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Fusion of the *COL4A5* Gene With *NR2F2-AS1* in a Hemangioma Carrying a t(X;15)(q22;q26) Chromosomal Translocation

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Abstract. *Background/Aim: Hemangiomas are benign neoplastic proliferations of blood vessels. Cytogenetic information on hemangiomas is limited to four tumors with abnormal karyotypes. We report here a solitary chromosomal translocation and its molecular consequence in a hemangioma. Materials and Methods: A cavernous hemangioma was extirpated from the foot of a 62 years old man and genetically studied with cytogenetic and molecular genetic methodologies. Results: G-Banding analysis of short-term cultured tumor cells yielded the karyotype 46,Y,t(X;15)(q22;q26)[4]/46,XY[12]. RNA sequencing detected fusion of the collagen type IV alpha 5 chain gene (COL4A5 on Xq22.3) with intronic sequences of nuclear receptor subfamily 2 group F member 2 antisense RNA 1 (NR2F2-AS1 on 15q26.2) resulting in a putative COL4A5 truncated protein. The fusion was verified by RT-PCR together with Sanger sequencing and FISH analyses. Conclusion: The involvement of COL4A5 indicates that some hemangiomas have pathogenetic similarities with other benign tumors such as leiomyomas and subungual exostosis.*

Hemangiomas are benign neoplastic proliferations of blood vessels that may develop in any vascularized tissue. Histologically, they are composed of multiple vascular channels lined with a single layer of endothelium and supported by a fibrous connective tissue scaffold (1). They occur most often in the skin or subcutaneous tissue but may also be found in skeletal muscle, bone, kidneys, lungs, colon, brain, spleen, liver, and pancreas (2-5). Hemangiomas are mostly solitary although multiple hemangioma lesions may occur in individual patients (1). The incidence and prevalence of hemangiomas are difficult to calculate since most lesions are small and asymptomatic. There are several clinical and histological subtypes (6-9).

In the present study, we report the genomic abnormalities of a cavernous hemangioma.

Ethics statement. The study was approved by the Regional Ethics Committee (Regional komité for medisinsk forskningsetikk Sør-Øst, Norge, http://helseforskning.etikkom.no, 2010/1389/REK sør-øst A). Written informed consent was obtained from the patient. The Ethics Committee's approval included a review of the consent procedure. All patient information has been de-identified.

Case Report

The patient was a 62-year-old man with a tumor, of 3 years' duration, on the first toe of the left foot. The tumor began as a pea-sized nodule but at the time of presenting to our Department measured 2.2×2×1.5 cm. It was not tender and moved freely against the skin and underlying structures. magnetic resonance imaging showed a cutaneous/subcutaneous nodule of unknown etiology. Upon extirpation, the tumor was found to be well-circumscribed, consisting of blood-filled, dilated vascular structures with inconspicuous endothelial cells (Figure 1A). Between the vessels was adipose tissue (Figure 1B). The endothelial cells were immunohistochemically positive for ETS transcription factor ERG, cluster of

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Table I. *Primers used for reverse transcription polymerase chain reaction and cycle (Sanger) sequencing. M13 forward primer (TGTAAAACGACGGCCAGT) and M13 reverse primer (CAGGAAACAGCTATGACC) sequences are in italics.*

Name	Sequence (5'->3')	Position (GRCh38/hg38Assembly)
M13For-COL4A5-F1	*TGTAAAACGACGGCCAGT*-CCTCCTGGATTACCTGGTCCTTCA	chrX:108681762-108681785
M13Rev-NR2F2AS1-S1R1	*CAGGAAACAGCTATGACC*-CCCAACTTTTCATGAGCGCAAGT	chr15:96227351-96227373
M13Rev-NR2F2AS1-S2R1	*CAGGAAACAGCTATGACC*-TTGCGGTAGAGGCTCCAAGATCA	chr15:96172670-96172692
M13Rev-NR2F2AS1-S3R1	*CAGGAAACAGCTATGACC*-CTTCATGCTTCCAGTCCAGTGCTG	chr15:96173857-96173880

differentiation 31 (CD31), and cluster of differentiation 33 (CD34) but negative for podoplanin (antibody D2-40). The diagnosis was cavernous hemangioma (Figure 1).

G-Banding and karyotyping. Fresh tissue from a representative area of the tumor was cultured short-term and analyzed cytogenetically as previously described (10). The karyotype was written according to the International System for Human Cytogenomic Nomenclature (11).

