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A novel specific genetic translocation in epithelioid hemangioendotelioma, showing a fusion of the WWTR1 and CAMTA1 genes, supports the monoclonality of multifocal epithelioid hemangioendotelioma.

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Esame finale anno 2012

To my wife, Daniela,

and my daughters, Olivia and Matilda,

for their loving support.

CONTENTS

Chapter 1

A novel specific genetic translocation in epithelioid hemangioendothelioma showing a fusion of the WWTR1 and CAMTA1 genes.

Introduction

Vascular tumors encompass a wide histologic spectrum and include hemangioma, hemangioendothelioma, angiosarcoma, and their epithelioid variants (Wenger and Wold, 2000; O'Connell et al., 2001). The vast majority of both benign and malignant vascular tumors are readily diagnosed based on their characteristic histologic features, such as the formation of vascular spaces and the expression of endothelial markers. However, some vascular tumors have atypical histologic features, such as a solid growth pattern, epithelioid change, or spindle cell morphology, which complicates their diagnosis (Folpe et al., 2001). For this rare subset of vascular tumors, there remains considerable controversy in regards to the terminology and the classification that should be used (O'Connell et al., 2001; Evans et al., 2003). For example, Evans et al. (2003) argued that epithelioid hemangioma is not a distinct tumor entity but rather a misdiagnosed hemangioendothelioma, a tumor that, unlike hemangioma, has metastatic potential. Furthermore, hemangioendothelioma of bone is not listed as a distinct diagnostic entity in current classification systems (World Health Organization Classification of Tumours, 2002).

The genetic hallmark of vascular tumors is still under investigation. To date, only a few cases of vascular tumors have been analyzed cytogenetically, reporting different chromosomal translocations (Boudousquie et al., 1996; He et al., 2006; Dunlap et al., 2009). However, Mendlick et al. (2001) found an identical chromosomal translocation involving

chromosomes 1 and 3 [t(1;3)(p36.3:q25)] in 2 cases of epithelioid hemangioendothelioma (EHE), which possibly represents a characteristic rearrangement for this histopathologic entity. Therefore, we undertook a systematic molecular analysis of a large spectrum of EHEs, including lesions from various anatomic locations and lesions with different biological potentials. We hypothesized that a better understanding of the molecular signature of vascular tumors may help to refine the present classification system based on immunophenotype alone.

Material and Methods

We retrieved 23 cases of EHE with tissue samples available for molecular analysis from the surgical pathology and consultation files of our institution. In each case, we confirmed the pathologic diagnosis and the histologic grade by reviewing the pathology slides and by immunostaining them for the following endothelial cell markers: CD31, CD34, FLI1, and von Willebrand factor. The tumors were assessed morphologically for growth pattern, vasoformative nature, epithelioid versus spindle cell composition, cellular pleomorphism, mitotic activity, and necrosis (Fig 1).

For each case, the location of the tumor was recorded, along with the anatomic structures involved. Based on their location, the lesions were classified into 4 groups: bone, soft tissue, intrathorax, and liver.

Because EHE, a low-grade tumor with metastatic potential, is intermediate between epithelioid hemangioma, a benign tumor, and epithelioid angiosarcoma, a high-grade malignant tumor, we included 15 cases of epithelioid hemangioma and 5 cases of epithelioid angiosarcoma to determine if there was any relationship between them. In addition, we included 3 cases of epithelioid sarcoma because this tumor has the same morphologic and immunophenotypic features as EHE.

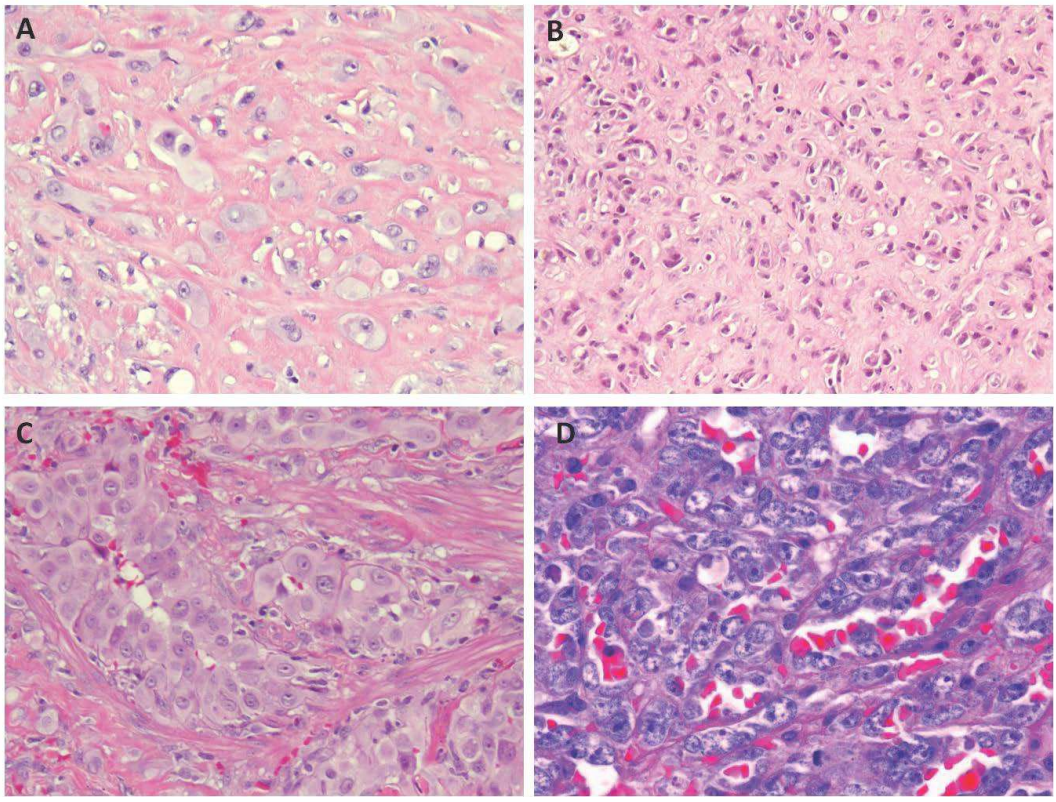


Fig 1: Morphological appearance of epithelioid vascular tumors analyzed in this study. (A) Malignant EHE of the arm, with cords and single cells of epithelioid cells with moderate nuclear atypia, embedded in a hyalinized stroma. (B) Classic EHE of the liver with bland epithelioid cells with readly visible intracytoplasmatic vacuoles. (C) EH of the penis in a 48-year-old man, who presented as multiple cutaneous and s.c. nodules, and showed large epithelioid cells with abundant eosinophilic cytoplasm surrounding vascular lumina. (D) Radiation-induced angiosarcoma of breast, composed of predominantly epithelioid morphology and showing high grade cytologic atypia, with prominent nucleoli, as well as vascular channel formation.

