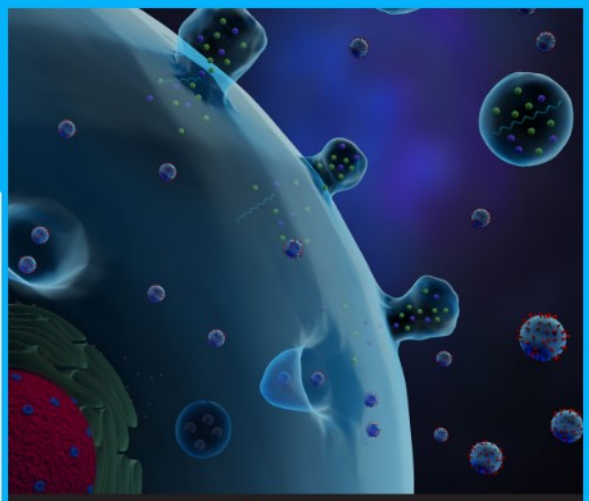
Exosome Production, Isolation and Applications: From Cancer Research to Theraputic Targets



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Exosomes play a crucial role in intercellular communication, including in cancer. Here we explore their potential in novel exosome production modulators, plant isolation and purification, in tumor immunology, and bioprocessing for diagnosis and treatment. Isolating exosomes is vital for developing tools and therapies.

This article collection presents innovative methods for exosome isolation and purification, emphasizing their role in developing new diagnostic tools and therapies for diverse diseases, with the goal of enabling researchers to explore their potential.

Key topics include:

- Isolation techniques of exosomes
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Fusion transcriptome profiling defines the monoclonal origin of multifocal epithelioid haemangioma of bone

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Fusion transcriptome profiling defines the monoclonal origin of multifocal epithelioid haemangioma of bone

Aims: Epithelioid haemangioma (EH) of bone remains a highly controversial entity. Indeed, the WHO classifies EHs of soft tissues as benign tumours, whereas bone EHs are considered intermediate–locally aggressive tumours due to common multifocal presentation and local destructive growth. To gain insights into the clinical behaviour and biology of EH of bone we retrospectively analysed 42 patients treated in a single institution from 1978 to 2021.

Methods and results: Multifocal presentation was detected in 17 of 42 patients (40%) primarily as synchronous lesions. Patients were treated with curettage (57%), resection (29%) or biopsy, followed by radiotherapy or embolisation (14%). Follow-up (minimum 24 months) was available for 38 patients, with only five local recurrences (13%) and no death of disease. To clarify whether the synchronous bone lesions in multifocal EH represent multicentric disease or clonal dissemination, four cases were profiled by RNA-sequencing. Separate lesions from the same patient, which showed a similar transcriptional profile, expressed the same fusion transcript (involving FOS or FOSB) with identical gene breakpoints.

Conclusions: These results indicate that, in EH of bone, multifocal lesions are clonally related and therefore represent the spread of a same neoplastic clone rather than simultaneous independent tumours. This finding is in apparent contradiction with the benign clinical course of the disease, and suggests that tumour dissemination in bone EH probably reflects a phenomenon of passive spreading, with tumour cells colonising distal sites while maintaining their benign biological nature.

Keywords: clonal analysis, epithelioid haemangioma of bone, FOS, FOSB, fusion transcript, RNA-sequencing

Introduction

Vascular bone tumours represent controversial entities because of their rarity, unusual morphology, variable classifications and unpredictable biological behaviour.[1,2] Epithelioid haemangioma (EH) is a tumour of unclear aetiology and pathogenesis that may arise in diverse anatomical sites, including bone.[1–4] The differential

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diagnosis of EH includes epithelioid haemangioendothelioma and epithelioid angiosarcoma, characterised by significantly different clinical courses.[1,5] Imaging is of limited help, as this tumour lacks characteristic imaging features.[6] The recent identification of FOS and FOSB gene rearrangements as a genetic hallmark of EH, and CAMTA1 and TFE3 gene rearrangements as recurrent alterations in epithelioid haemangioendothelioma, provides an objective and powerful diagnostic tool for distinguishing EH from other vascular tumours.[7–12] EH of soft tissues is classified by the most recent World Health Organisation (WHO) Classification of Soft Tissue and Bone Tumours as a benign tumour, while EH of bone is classified as intermediate and locally aggressive tumour.[1] Despite the benign histological appearance, EH of bone may show a locally destructive growth pattern, lymph node involvement and multifocal presentation.[1,6,13] These data would suggest an aggressive clinical behaviour and reinforce the concept that EH of bone is a controversial entity.[14,15] Moreover, whether multifocal lesions of a EH of bone, similar to multifocal epithelioid haemangioendothelioma, are the result of tumour spreading of a primary lesion, as proposed,[16] or rather represent independent tumours, due to a type of 'field effect', is still debated.[1,13,17,18] This uncertainty, which is particularly relevant when separate lesions affect different bones, impacts upon clinical decision-making and, indeed, there is no consensus regarding the treatment that ranges from intralesional curettage to *en-bloc* resection.[3,6,17]

In the attempt to shed light into the clinical and biological characteristics of EH of bone, we retrospectively analysed 42 patients treated in a single institution from 1978 to 2021.

Materials and methods

TUMOUR SERIES

The medical records of 42 patients with EH of bone treated in a single institution between 1978 and 2021 were retrospectively reviewed. Eight of these patients were described previously.[17] Medical records were available for 38 patients with a mean follow-up of 100 months (range = 24–314 months). Demographics, clinical data and follow-up information were retrieved from medical records (Table 1). The study was approved by the ethics committee of our institution and registered at ClinicalTrials.gov (identifier NCT03169595). Parents/guardians gave written informed consent for the retrospective analysis of clinical data according to the Institutional Review Board and before inclusion into ongoing protocols.

