DAPI (0.24 μg/mL) (Sigma) in Antifade (Vector Laboratories, CA, USA), whereas chromatin fibers were mounted with Antifade only.

Fluorescence analysis and scoring

The slides were observed at 100X magnification with an AX70 Olympus (Olympus Deuschland GmbH, Hamburg, Germany) fluorescence microscope equipped with DAPI, FITC and Texas Red (TXRD) specific filters. Digital images were captured using the CellP software ver. 2.6.

A total of 30 randomly selected metaphase cells were examined per normal control to ensure the reliability of the probe signals by FISH. The hybridization efficiency was calculated as follows: FISH efficiency (%) is equal to the number of cells with hybridization signals present at the 10q22 region of both chromosomes 10 divided by the number of cells examined. Distances between the probe signals were computed and further analyzed in a spreadsheet program according to Florijn et al. (1995).

Results

The four investigated sheep were karyotyped. The analysis of the RBA-banding pattern showed karyologically normal animals. Sheep chromosome 10 is reported in detail in Figure 2a.

High resolution FISH

Four PCR amplicons spanning the *EDNRB* gene and partially its promoter region were mixed together and used to set up a fluorescence in situ hybridization (FISH) method for the detection of the gene deletion. The specificity of the amplified probes was first verified by agarose gel electrophoresis (Figure 1b) and then by sequencing the corresponding recombinant clones for approximately 500 bp in each direction. The comparison with the homologous bovine sequence (EMBL acc. no. NC_007310) confirmed that the probes belonged to the *EDNRB* gene (Table 1).

To ensure the reliability of the detection of the gene signals by FISH, the probes were preliminarily tested on the two normal individuals (*EDNRB* +/+); then the probes were applied to the heterozygous *EDNRB* deleted samples. For all the analyzed samples, the FISH efficiency was 91% on average (range 83%-95%).

DAPI counterstained FISH analysis on heterozygous gene deleted animals showed two symmetrical spots on a single chromosome (Figure 2b), whereas the normal samples showed four distinct signals on the two homologous chromosomes. In the RBPI-FISH experiment, the *EDNRB* gene showed two symmetrical spots located on the chromosome 10q2.2 (Figure 2a) according to the standard ideogram. For the two investigated sheep (*EDNRB* +/-), the hybridization signal was visible only on one chromosome 10, whereas no signal was detected on the other homologous chromosome 10 (Figure 2c). In the normal sample (*EDNRB* +/+) instead two clearly visible spots were detected on both chromosomes 10 (Figure 2d). This finding confirmed that the deletion is located on chromosome 10q2.2 and that the two investigated ewes are heterozygous carriers of the *EDNRB* gene deletion.

Fiber-FISH

In order to estimate the length of the gene, the size of the gap and its position we set up a dual color high resolution fiber FISH analysis. A bovine BAC probe, including the *EDNRB* gene, was used in a ZOO-FISH experiment to cover approximately 200 kb of OAR

10q2.2. The BAC was labeled in red, whereas a mixture of 4 specific sheep *EDNRB* gene probes was labeled in green.

According to the length of the chromatin fiber, four distinct green spots (on average) were visible for the *EDNRB* gene probes for the normal chromosome, whereas no signal was detected on the homologous *EDNRB* deleted chromosome (Figure 3a and 3b). Overlapping of colors between BAC and *EDNRB* gene probes often gave yellow signals (Figure 3b).

The comparison of the fiber bar-codes from the homologous chromosomes indicated the physical location of the breakpoints and a deletion even larger than the only *EDNRB* gene. The analysis of the distance among the spots allowed us to estimate the length of the gene in the sheep to be about 21 kb, whereas the length of the entire micro-chromosomal deletion was calculated to be approximately 100 kb (Table 2 and Figure 4).

Discussion

The mating of genetically related animals is known to increase the homozygosity. Such condition can also increase the chances of offspring to be affected by recessive traits. In this study we carried out a cytogenetic investigation on two ewes belonging to a small flock of Cameroon sheep, mated to their father, and which produced hypopigmentated lambs with clinical signs similar to the lethal white foal syndrome (LWFS).

For the first time we produced DNA clones specific for the sheep *EDNRB* gene and hybridized them to metaphase chromosomes of cultured lymphocytes in experiments of high resolution FISH. The two ewes were heterozygous for the deletion of the entire *EDNRB* gene. The specific signals were located only on one of the homologous chromosomes evidencing the single copy of the gene (Figure 2b). Since the DAPI banding does not allow chromosome identification, we set up a RPBI-FISH experiment to physically map the *EDNRB* gene. By this method, it is possible in fact to visualize simultaneously R-banding (by late BrdU-incorporation and propidium iodide staining) and FITC-signals (by the specific probes)

providing immediate and clearer results. According to the standard ideogram, the position of the *EDNRB* gene was confirmed on chromosome 10q2.2 (Figure 2a), while the two investigated ewes were confirmed to be heterozygous carriers of the *EDNRB* gene deletion (Figure 2c) according to the molecular assessment reported by Lühken et al. (2012) and the preliminary cytogenetic analysis stated by Pauciullo et al. (2012a).