FISH was performed on paraffin-embedded 4-µm-thick tissue sections using custom-labeled FISH probes, as previously described (Antonescu et al., 2010). Each case was analyzed with 3 probes covering and flanking chromosomes 1p36.3 and 3q25. The rearranged regions of each chromosome were then evaluated using 3 new probes. This process was repeated as much as possible to zoom into the rearranged chromosomal regions (Fig. 2, 3).

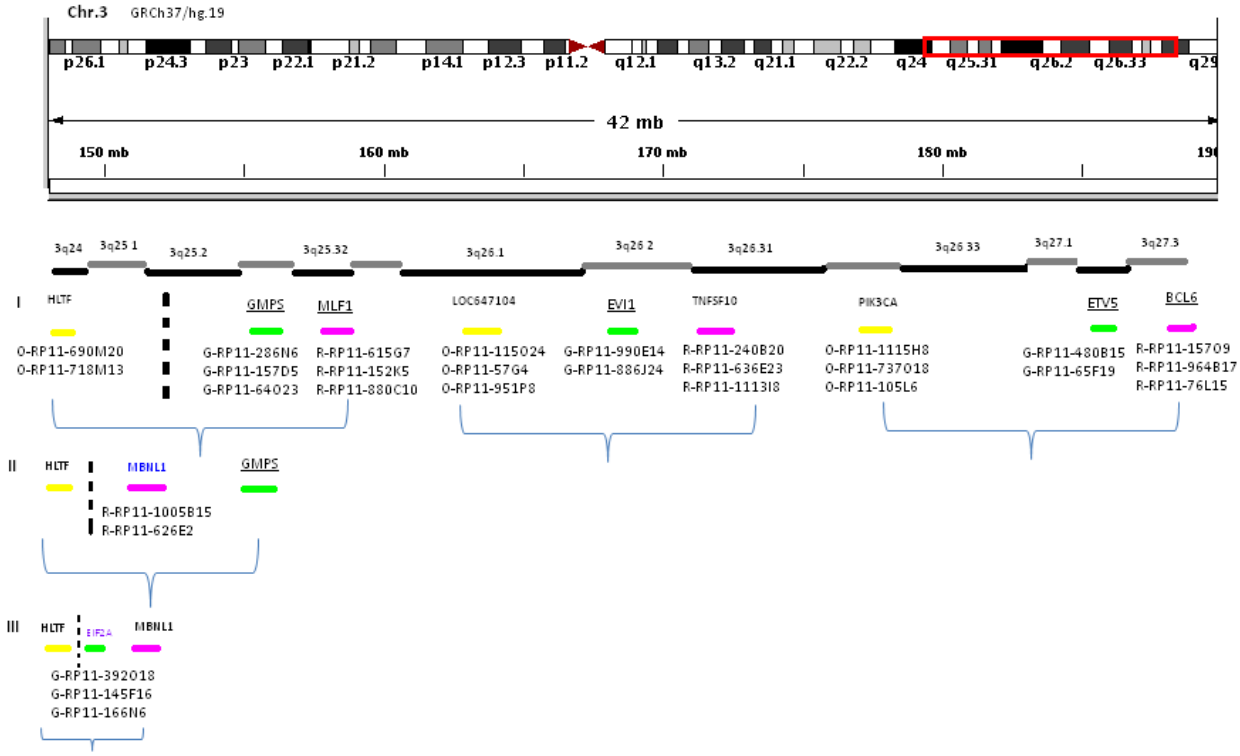


Fig 2: FISH positional cloning strategy using BAC probe sets on 1p36.33-1p36.11. Three sets of experiments identified the breakpoint in 1p36.23. Underlined genes have been previously reported in other chromosomal translocations.

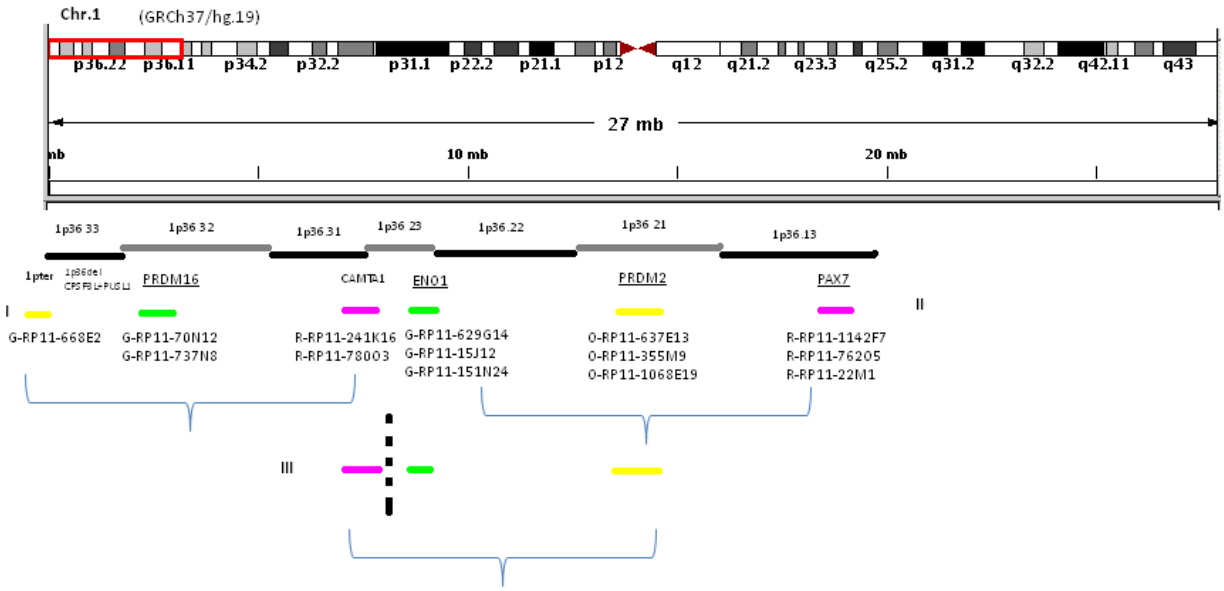


Fig 3: Distribution of BAC probe sets tested spanning the 3q24-27 region. Three rounds of FISH experiments illustrated in this diagram were able to narrow-down the break-apart region between 3q24-25.1. Underlined genes have been previously reported in other chromosomal translocations.