Diagnosis of EH of bone was based on morphological, immunohistochemical and molecular analysis. Histology was reviewed by musculoskeletal tumour pathology experts. Imaging was available for 23 patients at the onset of their disease [radiographs, 20 patients; computed tomography (CT), 16 patients; magnetic resonance (MR) imaging, 14 patients] and was reviewed by a musculoskeletal tumour radiologist. Surgical treatment ranged from biopsy alone to *en-bloc* resection.

IMMUNOHISTOCHEMISTRY (IHC)

Immunohistochemistry was performed as described previously.[19] The following antibodies were used: ERG (monoclonal antibody V9; Ventana, Tucson, AZ, USA), CD31 (monoclonal antibody O13; Ventana), CK AE1/AE3 (mouse monoclonal antibody, 6F-H2; Cell Marque, Rocklin, CA, USA), INI1 (mouse monoclonal antibody, clone BAF47; Cell Marque), CAMTA1 (rabbit polyclonal antibody, dilution 1:200; Novusbio, Centennial, CO, USA), TFE3 (rabbit monoclonal antibody, clone RQ-37; Cell Marque) and FOSB (rabbit monoclonal antibody, clone 5G4, 1:100 dilution; Cell Signalling Technology, Danvers, MA, USA). Antibody detection was performed using UltraView Universal DAB detection kit (Ventana). FOSB positivity was defined as moderate-to-strong nuclear staining in at least 50% of cells, as in Huang *et al.*[7] Samples were also stained with a FOS rabbit polyclonal antibody (ABE457; Millipore, Burlington, MA, USA), unfortunately providing unreliable results probably due to the decalcification procedure.

FISH ANALYSIS AND ZFP36::FOSB REVERSE TRANSCRIPTION-QUANTITATIVE POLYMERASE CHAIN REACTION (RT-QPCR) ANALYSIS

Fluorescence *in-situ* hybridisation (FISH) and ZFP36::FOSB RT-qPCR were performed as previously described[17] and detailed in the Supporting information. The following FISH probes were used: SPEC TFE3 (Xp11.23), SPEC WWTR1 (3q25.1) LSI dual colour break-apart DNA probes (Zytovision, Bremerhaven, Germany); FOS (14q24.3) dual colour break-apart probe (Empire Genomics, Williamsville, NY, USA).

TARGETED RNA-SEQUENCING AND WHOLE TRANSCRIPTOME ANALYSIS

Nucleic acids were extracted using the AllPrep DNA/RNA mini kit columns (Qiagen, Germantown, MD, USA) for frozen samples and the FFPE RNA/DNA

Table 1. Details of the 42 patients with epithelioid haemangioma of bone

Patient ID	Age (years)	Gender	Location	Presentation	Treatment	Outcome (FUmonths)
EH1	29	F	Bone (verterbra)	Solitary	Surgery (WM)	NED (276)
EH2	35	M	Bone (humerus)	Solitary	Surgery (WM) RXT	NED (84)
EH3	38	F	Bone (vertebra)	Solitary	Surgery (IL) RXT	NED (200)
EH4	12	M	Bone (humerus)	Solitary	Surgery (IL)	NED (44)
EH5	22	F	Bone (sacrum)	Solitary	Surgery (IL)	NED (211)
EH6	57	F	Bone (cuneiform)	Solitary	Surgery (IL)	NED (53)
EH7	34	M	Bone (clavicle)	Solitary	Surgery (WM)	NED (195)
EH8	42	M	Bone (metatarsal, cuneiform)	Multifocal	Surgery (WM)	NED (38)
EH9	60	M	Bone (sternum)	Solitary	Surgery (IL), SAE	NED1 (72)
EH10	31	M	Bone (humerus)	Solitary	Surgery (WM)	NED (126)
EH11	48	F	Bone (femur)	Solitary	Surgery (WM)	NED (166)
EH12	45	F	Bone (vertebra, rib)	Multifocal	Surgery (WM)	NED (52)
EH13	22	M	Bone (tibia)	Solitary	Surgery (IL)	NED (25)
EH14	60	F	Bone (metatarsal)	Multifocal	Surgery (IL), RXT	NED (106)
EH15	58	M	Bone (ulna)	Solitary	Surgery (WM)	NED (314)
EH16	28	F	Bone (pelvis)	Solitary	Surgery (IL)	NED (256)
EH17	42	M	Bone (femur)	Multifocal	Surgery (WM)	NED (32)
EH18	51	M	Bone (femur, tibia)	Multifocal	Surgery (IL)	DOO (96)
EH19	55	F	Bone (vertebra)	Solitary	Biopsy, RXT	NED (27)
EH20	39	F	Bone (vertebra, rib)	Multifocal	Biopsy, SAE	NED (70)
EH21	45	M	Bone (tibia, calcaneus)	Multifocal	Surgery (IL)	NED (24)
EH22	12	F	Bone (femur, tibia)	Multifocal	Surgery (WM, IL)	Lost
EH23	65	M	Bone (fibula, calcaneus)	Multifocal	Surgery (IL) RXT	NED (202)
EH24	33	M	Bone (tibia, cuboid, cuneiform)	Multifocal	Surgery (IL), RXT	NED (92)
EH25	28	M	Bone (vertebra)	Solitary	Biopsy	AWD (39)
EH26	83	M	Bone (tibia)	Multifocal	Surgery (IL)	DOO (94)
EH27	33	M	Bone (humerus)	Solitary	Surgery (WM)	NED (182)
EH28	25	M	Bone (femur, pelvis)	Multifocal	Biopsy, RXT	NED (79)
EH29	41	M	Bone (metacarpal)	Solitary	Surgery (IL)	NED1 (178)
EH30	44	M	Bone (humerus)	Solitary	Surgery (WM)	NED (185)
EH31	40	M	Bone (tibia)	Multifocal	Surgery (WM)	NED (202)
EH32	21	F	Bone (vertebra)	Solitary	Biopsy, SAE	NED (28)
EH33	20	F	Bone (humerus, radius, skull, sacrum)	Multifocal	Surgery (WM, IL)	NED2 (240)
EH34	40	M	Bone (calcaneus, fibula)	Multifocal	Surgery (IL)	NED (33)