The deletion of the *EDNRB* locus is a molecular event occurring also in other species. For instance in human, Lamont et al. (1989) reported two patients with multiple congenital anomalies/mental retardation syndromes whose findings included the Hirschsprung disease. The chromosomal aberration was interpreted as del(13)(q14.1q22.3), which includes the *EDNRB* locus. The same chromosome was found to be deleted in another clinical case of Hirschsprung disease by Bottani et al. (1991). Deletions of the distal long arm of human chromosome 13 were more recently investigated by high resolution Comparative Genomic Hybridization (CGH), and the Waardenburg-Shah syndrome (a disorder which combines the manifestation of Waardenburg syndrome and Hirschsprung disease) has been also mapped to the *EDNRB* locus (Shanske et al. 2001).

The total whiteness and megacolon associated with a naturally occurring deletion of the complete *EDNRB* locus in homozygous piebald-lethal mice is quite similar to the phenotype caused by a targeted disruption of the gene in *EDNRB* knock-out mice (Hosoda et al. 1994). A deletion of 301 bp, spanning from the distal half of the second exon to the proximal part of the adjacent intron of the *EDNRB* gene, results in the absence of a functional receptor protein in the spotting lethal rat (Gariepy et al. 1996).

Different is the case of lethal white foal syndrome in horse. In fact, this variant of the Hirschsprung disease is associated to missense mutations in the endothelin-B receptor gene (Young et al. 1998, Metallinos et al. 1998) and no cases of chromosomal deletions were reported so far.

Since in all the aforementioned cases, no high resolution investigation was performed, we decided to combine the use of *EDNRB* specific probes with a bovine BAC probe in order to develop a dual color high resolution fiber FISH method which would allow us to estimate the size of chromosomal deletion in the two ewes heterozygous carriers, and the length of the *EDNRB* gene in normal sheep.

This approach was chosen because chromatin fibers are much less condensed in the interphase nucleus, moreover, since fibers are stretched out in linear way on a microscope slide, the order of the genes can be maintained (Heng and Tsui 1998). Furthermore, Florijn et al. (1995) demonstrated that the availability of fiber-FISH dual color bar-codes allows the rapid visual identification of gene rearrangements such as deletions, translocations or duplications, due to the identification of the breakpoints in clinical DNA samples.

In the investigated sheep, the application of a dual color fiber-FISH and the comparison of the generated bar-code, allowed the identification of chromosomal breakpoints and the detection of an interstitial deletion of about 100 kb on chromosome 10 including the entire *EDNRB* gene (Figure 4). Although the degree of condensation of the hybridization tracks may vary by a factor of ~1.8, the normalized average fiber-FISH lengths were found to be fairly accurate with a standard error of 0.849 out of 10 measurements (Table 2). Our estimation of the chromosomal deletion (~100 kb) is slightly lower (~10 kb) than the finding of Lühken et al. (2012), however the order of magnitude of the micro-chromosomal deletion is the same.

The average length of the *EDNRB* gene was estimated to be ~21kb (Table 2 and Figure 4). A confirmation of the assessed size was found also in the virtual sheep genome browser ver. 1.2.1 (http://www.livestockgenomics.csiro.au/perl/gbrowse.cgi/vsheep1.2/). This size is similar to the horse *EDNRB* gene (~21kb EMBL acc. no. NC_009160), but it seems to be shorter than the homologous gene in human (~80kb, EMBL acc. no. NG_011630), mouse (~29kb, EMBL acc. no. AC_000036) and bovine (~33kb, EMBL acc. no. NC_007310).

However this result is not surprising. It is in fact known that the lengths of intergenic regions and genes can vary even among closely related species, as in the case of the ruminants. An example is given by casein genes, where the bovine *CSN1S1* locus is bigger than the caprine counterpart as consequence of retrotransposon insertions (Ramunno et al. 2004).

The orientation of the *EDNRB* gene along the fibers was not defined with certainty, but considering the structure of the gene and the physical location of the probes, it's more likely that a great part of the deletion belongs to the 3'-flanking region (~60kb), whereas the 5'-flanking region of the gene is missing only in a length of ~20kb (Figure 4).