FISH enabled us to focus on the 200-kb region in which the CAMTA1 and WWTR1 genes are located in chromosomes 1 and 3, respectively. Therefore, we performed reverse transcriptase-polymerase chain reaction (RT-PCR) on the 3 cases of EHE with frozen tissue available using housekeeping primers, as previously described (Antonescu et al., 2010). The RT-PCR products were analyzed by electrophoresis, and the RT-PCR-amplified products were sequenced using the Sanger method (Antonescu et al., 2010)**.**

Results

In this study, we included a total of 17 cases of immunohistochemically confirmed EHE with tissue available for molecular analysis (Table I).

EHE Case no.	Age	Sex	Location	IHC	WWTR1-CAMTA1 Fusion	Oncological Outcome	Months
1	52	F	Soft Tissue	CD 31 and CD 34	+	AWD	15
2	54	M	Soft Tissue	CD 31 and CD 34	+	NED	108
3	59	F	Soft Tissue	CD 31 and Factor VIII	+	NED	116
4	39	M	Soft Tissue	CD31, CD34 and FLI1	+	NED	14
5	68	M	Soft Tissue	CD31 and Factor VIII	+	NED	16
6	66	M	Soft Tissue	CD31 and CD34	+	NED	4
7	39	F	Soft Tissue	CD31 and CD34	+	DOD	43
8	56	M	Intra-thoracic	CD31 and CD34	+	NED	30
9	65	F	Intra-thoracic	CD31 and CD34	+	NED	20
10	61	M	Intra-thoracic	CD31, CD34 and Factor VIII	+	DOD	82
11	32	M	Intra-thoracic	CD34	+	AWD	7
12	29	F	Intra-thoracic	CD31 and CD34	+	Lost at FU	?
13	42	F	Intra-thoracic		+	NED	23
14	34	M	Intra-thoracic	CD31	+	DOD	4
15	48	F	Liver	CD31 and CD34	+	Lost at FU	?
16	41	F	Liver	CD31 and CD34	+	NED	7
17	25	M	Bone	CD31	+	DOD	24

Table I: EHE, epithelioid hemangioendotelioma; NED, no evidence of disease; AWD, alive with disease; DOD, dead of disease; FU, follow-up.

Six cases were excluded because of unsuccessful fluorescence in situ hybridization (FISH): 4 cases because of low cellularity and 2 cases because of decalcification. There were 8 women and 9 men, with a median age of 48 years (range, 25 to 68 years). The anatomic distribution of EHE was as follows: 7 cases in soft tissue, 7 in the intrathorax, 2 in the liver, and 1 in bone.

All cases had an identical chromosomal translocation involving chromosomes 1 and 3 [t(1;3)(p36.23:q25.1)]. Immunohistochemically, all tumors were positive for CD31, showing typically strong and diffuse staining, as well as for CD34 and/or Factor VIII or FLI1. The RT-PCR applied in the 3 tumors with available frozen tissue showed 3 different rearrangements: fragments of exons 8 and 9 of CAMTA1 were fused in-frame to a fragment of exon 2 of WWTR1 (Fig. 4).

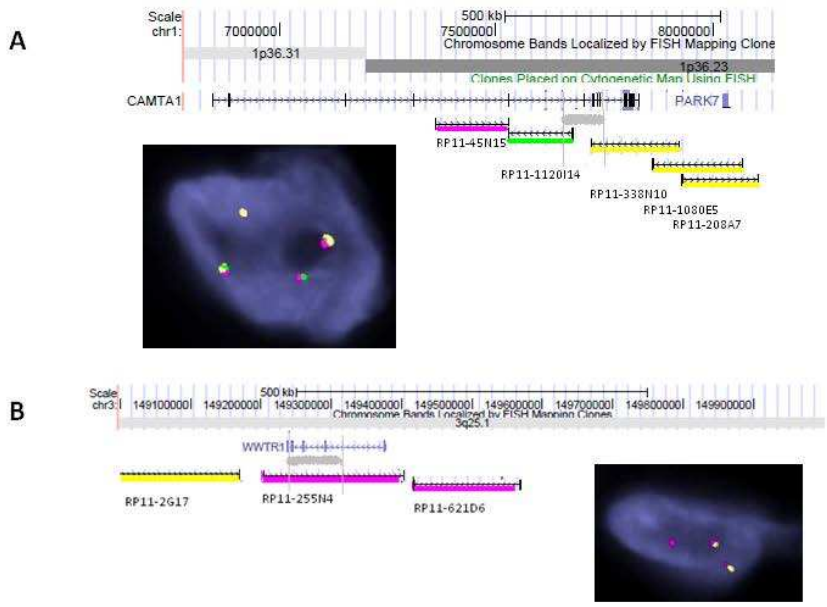


Fig. 4: Identification of candidate genes on 1p36.23 and 3q25 by FISH. (A) Gray are showed 1p36.23 breakpoint location within CAMTA1 gene. Three-color FISH showed a break-apart between green-RP11-1120114 and Orange-RP11-338N10 (inset). (B) Two-color FISH (orange-RP11-2G17 and Red-580-RP11-255N4) identified a split red signal associated with the orange signal (inset). This pattern narrowed the breakpoint at chr.3: 149270000 (hg. 19), which localized in WWTR1 exon 4 to exon 8.