RNA sequencing. Total RNA was extracted from frozen (–80˚C) tumor tissue adjacent to that used for cytogenetic analysis and histological examination using miRNeasy Mini Kit (Qiagen, Hilden, Germany). One microgram of total RNA was sent to the Genomics Core Facility at the Norwegian Radium Hospital, Oslo University Hospital (http://genomics.no/oslo/) for high-throughput paired-end RNA-sequencing. The software deFuse was used for detection of possible fusion transcripts (12).

Confirmation of fusion transcripts. The actual presence of the fusion transcripts (see below) was confirmed by reverse transcription (RT) polymerase chain reaction (PCR) and Sanger sequencing. One μg of total RNA was reverse-transcribed in a 20 μL reaction volume using iScript Advanced cDNA Synthesis Kit for RT-qPCR according to the manufacturer's instructions (Bio-Rad, Hercules, CA, USA). cDNA corresponding to 20 ng total RNA was used as template in subsequent PCR assays. The BigDye Direct Cycle Sequencing Kit was used to perform both PCR and cycle (Sanger) sequencing according to the company's recommendations (ThermoFisher Scientific, Waltham, MA, USA). The primer combinations for collagen type IV alpha 5 chain gene (COL4A5)/nuclear receptor subfamily 2 group F member 2 antisense RNA 1 (*NR2F2-AS1*) were M13For-COL4A5-F1/M13Rev-NR2F2AS1-S1R1, M13For-COL4A5-F1/M13Rev-NR2F2AS1-S2R1, and M13For-COL4A5-F1/M13Rev-NR2F2AS1-S3R1. The primers used for RT-PCR/cycle (Sanger) sequencing are listed in Table I.

Fluorescence in situ hybridization (FISH). BAC clones were retrieved from the RPCI-11 Human BAC library (Human 32K clone set, BACPAC Resources Center, https://bacpacresources.org/pHumanMinSet.htm). They were selected according to physical and genetic mapping data on chromosomes X and 15 (see below) as reported on the Human Genome Browser at the University of California, Santa Cruz website [https://genome.ucsc.edu/; 2013 (GRCh38/hg38) assembly]. For *COL4A5* on Xq22.3, the BAC clone used was RP11-815E21 (position: chrX:108600939-108762248). For the *NR2F2-AS1* gene on 15q26.2, the probe used consisted of the BAC clones RP11-4G2 (accession number: AC018574.6, position: chr15:95,875,102-96,046,959) and RP11-522B15 (accession number: AC087477.8, position: chr15:96,350,179-96,541,611). The *COL4A5* probe was labelled with Texas Red-5-dCTP (PerkinElmer, Boston, MA, USA) in order to obtain a red signal. The probe for *NR2F2-AS1* was labelled with fluorescein-12-dCTP (PerkinElmer) in order to obtain green signals. FISH mapping of the probes on normal controls was performed to confirm their chromosomal location. Chromosomal preparations were counterstained with 0.2 μg/ml 4',6-diamidino-2-phenylindole, and overlaid with a 24×50 mm^2 coverslip. Fluorescent signals were captured and analyzed using the CytoVision system (Leica Biosystems, Newcastle upon Tyne, UK). Detailed information on the FISH procedure is provided elsewhere (13).

Results

G-Banding analysis yielded a karyotype with a single chromosomal translocation: 46,Y,t(X;15)(q22;q26)[4]/46,XY[12] (Figure 2).

Using the deFuse software on the fastq files of the RNA sequencing data, three fusion transcripts of the *COL4A5* gene on Xq22.3 (chrX:108,439,924-108,697,545) with intronic sequences from the locus *NR2F2-AS1* on 15q26.2 were found (Seq1, Seq2, and Seq3 in Figure 3), resulting in a putative COL4A5 truncated protein.

RT-PCR/cycle (Sanger) sequencing verified the presence of the above-listed fusion transcripts (Figure 3).

Interphase FISH analyses showed the normal male pattern of one red (*COL4A5* probe, Xq22) and two green signals (*NR2F2-AS1* probe, 15q26) in 72 nuclei, whereas the abnormal fusion pattern of two yellow signals and one green signal was seen in 28 nuclei (Figure 4).