These results agree with the molecular data reported by Lühken et al. (2012). These authors used the bovine genomic sequence (EMBL acc. no. NC_007310.4) as reference to estimate the chromosomal deletion of the lambs affected by hypopigmentation. However, the comparison of sequences belonging to different species is theoretically not adequate for a precise evaluation of molecular differences. Due to the lack of a complete available sequence and the presence of many gaps also in the recent update of the sheep genome (http://www.livestockgenomics.csiro.au/sheep/oar3.1.php), the approach we used in the present study can be considered as definitely useful and precise.

To our knowledge, this is the first time that a deletion of the entire *EDNRB* locus has been described by fiber-FISH. The high resolution method developed in this study provides simple, rapid and precise detection of the chromosomal gap. Furthermore the evaluation of the size of the break can be considered definite because obtained directly in sheep.

It is also interesting to notice that the *EDNRB* gene is mapped in a chromosomal region considered as a fragile site, both in sheep (Ali et al. 2008) and in river buffalo (Nicodemo et al. 2008). Although the location and the distribution of fragile sites is species-specific, their cytogenetic expression is a consequence of genome instability at specific loci, then involved in chromosome breakage and recombination events (Svetlova et al. 2001). Chromosomal regions carrying evolutionary important genes should be less prone to break

than others (Nicodemo et al. 2008). However it is not possible to exclude that in sheep the deletion including *EDNRB* gene might be generated by an abnormal recombination event or by a chromosomal breakage, thus opening also further interesting perspectives of investigation also in the field of the fragile sites in sheep.

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Amplicon	Physical location	Primers	Sequence	Size
1	Promoter-Exon 1	*Forward*†	5'-gaggtaaccgttctgctt-3'	~1300 bp
		Reverse	5'-ggctggctatcaagatattt-3'	
2	Intron 1	*Forward**	5'-aaaatgctggtatgtcaaaatg-3'	~1800 bp
		*Reverse**	5'-tagcattaggcatgtttct-3'	
3	Exon 2-Exon 4	*Forward*	5'-gagatgtgtaagctggtg-3'	~2000 bp
		Reverse	5'-caatttgcataccactcttct-3'	
4	Intron 5-Exon 7	*Forward**	5'-gagagagacagaagcaaagagaaa-3'	~2300 bp
		Reverse	5'-tcaagatgagctgtatttattac-3'	

Table 1. Primer sequences and amplicon size used for the preparation of the probes covering the endothelin receptor B (*EDNRB*) gene in *Ovis aries*. * and † designed on bovine *EDNRB* gene (EMBL acc. no. NC_007310) and promoter region (EMBL acc. no. D10994), respectively. The other primers were designed on sheep *EDNRB* gene sequence (GenBank acc. no. JQ937242).

Measurement	Deletion (kb)	SE	*EDNRB* gene (kb)	SE
1	97.638		20.472	
2	98.462		21.538	
3	101.695		20.339	
4	94.949		24.242	
5	97.561	***0.849***	19.512	***0.509***
6	103.636		21.818	
7	97.561		22.748	
8	96.098		21.951	
9	99.048		20.952	
10	101.333		18.667	
Average	***98.798***		***21.226***	

Table 2. Singles and average values of the chromosomal deletion and the *EDNRB* gene length estimated out of 10 fibers normalized for the degree of DNA condensation and computed according to Florijn et al. (1995).

Figure 1. a) Chromatin fibers released from fixed lymphocyte cells (magnification 20X); b) PCR amplification of specific DNA probes belonging to *EDNRB* gene in sheep. Line 1: promoter region and partial exon 1 (~1300bp); line 2: partial intron 1 (~1800 bp); line 3: probe covering the DNA region between exon 2 and exon 4 (~2000 bp); line 4: probe covering the DNA region between intron 5 and exon 7 (~2300 bp); M: 2-Log DNA ladder 0.1-10kb (New England Biolabs, MA, USA).

Figure 2. Fluorescence in situ hybridization (FISH) obtained by using specific *EDNRB* gene probes on sheep metaphases shows specific signal on OAR chromosome 10q2.2. a) diagrammatic representation of OAR 10, RBA-banding, RBPI and DAPI staining; b) Heterozygous sample for EDNRB gene deletion (DAPI staining); c) Heterozygous sample for EDNRB gene deletion (RBPI staining); d) normal sheep sample. FITC signals were superimposed on RBPI-banding (R-banding using early BrdU-incorporation and propidium iodide staining).