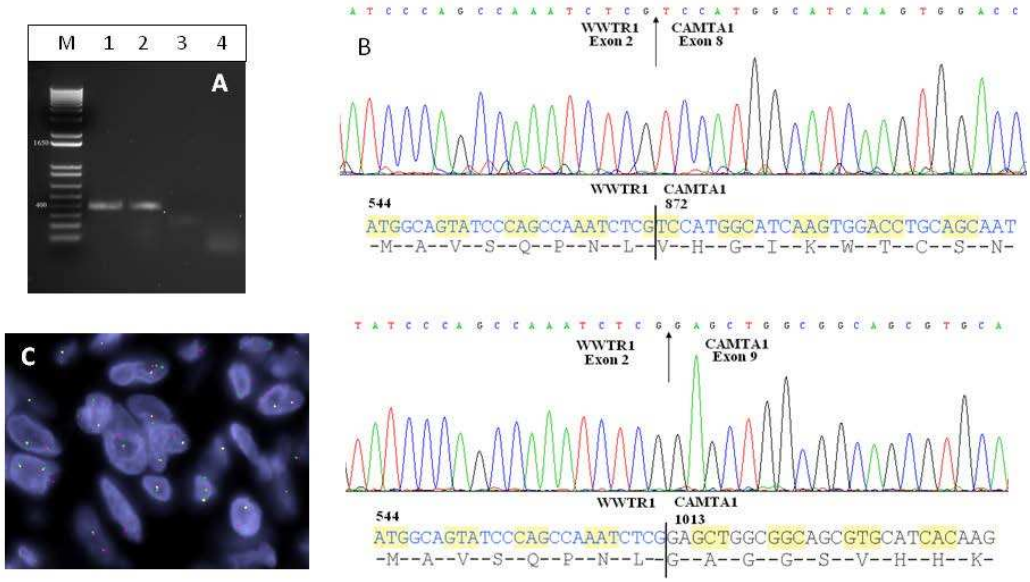


Fig. 5: RT-PCR detection of WWTR1-CAMTA1 fusion transcript variants and FISH demonstration of fused CAMTA1 and WWTR1 signals. (A) Gel electrophoresis showing amplified products in lanes 1-3, of two distinct sizes (M, size marker, lane 1; EHEs line 1, 2 and 3; negative control, lane 4). (B) Sequencing of three amplicons identified two molecular variants, with exon 4 of WWTR1 being fused in-frame to either exon 8 (variant 1, upper panel) or exon 9 (variant 2, lower panel) of CAMTA1. (C) FISH demonstration of fused signals, using probes centromeric to CAMTA1 and telomeric to WWTR1.

In terms of survival outcome, at follow-up, 9 patients were alive with no evidence of disease, 2 were alive with disease, 4 had died of disease, and 2 were lost to follow-up.

None of the other vascular tumors (13 cases of epithelioid hemangioma, 5 of epithelioid angiosarcoma, and 3 of epithelioid sarcoma) had a WWTR1-CAMTA1 fusion. Two epithelioid hemangiomas of bone were excluded because of unsuccessful FISH due to decalcification.

Discussion

One of the most confusing issues related to vascular tumors is the myriad of names that are used to describe them. Pathologically, these tumors are remarkably similar, which makes differentiating them from each other very difficult (Wenger and Wold, 2000). This issue is compounded by the fact that current surgical pathology textbooks inadequately

describe and classify these tumors. Moreover, most of these textbooks do not even acknowledge the existence of the 3 subtypes of epithelioid neoplasms. Not surprisingly, epithelioid hemangioma continues to be confused with EHE (O'Connell et al., 2001). In a series of 13 patients with so-called hemangioendothelioma reported by Evans et al. (2003), 3 of their patients were treated with chemotherapy, and another 3 underwent amputation. However, none of the patients in their series died. Furthermore, Rosenberg has argued that Evans et al.'s illustrations of the tumors show characteristics of epithelioid hemangioma, a benign neoplasm (Floris et al., 2006). This example illustrates the danger inherent in using poorly defined and inappropriate terminology to classify vascular tumors. Because clinical behavior and, consequently, treatment and prognosis vary significantly among vascular tumors, it is important to effectively and accurately distinguish them from each other.

Currently, we are limited to our subjective interpretations, so molecular analysis may help provide an objective answer. Prior to the current study, an identical chromosomal translocation [t(1;3)(p36.3:q25)] was identified in 2 cases of EHE arising in 2 distinct anatomic locations, the liver and soft tissue (Mendlick et al., 2001).

In our study, an in-depth molecular analysis of 17 cases of EHE arising in different anatomic locations revealed an identical genetic translocation [t(1;3)(p36:q25)] involving the CAMTA1 and WWTR1 genes on chromosomes 1 and 3, respectively. As a result of the translocation, 2 protein-coding regions were fused in-frame, producing a chimeric protein. To our knowledge, this is the first time that a CAMTA1-WWTR1 fusion has been reported. This is especially important because the CAMTA1 and WWTR1 genes have been shown to play an important role in oncogenesis (Barbashina et al., 2005; Henrich et al., 2006; Lei et al., 2008; Chan et al., 2009; Zhang et al., 2009).

CAMTA1

CAMTA1 is a member of a recently described protein family designed as calmodulin-binding transcription activators (CAMTAs) (Bouché et al., 2002). Its primary structure contains a nuclear localization signal, 2 DNA-binding domains (CG-1 and TIG), calmodulin-binding motifs, and ankyrin repeats. CAMTA1 is a transcription activator potentially involved in cell cycle regulation (Nakatani et al., 2004) that may interact with Ca^{2+}/calmodulin and be engaged in Ca^{2+} signaling (Bouché et al., 2002).

In mammalian cells, Ca^{2+} and the Ca^{2+} receptor calmodulin are involved in the regulation of gene transcription; nuclear and cytoplasmic Ca^{2+} control transcription by distinct mechanisms. Indeed, certain transcription factors are selectively activated in response to distinct Ca^{2+} signal duration and amplitude (Bouché et al., 2002; Lipskaia and Lompré, 2004; Munaron et al., 2006). A sustained increase in cytosolic Ca^{2+} is necessary to activate calcineurin, a Ca^{2+}/calmodulin–dependent phosphatase, which dephosphorylates many proteins including the transcription factor NFAT (nuclear factor of activated cells) and induces its translocation to the nucleus (Lipskaia and Lompré, 2004; Munaron et al., 2006).