Table 1. (*Continued*)

Patient ID	Age (years)	Gender	Location	Presentation	Treatment	Outcome (FUmonths)
EH35	34	F	Bone (talus, tibia)	Multifocal	Surgery (IL)	Lost
EH36	28	M	Bone (tibia)	Solitary	Surgery (IL)	NED (102)
EH37	28	F	Bone (humerus)	Solitary	Surgery (IL)	NED1 (239)
EH38	39	F	Bone (tibia, rotula)	Multifocal	Surgery (IL)	Lost
EH39	39	F	Bone (pelvis)	Solitary	Biopsy	AWD (30)
EH40	46	F	Bone (cervical vertebra)	Solitary	Surgery (IL)	NED (24)
EH41	58	F	Bone (distal phalanx, second finger, foot)	Solitary	Surgery (IL)	NED1 (29)
EH42	28	M	Bone (clavicle)	Solitary	Surgery (IL)	Lost

AWD, Alive with disease; DOO, Dead of other cause; F, Female; FU, Follow-up; IL, Intralesional curettage; M, Male; NED, No evidence of disease; NED1, No evidence of disease after one local recurrence; NED2, No evidence of disease after two local recurrence; RXT, Radiation therapy; SAE, Selective arterial embolisation; WM, Wide margin.

purification plus kit (Norgen Biotek Corporation, Ontario, Canada) for formalin-fixed paraffin-embedded (FFPE) specimens.

A customised Archer FusionPlex sarcoma RNA-sequencing panel version 1.1 (ArcherDX, Boulder, CO, USA), supplemented with spike-ins primers for FOS (exon 4, forward primer) and FOSB (exons 1 and 2, reverse primers) was employed for library generation. Libraries were run on an Illumina MiSeq sequencing platform and were first analysed with the Archer Analysis suite software version 6.0.4 (January 2022). Subsequently (June 2023), raw data were re-analysed with the most recent version 7.1.0 release. Raw data were also analysed with the Arriba fusion caller, as previously described.[20] RT-PCR and RT-PCR/Sanger sequencing were employed for orthogonal validations. Primer sequences are provided in the Supporting information.

For whole transcriptome analysis, libraries were generated with the Illumina Stranded Total Ribo-Zero Plus RNA library prep kit (Illumina, San Diego, CA, USA) and run on an Illumina Hiseq 1000 platform. Sequencing depth was ≥ 50 million paired-end reads per sample. RNA-sequencing data were analysed as in Gasparotto *et al.*[21] with minor modifications. Arriba (version 2.3.0)[22] and FusionCatcher tools were used for fusion transcript identification (see Supporting information for details).

Results

TUMOUR SERIES: DEMOGRAPHIC AND CLINICAL FEATURES

The analysis of medical records of 42 patients with EH of bone (Table 1) showed no age predilection, with patients' ages ranging between 12 and 83 years (mean age = 39 years). Twenty-three patients were male and 19 were female. Most EHs occurred in the extremities (seven humerus; one ulna; one radius; one hand; nine tibia; two fibula; five femur; eight foot) followed by the trunk (two rib; 10 vertebra; three pelvis; one clavicle; one sternum). Seventeen of 42 patients (40%) presented with multifocal bone involvement (13 lower limb; one pelvis and femur; one upper limb, sacrum and skull; two vertebra and rib; Figure 1). Except for one patient (EH33), multifocality was synchronous. In most cases the same or contiguous bones were involved, except in patients EH21, where non-contiguous bones were affected (tibia and calcaneus) and EH33 (see below).

Imaging data were available for 23 patients. All lesions were well-defined and lytic, associated with sclerosis in only two patients. The cortex was thin, with a calcified periosteal limitation in three patients (13%) and completely missing in six patients (26%). Soft tissues were involved in four patients (17%). Two patients presented with surface lesions (9%). The mean size of the bone lesions was 4.5 cm (range = 1.4–11.0 cm). On MR imaging all lesions showed a high signal on T2W images, but the signal was variable on T1W images (low in five patients, intermediate six patients and high in three patients). One vertebral lesion had MR imaging features similar to a typical haemangioma.

Twenty-four patients (57%) were treated with intralesional curettage, 12 patients (29%) underwent *en-bloc* resection with wide margins and six patients (14%) were treated with biopsy only, followed by radiation therapy or selective arterial embolisation.