Figure 3. Fiber-FISH mapping of the *endothelin type-B receptor* gene. a) High-resolution fibers outgoing a lymphocyte cell of sheep heterozygous for a chromosomal deletion of about 100 kb. The BAC probe is in red (~200 kb) and the *EDNRB* gene specific probes are in green (~21 kb). The alignment among the spots of the two fibers (color bar-code) occurred naturally during the stretching and no normalization was necessary; b) bundle of chromatin fibers with and without specific green signal for *EDNRB* gene. Overlapping of colors between BAC and gene probes gave often yellow signals according to the degree of decondensation of the fibers.

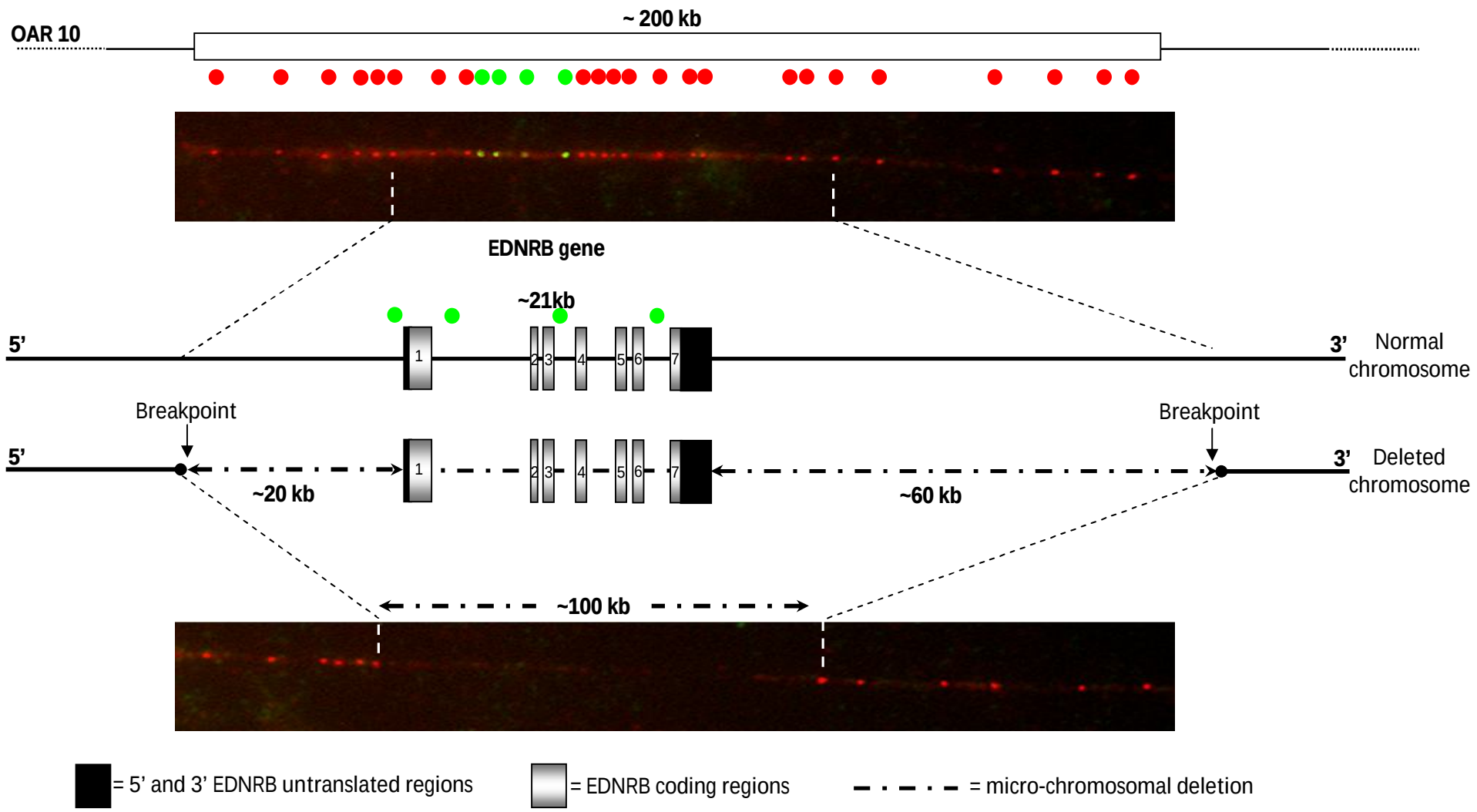

Figure 4. FISH on the homologous chromatin fibers of the sheep chromosome 10 (OAR 10) for the heterozygous carrier of the micro-chromosomal deletion and schematic representation of the genetic defect. The BAC probe is in red (~200 kb) and the *EDNRB* gene specific probes are in green (~21 kb). Dashes indicate the deleted fragment of about 100 kb including the *EDNRB* gene.