By contrast, transient Ca^{2+} influx is particularly effective in activating CREB, the cAMP-responsive element binding protein, via Ca^{2+}/calmodulin–dependent phosphorylation by Ca^{2+}/calmodulin–dependent protein kinase (CaMK) or by mitogen-activated protein kinase (Lipskaia and Lompré, 2004; Munaron et al., 2006). Phosphorylation of CREB by Ca^{2+} facilitates its interaction with the co-activator CREB-binding protein (CBP) or the related protein p300. There is substantial evidence suggesting that the p300/CBP transcriptional co-activators play a critical role in the transactivation of the tumor suppressor p53 and on downstream effects of p53 on growth arrest and apoptosis. Therefore, one of the functions of CREB phosphorylation via Ca^{2+} entry might be the maintenance of a quiescent

state, at least in excitable cells (Lipskaia and Lompré, 2004; Finkler et al., 2007).

A possible correlation between CAMTA1 and growth control is further supported by Nakatani et al. (2004), who examined the expression of CAMTA1 mRNA and protein during cell cycle progression in human neuroblastoma cells. Because the expression of CAMTA1 was found to be similar to that of p53 in neuroblastoma cell lines, they speculated that CAMTA could be involved in cell cycle regulation in the same way as p53 (Nakatani et al., 2004).

Bouché et al. (2002) investigated the properties of members of the CAMTA family from Arabidopsis and humans and demonstrated the ability of both to interact with DNA in vitro and activate transcription in yeast cells. Using the fly CAMTA, Gong et al. (2007) further demonstrated that CAMTAs may function as a dimer, both in vitro and in fly photoreceptor neurons, and that the CG-1 domain may mediate the potential dimerization of CAMTA transcription factors. Therefore, in organisms with multiple CAMTAs, the possibility of homo- and heterodimerization exists with further functional implications (Finkler et al., 2007; Gong et al., 2007).

A possible role for human CAMTA1 in cell proliferation and tumor suppression has recently been put forward by several research groups (Katoh and Katoh, 2003; Attiyeh et al., 2005; Barbashina et al., 2005; Henrich et al., 2006).

Loss of genetic material on the short arm of chromosome 1 occurs in many human cancers. In a study of 683 solid tumors arising at different anatomic locations, the prevalence of loss of heterozygosity on 1p ranged from 30% to 64%, depending on tumor location (Ragnarsson et al., 1999). However, the most extensive 1p deletion mapping in search of tumor suppressors has been done in neuroblastomas, which are known to have 1p losses in about 30% of cases (Maris et al., 2000; Attiyeh et al., 2005; White et al., 2005).

Although the expression of CAMTA1 is seen in various organs, low CAMTA1 expression seems to be significantly associated with poor outcome in neuroblastoma (Attiyeh et al., 2005; Henrich et al., 2006). In addition, Katoh and Katoh (2003) showed that the CAMTA1 gene was located within the commonly deleted region of neuroblastoma. The potential role of CAMTA1 in tumor development is also supported by Barbashina et al. (2005) who showed, in a subset of gliomas, that a deleted region on 1p36 involved the CAMTA1 gene.

Taken together, these data strongly suggest that CAMTA1 is involved in the development of neuroblastoma and other tumors with 1p mutations. Identifying CAMTA1 downstream target genes and interacting proteins are among the major tasks ahead. Such studies should provide important information to elucidate the role of CAMTA1 in oncogenesis and, consequently, improve diagnostic tools and therapies.

WWTR1

WWTR1, also called TAZ, is a transcriptional co-activator with PDZ-binding motif that was initially identified by its ability to interact with 14-3-3 proteins. Sharing amino acid sequence homology with YAP (Yes-associated protein), TAZ contains a conserved WW domain capable to interact with the PDZ domain (Kanai et al., 2000). Lei et al. (2008) reported that TAZ is negatively regulated by LATS tumor suppressor kinase, which is a component of the Hippo pathway initially defined by genetic studies in *Drosophila melanogaster* (Justice et al., 1995; Xu et al., 1995; Tapon et al., 2002).

The Hippo pathway controls organ size and contact inhibition by regulating cell proliferation and apoptosis (Chan et al., 2010a). It is conserved from fly to human and its deregulation in mammals often leads to tumorigenesis (Chan et al., 2010a). The downstream

effectors of the Hippo pathway in mammals are the transcriptional co-activators YAP and TAZ.

The transcription factors TEAD1-4 (TEADs) in mammals are major interacting partners for functional outcome. When TAZ is not inhibited by the Hippo pathway and remains in the nucleus, it interacts with TEADs and activates expression of genes such as CTGF, IGFBP3, ITGB2, Birc5/Survivin, Gli2, and Axl (Chan et al., 2010a). Phosphorylation of TAZ by LATS leads to 14-3-3 binding and translocation from the nucleus to the cytoplasm, resulting in functional inactivation of this transcription co-activator. TAZ itself has no DNA-binding domain, and so it must bind to DNA-binding transcription factors to stimulate downstream target gene expression (Lei et al., 2008).

TAZ has been shown to interact not only with TEADs (Chan et al., 2009) but also with many other proteins, such as EphrinB1 (Xing et al., 2010), Cbfa1/Runx2 (Hong et al., 2005), Wbp2 (Chan et al., 2010b), and PAX3 (Marukami et al., 2005). The identification of a myriad of TAZ-interacting transcription factors participating in various cellular and development processes raises an important question as to which protein is most relevant to the role of TAZ in oncogenesis and what is the underlying molecular mechanism (Chan et al., 2009).

Chan et al. (2009) presented evidence supporting a novel mechanism for TEADs to mediate nuclear accumulation of TAZ to promote oncogenic transformation. They suggested that TAZ distribution is regulated by 2 major regulatory mechanisms. The first is the well-defined cytoplasmic sequestration by interaction with 14-3-3 proteins upon its phosphorylation by the Hippo pathway; the other is nuclear retention mediated by its interaction with TEADs. Their results suggest that endogenous TEADs, and especially TEAD4, are important for TAZ to promote oncogenic transformation of MCF10A cells (Chan et al., 2009).

Zhang et al. (2009) supported these results and identified TEADs transcription factors as the major TAZ-interacting transcription factors in HEK293 cells. They further demonstrated that TEADs are indispensable for TAZ function in promoting cell proliferation and cell migration and in inducing epithelial mesenchymal transition, which are all involved in cancer initiation and progression (Zhang et al., 2009).