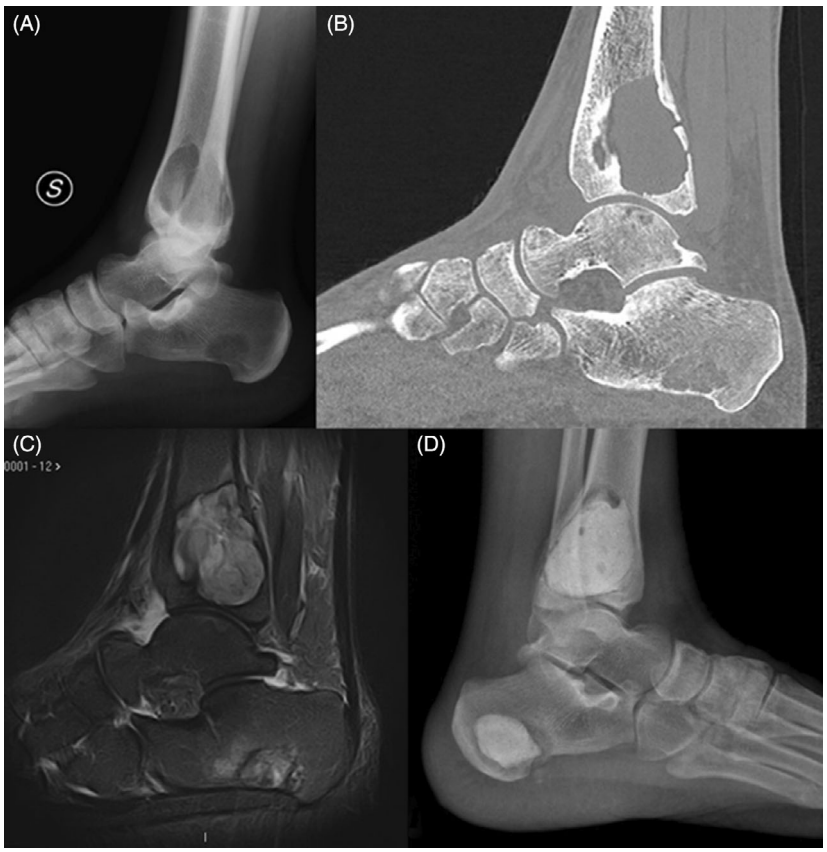

Figure 1. Multifocal EH of the left lower limb of patient EH21. **A,** Laterolateral radiograph, **B,** sagittal CT and **C,** MRI show osteolytic lesions involving the distal tibia and calcaneus. **D,** Laterolateral radiograph showing the result after curettage and filling the bone defects with cement.

Follow-up information was available for 38 patients: five of 38 patients (13%) had a local recurrence at 12, 26, 28, 48 and 120 months, respectively. Four of these patients were treated with curettage and only one with resection. At the last follow-up, no patient was dead of disease. The only patient with metachronous presentation (EH33) underwent resection of lesions involving skull and proximal humerus and a curettage of proximal radius, distal humerus and sacrum. The patient had a favourable prognosis at 19-years' follow-up.[23]

HISTOLOGICAL AND IMMUNOHISTOCHEMICAL FINDINGS

IHC showed a strong expression of vascular markers (CD31 and ERG), retained INI1/SMARCB1 expression and negativity for pan-keratin AE1/AE3, CAMTA1 and TFE3 in all patients. The diagnosis of EH of bone with exclusion of EH mimics was supported by molecular analyses (FISH or RT-PCR) whenever the quality and quantity of biological material allowed. According to morphological, IHC and molecular features, 34 EHs were classified as classic variant and eight as atypical/cellular variant. On haematoxylin and eosin, classic EHs showed no significant cytological atypia (Figure 2). Focal tumoral necrosis was detected in three patients. The mitotic rate was low, with fewer than two mitoses per 10 high-power fields in all patients. The eight atypical/cellular EH presented solid neoplastic areas constituted of endothelial cells, and in six of these patients a strong nuclear immunoreactivity for FOSB was detected (Figure 2, Table 2). Unfortunately, in our series the FOS staining was unreliable, probably due to the decalcification procedure.

MOLECULAR ANALYSES AND CLONALITY ASSESSMENT

Molecular analyses were conducted in 15 patients for which biological material was available (Table 2).