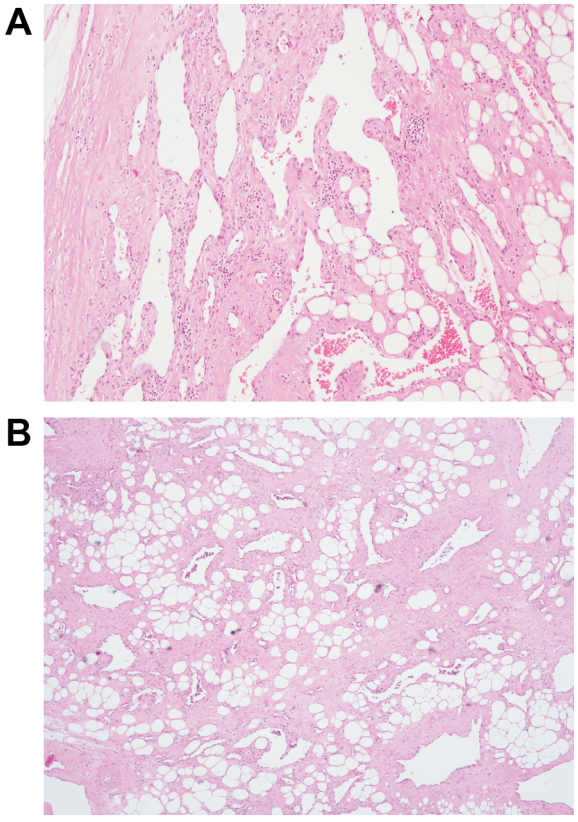

Figure 1. *Hematoxylin and eosin staining of the hemangioma. A: Vessels of various size are apparent, with erythrocytes in the lumen. The endothelium is a single layer and without atypia, and there is a muscular layer, ×100. B: Thin-walled vessels can be seen intermingled with fat against a background of fibrous tissue, ×40. The histological picture is consistent with the diagnosis of cavernous hemangioma.*

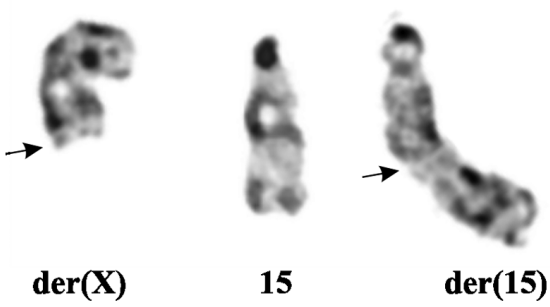

Figure 2. *Partial karyotype showing the der(X)t(X;15)(q22;q26), der(15)t(X;15)(q22;q26), and the normal chromosome 15. Breakpoint positions are indicated by arrows.*

Discussion

We present here a hemangioma carrying a t(X;15)(q22;q26) as the sole chromosome abnormality. This translocation, which to our knowledge has never been described in neoplasia (14), recombined *COL4A5* from Xq22 with *NR2F2-AS1* from 15q26 generating a *COL4A5–NR2F2-AS1* fusion gene. Rearrangements of the *COL4A5* gene were, however, found in five subungual exostoses carrying the translocation t(X;6)(q13-14;q22) (15). In four of those cases, the breakpoint mapped to the 3'-region of *COL4A5,* whereas in the fifth tumor, it was slightly telomeric of *COL4A5* (15).

The *COL4A5* gene is transcribed from centromere to telomere and encodes one of the six subunits of type IV collagen, the major structural component of basement membranes (16, 17). *COL4A5* is paired head-to-head with *COL4A6,* sharing a bidirectional promoter (18, 19). Mutations in this gene are associated with the X-linked Alport syndrome, also known as hereditary nephritis (16, 17, 20, 21). Deletions of the 5'-ends of both *COL4A5* and *COL4A6*, including the intergenic region, were found in Alport syndrome associated with diffuse leiomyomatosis (22-26). Furthermore, whole-genome sequencing analysis showed that a subset of uterine leiomyomas harbored somatic deletions within the *COL4A5–COL4A6* locus (27). Somatic deletion of the 5'-ends of both *COL4A5* and *COL4A6* was also found in an esophageal leiomyoma (28).