TAZ is highly expressed in a wide spectrum of human cancer cell lines and various primary tumors, suggesting that this protein has oncogenic potential (Chan et al., 2008; Balasenthil et al., 2010). Chan et al. (2008) reported that TAZ was highly expressed in invasive breast cancer cell lines and in a significant fraction of primary breast cancers. They also reported that TAZ overexpression induced morphologic changes characteristic of cell transformation and enhanced cell migration and invasion (Chan et al., 2008).

In addition, Balasenthil et al. (2010) found TAZ overexpression in pancreatic cell lines. Ectopic TAZ expression also induced cell proliferation, overcame contact inhibition, and led to tumorigenesis in nude mice (Lei et al., 2008).

Taken together, these finding advance our understanding of the role of TAZ in cancer development and provide a potential therapeutic target for cancer treatment. Chan et al. (2011) recently showed that angiomotin, a novel regulator of endothelial cell migration (Troyanovsky et al., 2001), can interact with TAZ, leading to its cytoplasmic retention and inhibiting its transcriptional outcome and oncogenic property. This interaction causes cytoplasmic sequestration of TAZ in a manner similar to, but independent of, TAZ interaction with the Hippo pathway. Along with this study, future experiments should further our understanding of the possible use of angiomotin as a targeted therapy for EHE.

Acquired chromosome abnormalities were first suggested to be casual factors in the origin of cancer by Boveri in 1902 (Boveri, 2008). However, the first specific translocation identified in human neoplasia was t(9;22)(q34;q11), resulting in the Philadelphia

chromosome (Rowley JD, 1973). There is now a general agreement that cancer is a genetic disease with 2 types of initiating genetic events having been identified: the inactivation of genes by deletion, mutation, or epigenetic mechanism, and the activation or deregulation of genes as a consequence of point mutation, amplification, or balance cytogenetic abnormalities (Mitelman F et al., 2007). It is also should be noted that there are a few examples of balanced translocations leading to a loss of gene function (Popovici et al., 2002; Belloni et al., 2004).

Compared with haematological disorders, our knowledge of the karyotype of solid tumors is limited. All solid tumors make up only 27% of the total number of cases with an abnormal karyotype reported in the literature (Mitelman F et al., 2007). In fact, we know less about cytogenetics of the most common malignant tumors becasuse the chromosome morphology is often poor and the karyoptype is usually complex. However, molecular and cytogenetic studies performed over the past decades have had a major impact on the identification and classification of a large variety of sarcomas (Bovée and Hogendoorn, 2010).

Non-random chromosomal translocations have been detected in about 15% to 20% of mesenchymal tumors, and they are restricted to specific tumor types (Mitelman et al., 2007; Bovée and Hogendoorn, 2010). Tumor specific molecular changes can be useful for several reasons. First, the identification of chromosomal translocations helps the pathologist in diagnosing these lesions. Second, these tumor-specific molecular changes may serve as markers to detect minimal residual disease. Third, these molecular data increase our understanding of the pathogenesis of cancer. Finally, recurrent fusion oncogenes offer the best potential targets for therapeutics strategies (Kaye, 2009; Bovée and Hogendoorn, 2010).

Since vascular tumors are uncommon neoplasms, they are generally regarded as difficult to classify by surgical pathologists. The differential diagnosis of these tumors can be very difficult because of their remarkably similar histopathologic and morphologic features.

Although morphologic and immunohistochemical features remain the cornerstone of diagnosis, tumor-specific genetic alterations can be very helpful in diagnosis-making (Bovée JV and Hogendoorn PC, 2010). Indeed, there is a strong sentiment to reclassify solid tumors on the basis of their pathogenetic fusion translocations (Kaye, 2009).

It is important to emphasize that the rearrangenets might not be the sole anomaly. In fact, tumor developmemt is usually clonal evolution process driven by the accumulation of new genetic changes. However, recurrent balanced aberrations represent often an initial event in oncogenesis. Moreover, there is some evidence that the expression of certain sarcoma gene fusions is sufficient for the cell differentiation and tumorigenicity (Riggi et al. 2006; Riggi et al 2010).

Most balanced structural rearrangements have been found to exert their tumorigenic action by 2 alternative mechanisms: overexpression of a gene in one of the breakpoints, or the creation of a hybrid gene through the fusion of two genes, one in each breakpoint. Therefore, the identification of structural chromosome changes is important because the breakpoints involved point to the location of cancer-relevant genes (Mitelman et al., 2007). Specific translocations can also reveal targets for therapy. A fusion product involving the collagen type 1, α1 gene and the platelet-derived growth factor B gene (COL1A1-PDGFB) in dermatofibrosarcoma protuberans can be blocked using tyrosine kinase inhibitors at PDGFR, such as imatinib (Bovée and Hogendoorn, 2010).

In summary, we identified a novel specific chromosomal translocation [t(1;3)(p36:q25)] in 17 cases of EHE arising in distinct anatomic locations and involving the CAMTA1 and WWTR1 genes. This chromosomal translocation may serve as the ultimate biomarker, as it is specific for this distinct histopathologic tumor type, so it may be helpful to refine the classification of vascular neoplasms.

Furthermore, it is widely accepted that fusion proteins resulting from chromosome

translocations are oncogenic, based on evidence that they are able to transform cells in culture (Xia and Barr, 2005). As more oncogenic properties of the fusion protein and cooperative events are elucidated, therapeutic strategies can be further developed to interrupt these oncogenic processes.

Chapter 2

Monoclonality of multifocal epithelioid hemangioendothelioma: confirmation by analysis of WWTR1-CAMTA1 rearrangements.

Introduction

Epithelioid haemangioendothelioma (EHE), similar to other vascular tumors, presents with multiple non-contiguous tumors in approximately 50% of cases, and it is unclear whether the separate lesions represent multicentric disease or metastases (Deyrup and Montag, 2007; O'Connell et al., 2001). Multicentricity in mesenchymal neoplasms is defined the presence of tumor at two or more anatomically separated sites, before the manifestation of disease in sites where sarcomas most commonly metastasize, such as the lungs (Antonescu et al., 2000). Because the clinical course of EHE is frequently indolent, the concept that different lesions are independent primary tumors often prevails (Gupta et al., 2006; O'Connell et al., 2001).