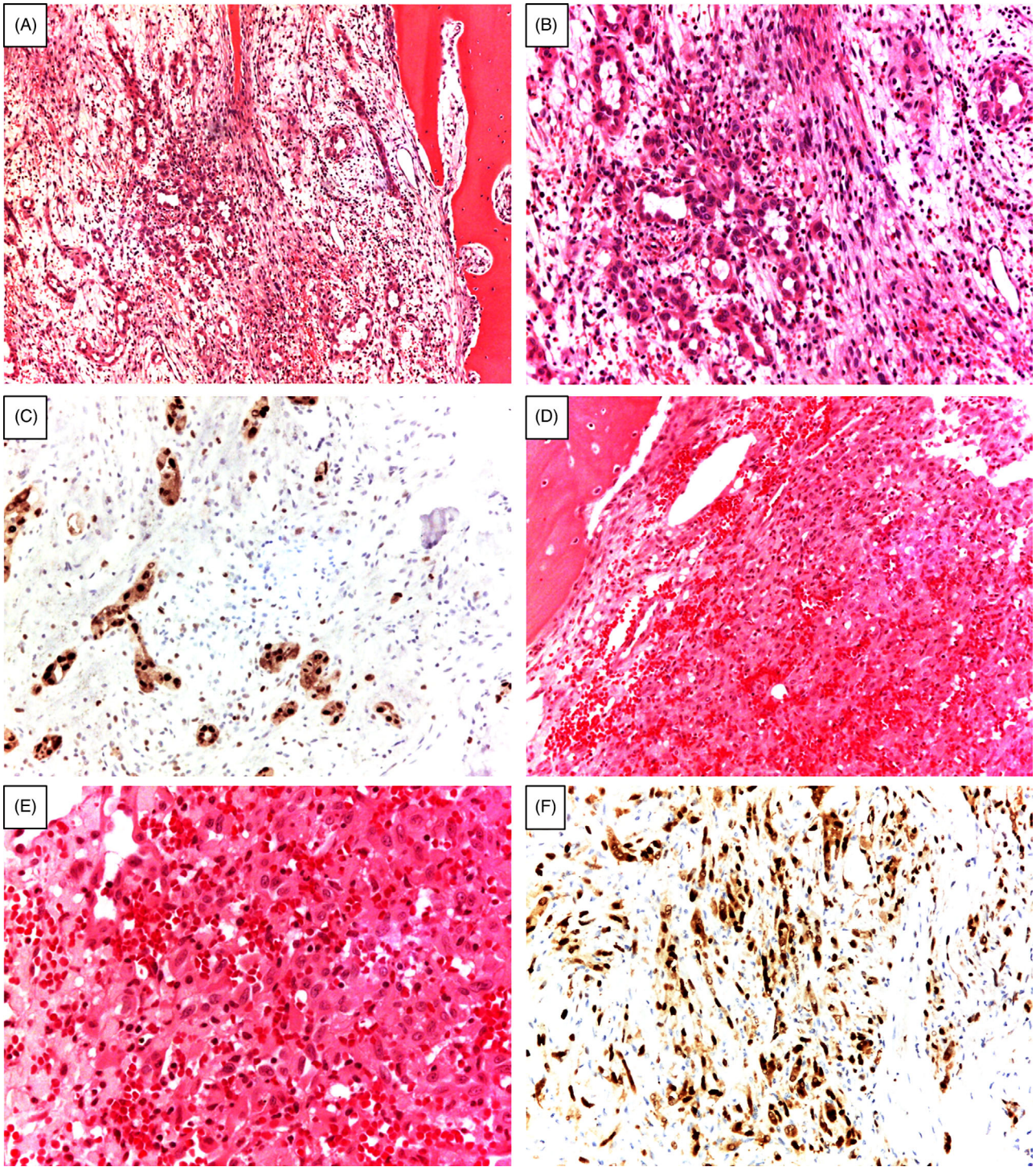

Figure 2. An example (EH22) of classic variant of epithelioid haemangioma composed of well-formed vascular channels lined by plump epithelioid endothelial cells, featuring moderate to abundant eosinophilic cytoplasm associated with eosinophils-rich inflammatory infiltrate: **A,** stain, haematoxylin and eosin, magnification; **B,** stain, haematoxylin and eosin, magnification; **C,** a nuclear immunohistochemical expression for FOSB antibody was observed in endothelial cells (magnification). An example (EH38) of atypical/cellular variant of epithelioid haemangioma of bone that shows solid neoplastic areas constituted of endothelial cells with abundant eosinophilic cytoplasm associated with extravasated red blood cells: **D,** stain, haematoxylin and eosin, magnification; **E,** stain, haematoxylin and eosin, magnification; **F,** nuclear FOSB immunoreactivity (magnification).

RT-qPCR indicated that 2/4 FOSB IHC-positive tumours (both atypical/cellular EH) expressed ZFP36::FOSB fusion; no ZFP36::FOSB fusion was detected in six FOSB IHC-negative EHs. An additional FOSB fusion (WWTR1::FOSB) was identified by both targeted and whole RNA-sequencing in a FOSB IHC-positive atypical/cellular EH. FISH highlighted FOS rearrangements in three of the four patients analysed, all classic EHs. RNA-sequencing revealed two further FOS rearrangements in classic EHs.

To determine the clonality relationship between separate EHs in the same patient, multifocal synchronous lesions of four patients were analysed for the expression of fusion transcripts. In these patients the separate lesions involved the same bone, contiguous bones (EH14, EH24, EH38) or non-contiguous bones (EH21). A targeted RNA-sequencing approach with a customised Archer fusion panel revealed the expression of a WWTR1::FOSB fusion with identical breakpoints in all three lesions of patient EH38 (Figure 3A, Table 3). Conversely, no high confidence fusions were detected by the Archer-suite software version 6.0.4 in cases EH14, EH21 and EH24. However, potential fusions involving FOS were included in the list of discarded/low-confidence calls. Raw sequencing data were then reanalysed with a more recent Archer-suite release (version 7.1.0) and with the Arriba fusion caller.[22] The potential fusions reported as discarded/low-confidence calls by the Archer-suite version 6.0.4 were erroneous, while the Archer-suite version 7.1.0 detected, with high confidence, a fusion of FOS with a sequence located on chromosome 11 in both lesions of case EH21. The Arriba tool not only confirmed the expression of this chimeric transcript, involving FOS and a long non-coding RNA in opposite orientation (FOS::ENSG00000255202 fusion; Figure 3B–D, Table 3), but efficiently captured the fusion events in all cases and indicated that synchronous EHs of the same patient shared FOS fusions with identical breakpoints. A FOS::VIM fusion had been previously reported in a metatarsal lesion of patient EH14.[16] Arriba analysis highlighted that both EH lesions of this patient carried indeed an identical FOS::chr10 fusion, involving FOS and an intergenic region on chromosome 10 located between VIM and ST8SIA6. An identical fusion of FOS with an intergenic region of chromosome 21, located between ADAMTS1 and CYYR1 genes, was also detected in the multiple EHs of patient EH24 (FOS::chr21 fusion).