NR2F2-AS1 was found to promote cell proliferation in prostate carcinoma and lung cancer (29, 30). Down-regulation of *NR2F2-AS1* induced G_1 arrest of colorectal cancer cells and inhibited proliferation/induced apoptosis of nasopharyngeal carcinoma cells (29, 30). Next and telomeric to *NR2F2-AS1* is the *NR2F2* gene, which is transcribed from centromere to telomere and in which alternate splicing results in multiple transcript variants (https://www.ncbi.nlm.nih.gov/gene/7026). *NR2F2* codes for a member of the steroid thyroid hormone superfamily of nuclear receptors, a ligand-inducible transcription factor involved in the regulation of many different genes (31, 32). A possible consequence of the t(X;15)(q22;q26) might also be deregulation of *NR2F2* in a manner similar to that which occurs with the fusion of the collagen type I alpha 1 chain gene (*COL1A1*) with the platelet-derived growth factor subunit B gene (*PDFGB*) , the ubiquitin specific peptidase 6 gene (*USP6*), and FYN proto-oncogene, Src family tyrosine kinase gene (*FYN*) in dermatofibrosarcoma protuberans, aneurysmal bone cyst, and epithelioid osteoblastoma, respectively (33-37). Thus, the expression of *NR2F2* is under control of the *COL4A5* promoter.

There are several different clinical and histological subtypes of hemangioma (6-8, 38, 39), some of which were also known to have genetic aberrations. Rearrangements of the Fos proto-oncogene, AP-1 transcription factor subunit (*FOS*) gene (14q24) were found to be frequent in epithelioid hemangiomas (40, 41), whereas a fusion of ZFP36 ring finger