However, it seems that we are limited to our subjective interpretations and that we must wait for molecular analysis of vascular tumors before a more definitive and objective answer becomes apparent.

In this study, we examined the question of whether EHE is a metastatic or multicentric disease. The recent identification of *WWTR1-CAMTA1* fusion, as the genetic hallmark of EHE irrespective of anatomic location, provides an objective and powerful diagnostic tool that can be used to distinguish if multifocal EHE has a monoclonal origin. In fact, as expected, in our previous study the genomic breakpoints of the t(1;3)(p36;q25) differed from one patient to others (Errani et al., 2011).

Therefore, we undertook a molecular analysis of 2 multicentric EHEs of the liver, including separate tumor samples from each patient. Our hypothesis is that the identification of an identical *WWTR1-CAMTA1* rearrangement in different lesions from each patient could explain the monoclonal origin of EHE.

Material and Methods

We retrieved 2 cases of EHE from the surgical pathology files of our institution with available tissue for molecular analysis. In each case, the diagnosis and histologic grade was confirmed by reviewing the H&E slides. All tumors included for analysis were positive for the CD31 endothelial marker. The tumors were assessed morphologically for growth pattern, vasoformative nature, epithelioid versus spindle cell composition, cellular pleomorphism, mitotic activity, and necrosis (Fig. 1).

For each case, the location of the tumor was recorded, along with the anatomic structures involved. Both patients presented with multiple sites in the liver, two lesions and three lesions, respectively (Fig. 2).

Fluorescence in situ hybridization (FISH) positional cloning of the t(1;3)(1p36.23;3q25.1).

FISH was performed in both cases for the presence of *WWTR1/CAMTA1* rearrangement to confirm the histologic diagnosis (Errani et al., 2011).

As previously reported, BAC clones were obtained from the BACPAC Resources Center of the Children's Hospital of Oakland Research Institute (http://bacpac.chori.org) (Errani et al., 2011). Probe preparation and FISH analysis were performed on paraffin-embedded, 4-μm-thick tissue sections, as previously described (Antonescu et al., 2010).

In brief, BAC DNA was isolated using phenol-chloroform, labeled with different fluorochromes (Enzo, PA, USA) in a nick translation reaction, and validated on normal

metaphases. Probe mixtures were co-denatured, and hybridized to pretreated slides. Slides were incubated, washed and mounted with DAPI in an antifade solution. At least two hundred successive non-overlapping nuclei were examined using a fluorescence microscope.

A case was confirmed as positive for rearrangement of a given gene when ≥ 20% of the nuclei examined showed a break-apart signal pattern using its respective BAC probes.

Reverse transcriptase-polymerase chain reaction (RT-PCR).

In both EHE tumors adequate RNA extracted from frozen tissue (Trizol Reagent; Invitrogen, USA) was available to investigate possible fusion transcripts from each different lesion in each patient. RNA quality was determined by Eukaryote Total RNA Nano Assay and cDNA was tested by RT-PCR for PGK housekeeping gene. A two-step RT-PCR was used, with Oligo(dT)20 primer under SuperScript® III system (Invitrogen, USA) being applied for first-strand cDNA synthesis, followed by a second-step PCR, using the HotStar Taq Master Mix (Qiagen, Valencia, CA). The RT-PCR products were analyzed by electrophoresis and the RT-PCR amplified products were sequenced using the Sanger method**.** Primers used for the RT-PCR detection of *WWTR1-CAMTA1* fusion are listed in Table 1.

Results

FISH analysis for the presence of a *WWTR1* and *CAMTA1* gene rearrangements showed signal abnormalities in both *WWTR1* and *CAMTA1*. Combined results confirmed the translocation t(1;3)(1p36.23;3q25.1) in both EHE cases (Fig. 3).

The RT-PCR applied in both cases identified an amplified product in each case, but of two different sizes. However, the size of the rearranged bands from multifocal tumors in each individual patient was identical (Fig. 4). RT-PCR amplified two 5'*WWTR1-CAMTA1*3' variant transcripts from both EHE cases. The 5'*WWTR1* showed a consistent breakpoint

within intron 3 and intron 4 respectively, while another 2 different breakpoints were seen in exon 9 by 3'*CAMTA1*. Exon 3 (variant 1) and exon 4 (variant 2) of WWTR1 were fused to CAMTA1 exon 9.

The sequence of the fusion gene confirmed a different *WWTR1-CAMTA1* rearrangement in each patient, but an identical *WWTR1-CAMTA1* rearrangement from different lesions in each individual patient (Fig. 5).