These rearrangements, which were orthogonally validated by PCR-Sanger sequencing (Figure 3A–D), involved exon 4 of FOS and yielded a truncated FOS protein, due to the generation of a *de-novo* stop codon a few amino acids downstream from the breakpoint. No recurrent fusion partner was identified, in line with the fact that the biological significance of these truncating fusions is the hyperactivation of FOS via the removal of the C-terminal regulatory region.[16,24] The correspondence in the breakpoint sequence was also confirmed at the level of genomic DNA in the multiple lesions of cases EH14 and EH24 (not shown), and the presence of identical fusion events in separate EHs of the same patient strongly indicated a clonal origin.

We also analysed the global transcriptional profile of the multiple tumours of patients EH21, EH24 and EH38. Separate EHs of a same patient showed similar transcriptomes (Figure 3E,F). More importantly, RNA-sequencing not only confirmed the presence of the FOS or FOSB fusions but identified further identical fusion events shared by the tumour lesions of the same patient (Table 3). We focused upon the ones picked up by both Arriba and FusionCatcher tools and orthogonally validated by RT-PCR the expression of PSME3IP1::WWOX and TFG::ADGRG7 chimeras in cases EH24 and EH38, respectively (not shown). Both are intrachromosomal events and the TFG::ADGRG7 fusion was reported previously.[25]

Discussion

The WHO ambivalently classifies EH of soft tissue and EH of bone. The former are classified as benign tumours, the latter as intermediate, locally aggressive tumours.[1] Indeed, in the absence of objective criteria, the classification of EH of bone remains controversial.[1,2] Some authors consider EH a benign tumour, as none of the patients reported in the literature experienced an adverse outcome, while others argue that EH is an aggressive tumour because of its multifocal presentation and frequent local destructive growth with destruction of cortex and extension to soft tissues.[1,14,15,26,27]

In an attempt to resolve this ambiguity, we studied the clinical and biological characteristics of 42 patients with EH treated at a single institution. Although the retrospective design could be considered a limitation, the rarity of EH mandates that such a study be retrospective to have sufficient patients for analysis[28] and the relatively large case series is an advantage of the present study.

The retrospective analysis of 38 patients with EH of bone showed that prognosis was excellent, with no death of disease. Most patients were treated with

Table 2. Immunohistochemical and molecular data of the 42 patients with EH of bone

Patient ID	Histological variant	TFE3 and WWTR1 FISH analysis	FOSB IHC	ZFP36::FOSB RT-PCR analysis	FOS FISH analysis	FOS and FOSB fusions identified by NGS in paired lesions
EH1	Classic	–	+	ND	ND	
EH2	Classic	ND	–	–	ND	
EH3	Classic	ND	+	ND	ND	
EH4	Classic	ND	–	–	ND	
EH5	Classic	ND	–	ND	ND	
EH6	Classic	ND	+	ND	ND	
EH7	Classic	ND	+	ND	ND	
EH8	Classic	ND	+	ND	ND	
EH9	Classic	ND	–	ND	ND	
EH10	Atypical/ cellular	–	+	ND	ND	
EH11	Classic	–	–	–	ND	
EH12	Atypical/ cellular	ND	+	ND	ND	
EH13	Classic	ND	–	–	ND	
EH14	Classic	–	–	ND	+	FOS::chr10
EH15	Classic	ND	+	ND	ND	
EH16	Classic	ND	–	ND	ND	
EH17	Classic	–	–	ND	+	
EH18	Classic	ND	+	–	ND	
EH19	Classic	ND	–	ND	ND	
EH20	Atypical/ cellular	–	–	ND	ND	
EH21	Classic	ND	–	ND	ND	FOS::ENSG00000255202
EH22	Classic	ND	+	ND	ND	
EH23	Classic	–	–	ND	ND	
EH24	Classic	ND	–	–	ND	FOS::chr21
EH25	Classic	–	–	ND	ND	
EH26	Atypical/ cellular	ND	+	+	ND	
EH27	Classic	–	–	ND	ND	
EH28	Classic	ND	+	ND	ND	
EH29	Classic	–	–	–	+	
EH30	Classic	ND	+	ND	ND	
EH31	Classic	ND	–	ND	ND	

Table 2. (*Continued*)

Patient ID	Histological variant	TFE3 and WWTR1 FISH analysis	FOSB IHC	ZFP36::FOSB RT-PCR analysis	FOS FISH analysis	FOS and FOSB fusions identified by NGS in paired lesions
EH32	Classic	ND	–	ND	ND	
EH33	Atypical/ cellular	–	+	+	ND	
EH34	Classic	ND	+	ND	ND	
EH35	Classic	ND	+	–	ND	
EH36	Atypical/ cellular	–	+	ND	–	
EH37	Atypical/ cellular	ND	–	ND	ND	
EH38	Atypical/ cellular	ND	+	ND	ND	WWTR1::FOSB
EH39	Classic	–	+	ND	ND	
EH40	Classic	–	+	ND	ND	
EH41	Classic	–	+	ND	ND	
EH42	Classic	ND	+	ND	ND	

ND, Not done or not feasible; +, Positive; –, Negative.

curettage or resection. Local recurrence occurred in five patients (13%) treated with curettage. Multifocal presentation was detected in 17 of 42 patients (40%), in all but one as synchronous EH lesions. The same contiguous, but in some cases also non-contiguous, bones were involved.