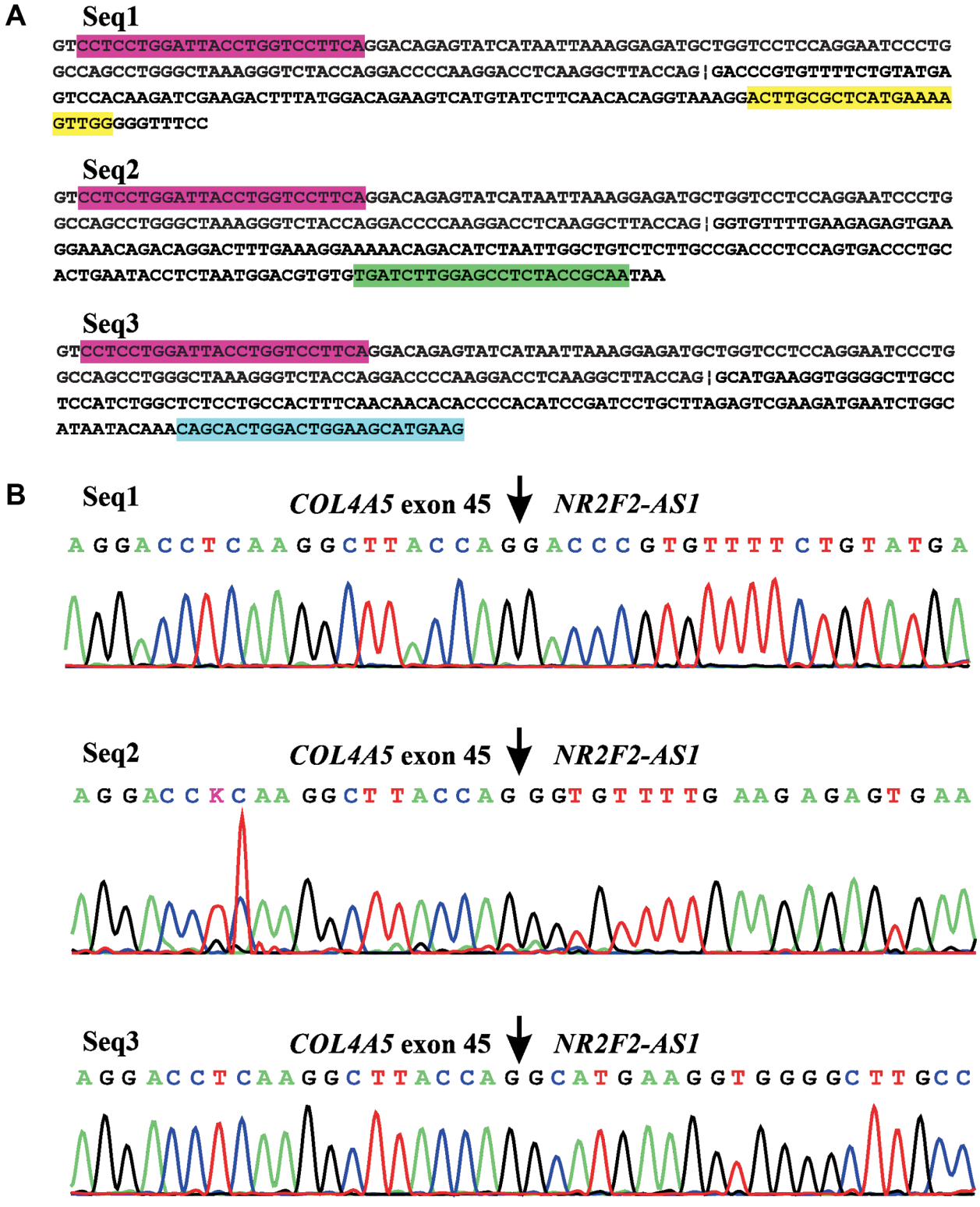

Figure 3. *Results of the RNA and Sanger sequencing. A: The three collagen type IV alpha 5 chain–nuclear receptor subfamily 2 group F member 2 antisense RNA 1 (COL4A5–NR2F2-AS1) fusion sequences obtained from the RNA sequencing data after analysis using the deFuse software package. The primers used are in color. B: Partial sequence chromatograms of the cDNA amplified fragment showing the junction position of COL4A5 and the three sequences from NR2F2-AS1 (arrow).*

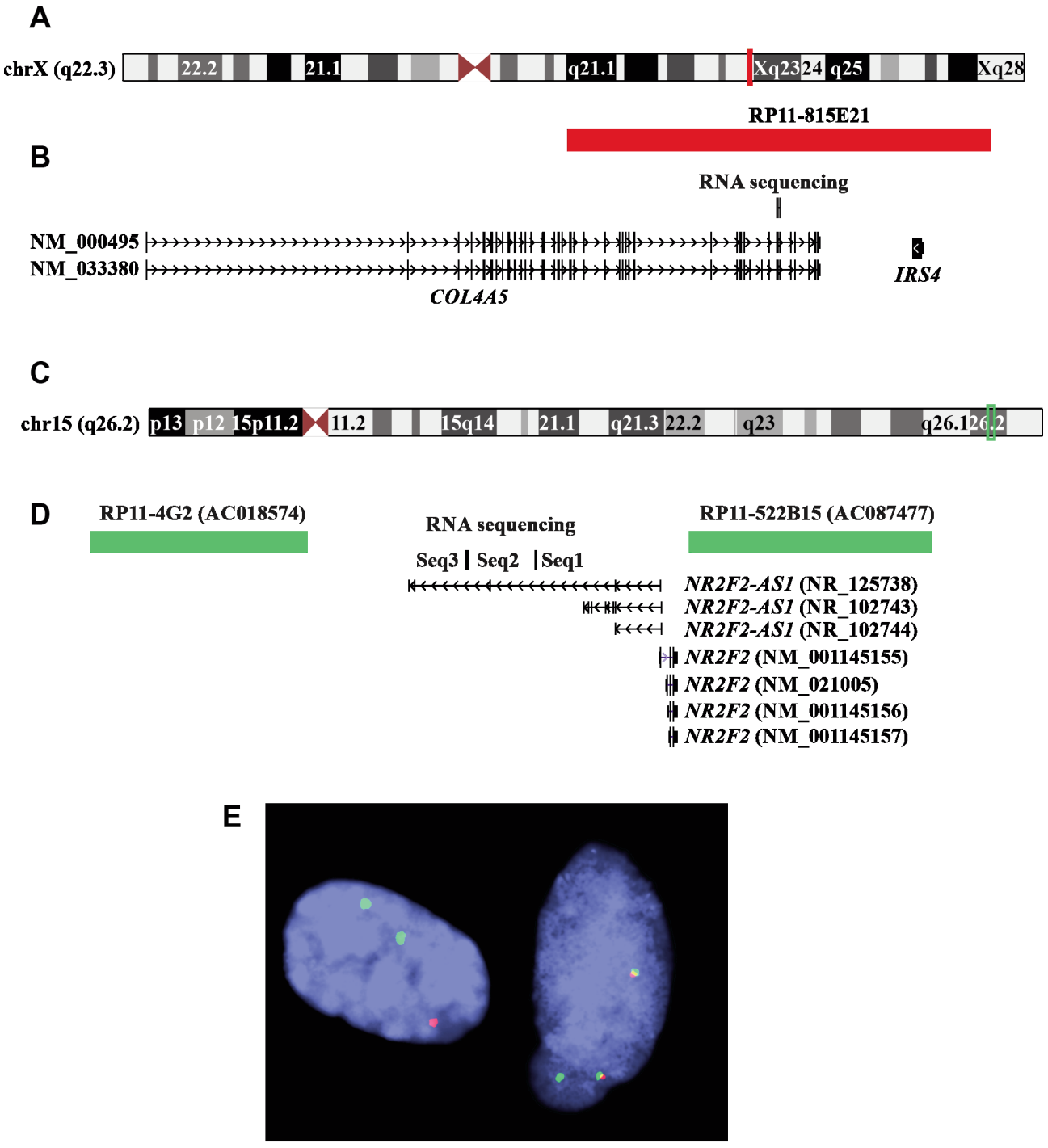

Figure 4. *Fluorescence in situ hybridization (FISH) analysis of the hemangioma using a home-made, dual color fusion probe for the detection of the chimeric gene collagen type IV alpha 5 chain–NR2F2 antisense RNA 1 (COL4A5–NR2F2-AS1). A: Ideogram of the X chromosome showing the mapping position of the COL4A5 gene at Xq22.3 (vertical red line). B: Diagram showing the FISH probe RP11-815E21 for COL4A5. The neighboring insulin receptor substrate 4 gene (IRS4) is also shown. The exons of COL4A5 found in fusion sequences using the deFuse software are shown (RNA sequencing). C: Ideogram of chromosome 15 showing the mapping position of the NR2F2-AS1 gene at 15q26.2 (green box). D: Diagram showing the FISH probes RP11-4G2 and RP11-522B15 for NR2F2-AS1. The neighboring NR2F2 gene in this region is also shown. Seq1, Seq2, and Seq3 (vertical lines) show the positions of the sequences which were found in the COL4A5–NR2F2-AS1 fusion. E: FISH results with the COL4A5 (red signal) and NR2F2-AS1 (green signal) probes on interphase nuclei. A nucleus with normal male pattern (one red and two green signals) and a nucleus with COL4A5–NR2F2-AS1 fusion (one green and two yellow/fusion signals) are shown.*

protein gene (*ZFP36*) with FosB proto-oncogene, AP-1 transcription factor subunit gene (*FOSB*) was shown to define a subset of epithelioid hemangioma with atypical features (42). *ZFP36* and *FOSB* map to 19q13.2 and 19q13.32, respectively. Somatic mutations in the G protein subunit alpha q (*GNAQ* on 9q21.2) and G protein subunit alpha 11 (*GNA11* on 19p13.3) genes were found in congenital hemangioma (43) whereas somatic mutations of isocitrate dehydrogenase 1 (*IDH1*) and isocitrate dehydrogenase 2 (*IDH2*) genes were found in spindle-cell hemangiomas (44-46). A fusion of the EWS RNA binding protein 1 gene (*EWSR1*) with the nuclear factor of activated T-cells 1 gene (*NFATC1*) was found in a hemangioma of the bone carrying a t(18;22)(q23;q12) translocation as the sole karyotypic change (47), and a fusion of the TBL1X receptor 1 gene (*TBL1XR1*) with the high mobility group AT-hook 1 gene (*HMGA1*) was detected in a splenic hemangioma with the translocation t(3;6)(q26;p21) (10). Two other hemangiomas of the nasal cavity/paranasal sinuses also had chromosomal aberrations. The first of those two was a cavernous hemangioma that underwent transformation to an angiosarcoma; it showed trisomy 5 together with loss of the Y chromosome upon karyotyping (48). The second, a lobular capillary hemangioma of the nasal cavity, carried a del(21)(q21q22) as the only cytogenetic aberration (49).

The data already published together with what we describe here therefore indicate that hemangiomas generally are characterized by simple chromosomal aberrations which sometimes generate fusion genes. The available information does not yet allow more specific conclusions as to how these tumors develop, however. Nevertheless, the involvement of *HMGA1* and *COL4A5* does indicate that, at least in some hemangiomas, the pathogenetic mechanisms are similar to those of other benign connective tissue tumors such as leiomyomas and subungual exostoses.

Conflicts of Interest

The Authors declare that they have no potential conflicts of interest in regard to this study.

Authors' Contributions

IP designed and supervised the research, performed molecular genetic experiments and bioinformatics analysis, and wrote the article. LG performed cytogenetic analysis and evaluated the FISH data. IL performed pathological examination. KA performed molecular genetic experiments, FISH analyses, and evaluated the data. ML-I performed pathological examination. FM supervised the research. SH assisted with experimental design and writing of the article. All authors read and approved of the final article.

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