Molecular characterisation of multifocal tumours indicated a clonal origin. Synchronous lesions affecting the same patient expressed the same fusion transcripts with identical gene breakpoints. These results are in keeping with the work by van Ijzendoorn and co-workers[16] who, by analysing two cases of multifocal EH, concluded that tumour foci affecting adjacent bones represent multifocal regional spread. Our work extends this observation also to EH lesions involving non-contiguous bones and supports the concept that multifocal presentations in this tumour represent the spread of a same neoplastic clone rather than simultaneous independent tumours.

Taken together, our data indicate that EH of bone is a tumour with a benign clinical course despite a disseminative potential. These two concepts are only seemingly contradictory. It has been estimated that millions of cells are shed by a tumour every day, although only a minute fraction of these will eventually seed into secondary colonies.[29] Metastasisation imposes that a tumour cell bypasses a number of roadblocks: to detach from the primary site, to survive in the lymphatic/circulatory system and to colonise secondary sites with induction of neoangiogenesis for tumour support.[30,31] Intriguingly, vascular tumours seem to be facilitated in some of these steps. Indeed, the multifocal presentation is somehow a distinctive feature of vascular tumours of bone, including classical haemangioma, haemangioendothelioma and angiosarcoma. Despite some cases of lymph node invasion and distal metastasis,[9,13,23] EH primarily demonstrates regional reseeding, suggesting that the capacity of EH cells to survive in the lymphatic/blood stream is somehow limited, and that their congenial soil is the organ of origin. In multifocal EH, the separate tumour lesions maintain their intrinsic 'benign' nature, as the presence of secondary seedings does not correlate with poor outcome. Thus, EH dissemination does not appear to be associated with aggressive biological traits; rather, having features of a passive phenomenon. Indeed, it has been previously reported that tumours may shed passively into the blood or lymphatic vessels in the absence of active cell migration.[31]

In conclusion, the excellent prognosis of EH of bone supports the contention that it is indeed a clinically benign tumour. Like other vascular tumours, EH of bone may be multifocal. Although tumour multifocality in EH of bone is the manifestation of a disseminative process, as established by clonality

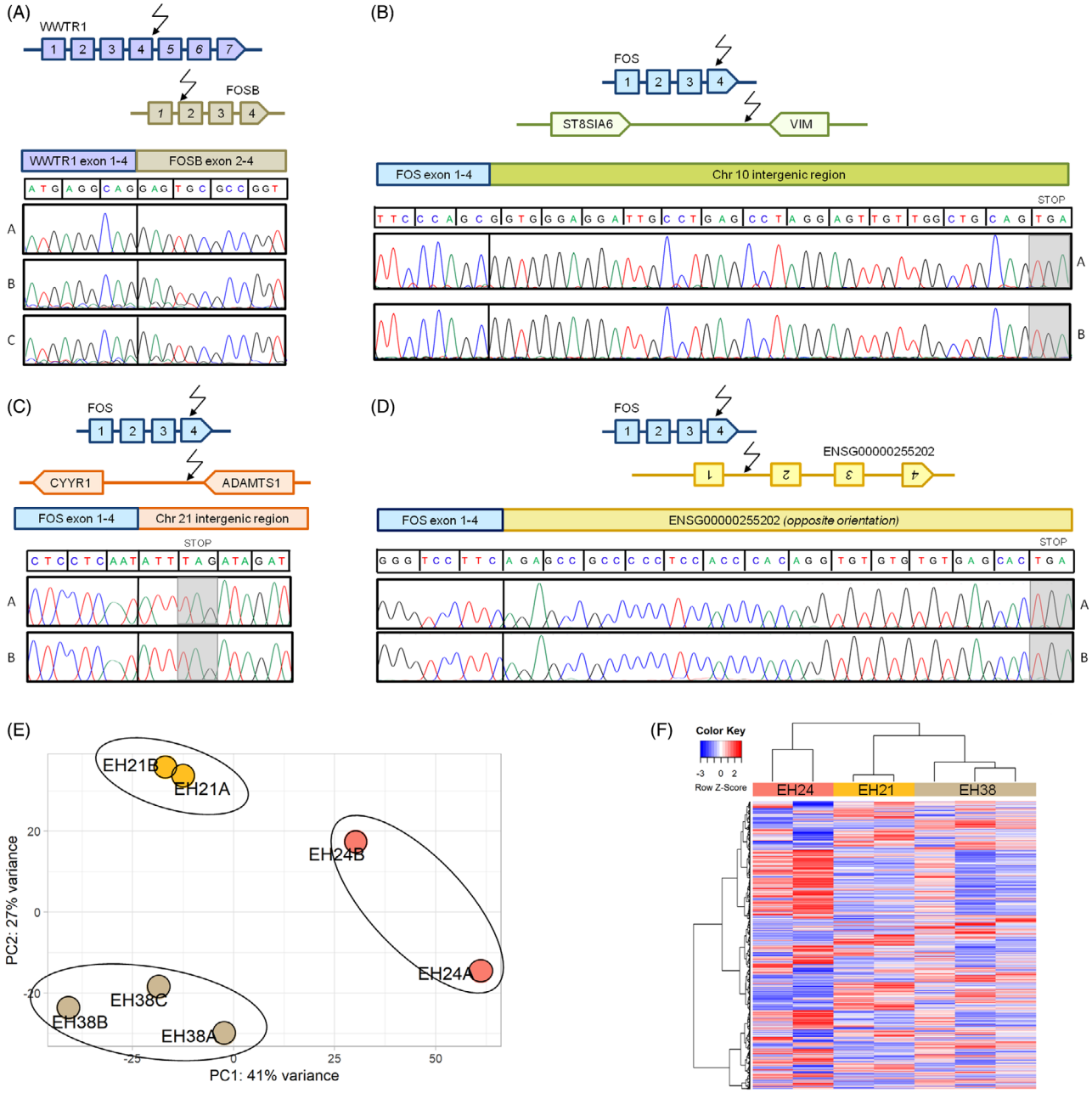

Figure 3. Fusion analysis of multifocal lesions indicates clonal relationship. **A,** Schematic representation of the WWTR1::FOSB fusion detected in the multifocal EHs of patient EH38. The arrows indicate the location of the breakpoints in the cDNA. The chromatogram in the lower panel confirms that the three EH lesions of this patient (**A,** left tibia; **B,** rotula; **C,** proximal tibia) share the same breakpoint. **B,** Schematic representation of the FOS::chr10 fusion detected in both EHs of case EH14. The fusion involved FOS exon 4 and an intergenic region of chromosome 10, close to the VIM gene. The chromatograms in the lower panel show that lesion A (IV metatarsus) and lesion B (II metatarsus) carry an identical breakpoint. The greyish area indicates the *de-novo* STOP codon provided by the 3′ partner. **C,** Illustration of the FOS::chr21 fusion detected in case EH24 involving FOS exon 4 and an intergenic region of chromosome 21 close to the ADAMTS1 gene. The same breakpoint sequence was detected in both lesions of this patient (**A,** distal tibia; **B,** III cuneiform). **D,** Case EH21 carried an identical FOS fusion (FOS exon 4 and lncRNA ENSG00000255202) in the lesions of the tibia and calcaneus (**A** and **B,** respectively). PCA (**E**) and unsupervised hierarchical clustering (**F**) of the multifocal EHs of patients EH21, EH24 and EH38 show co-clustering of paired lesions of the same patient.

Table 3. Recurrent fusions identified by RNA-sequencing in the separate tumour lesions of cases EH21, EH24 and EH38

Patient ID	Gene 1	Gene 2	Breakpoint_1	Site1	Breakpoint_2	Site2	Arriba (confidence)	FusionCatcher (confidence)
EH21A	FOS	ENSG00000255202	chr14:75281167	CDS	chr11:33694437	Intron	High	–
EH21B	FOS	ENSG00000255202	chr14:75281175	CDS	chr11:33694437	Intron	Low	–
EH21A	SYN2	ACTG1	chr3:12071910	Intron	chr17:81510151	3′ UTR	High	–
EH21B	SYN2	ACTG1	chr3:12071910	Intron	chr17:81510151	3′ UTR	Low	–
EH24A	FOS	chr21 (~ADAMTS1)	chr14:75281016	CDS	chr21:26829622	Intergenic	High	–
EH24B	FOS	chr21 (~ADAMTS1)	chr14:75281016	CDS	chr21:26829622	Intergenic	High	–
EH24A	PSME3IP1	WWOX	chr16:57185821	5′UTR/ splice	chr16:78164183	CDS/ splice	High	High
EH24B	PSME3IP1	WWOX	chr16:57185821	5′UTR/ splice	chr16:78164183	CDS/ splice	High	High
EH24A	NDUFS8	chr11 (~GSTP1)	chr11:68033283	CDS/ splice	chr11:67577746	Intergenic	High	–
EH24B	NDUFS8	chr11 (~GSTP1)	chr11:68033283	CDS/ splice	chr11:67577746	Intergenic	High	–
EH24A	PMEPA1	PURB	chr20:57651860	UTR	chr7:44879426	UTR	–	High
EH24B	PMEPA1	PURB	chr20:57651861	UTR	chr7:44879426	UTR	–	High
EH38A	WWTR1	FOSB	chr3:149542335	CDS/ splice	chr19:45470629	CDS/ splice	High	High
EH38C	WWTR1	FOSB	chr3:149542335	CDS/ splice	chr19:45470629	CDS/ splice	High	High
EH38A	WWTR1[1]	FOSB[1]	chr3:149528238	Intron	chr19:45469785	Intron	High	–
EH38B	WWTR1[1]	FOSB[1]	chr3:149528238	Intron	chr19:45469785	Intron	High	–
EH38C	WWTR1[1]	FOSB[1]	chr3:149528238	Intron	chr19:45469785	Intron	Low	–
EH38A	TFG	ADGRG7	chr3:100720058	CDS/ splice	chr3:100629598	CDS/ splice	Low	High
EH38B	TFG	ADGRG7	chr3:100720058	CDS/ splice	chr3:100629598	CDS/ splice	Low	High
EH38C	TFG	ADGRG7	chr3:100720058	CDS/ splice	chr3:100629598	CDS/ splice	High	High

Splice, Splice-site.
[1]Presplicing transcript.

analysis, this has no major impact on the clinical course of the disease, even for patients treated exclusively by surgery or biopsy. Therefore, EH of bone is a tumour with disseminative potential but of benign nature.

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Conflicts of interest

The authors declare that they have no conflicts of interest directly related to the topic of this article.

Clinical trial registration number

This study was registered at ClinicalTrials.gov (identifier NCT03169595).

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request. The data are not publicly available due to ethical restrictions.

Ethics approval

The study was approved by the ethics committee of IRCCS Istituto Ortopedico Rizzoli (Nr0030451del 10/09/2015).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Supporting Information S1. Materials and methods details.