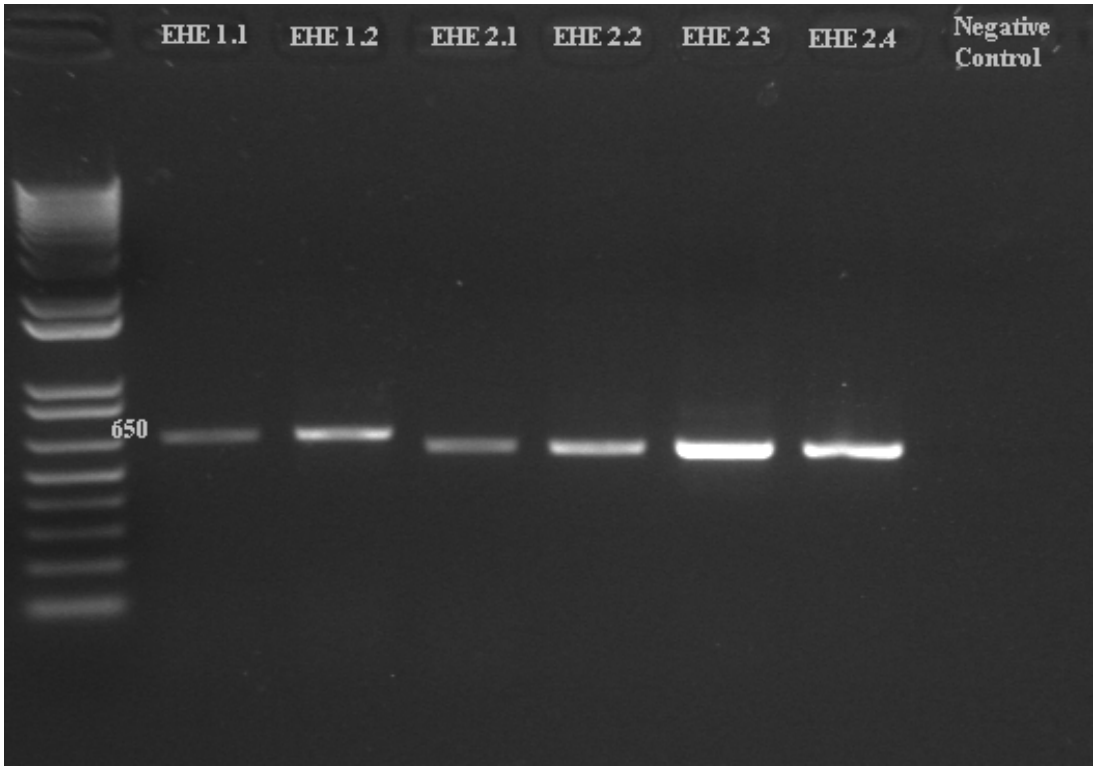
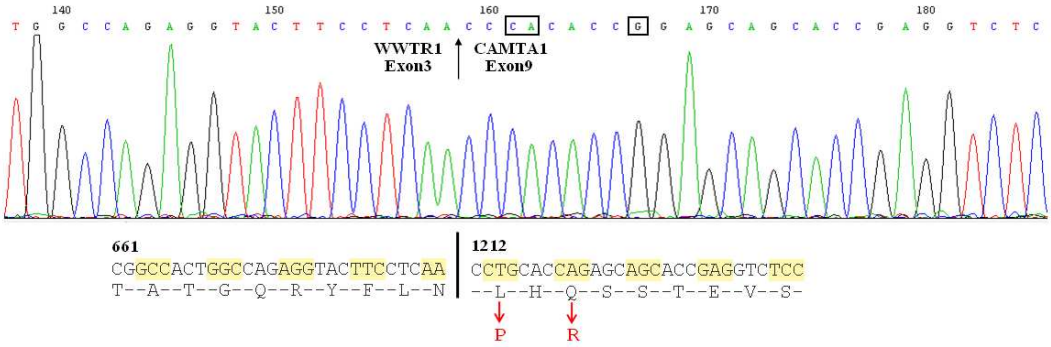
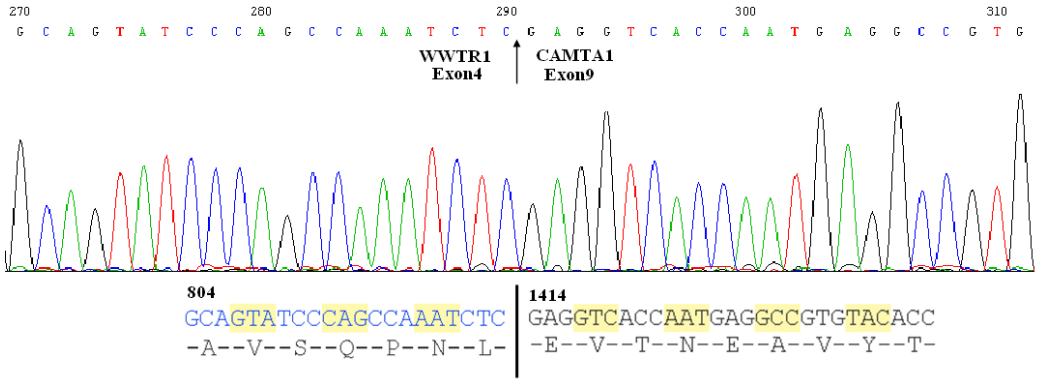


Fig. 5: Gel electrophoresis showing amplified products from two different cases of two distinct sizes.





CAMTA1

Ex9

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1013 GAGCTGGCGGCAGCGTGCATCACAAGTGTAACAGCGCCAAACACCGCATCATCTCGCCCA
     AGGTGGAGCCACGGACAGGGGGGTACGGGAGCCACTCGGAGGTGCAGCACAATGACGTGT
     CGGAGGGCAAGCACGAGCACAGCCACAGCAAGGGCTCCAGCCGTGAGAAGAGGAACGGCA
                    Break point of EHE 1
     AGGTGGCCAAGCCCGTGCTCCTGCACCAGAGCAGCACCG AGGTCTCCTCCACCAACCAGG
     TGGAAGTCCCCGACACCACCCAGAGCTCCCCTGTGTCCATCAGCAGCGGGCTCAACAGCG
     ACCCGGACATGGTGGACAGCCCGGTGGTCACAGGTGTGTCCGGTATGGCGGTGGCCTCTG
                                                  Break point of EHE 2
     TGATGGGGAGCTTGTCCCAGAGCGCCACGGTGTTCATGTCAGAGGTCACCAATGAGG CCG
     TGTACACCATGTCCCCCACCGCTGGCCCCAACCACCACCTCCTCTCACCTGACGCCTCTC
     AGGGCCTCGTCCTGGCCGTGAGCTCTGATGGCCACAAGTTCGCCTTTCCCACCACGGGCA
     GCTCGGAGAGCCTGTCCATGCTGCCCACCAACGTGTCCGAAGAGCTGGTCCTCTCCACCA
     CCCTCGACGGTGGCCGGAAGATTCCAGAAACCACCATGAACTTTGACCCCGACTGTTTCC
     TTAATAACCCAAAGCAGGGCCAGACGTACGGGGGTGGAGGCCTGAAAGCCGAGATGGTCA
     GCTCCAACATCCGGCACTCGCCACCCGGGGAGCGGAGCTTCAGCTTTACCACCGTCCTCA
     CCAAGGAGATCAAGACCGAGGACACCTCCTTCGAGCAGCAGATGGCCAAAGAAGCGTACT
     CCTCCTCCGCGGCGGCTGTGGCAGCCAGCTCCCTCACCCTGACCGCCGGCTCCAGCCTCC
     TGCCGTCGGGCGGCGGCCTGAGTCCCAGCACCACCCTGGAGCAGATGGACTTCAGCGCCA
     TCGACTCCAACAAGGACTACACGTCCAGCTTCAGCCAGACGGGCCACAGCCCCCACATCC
     ACCAGACCCCCTCCCCGAGCTTCTTCCTGCAGGACGCCAGCAAACCCCTCCCCGTCGAGC
     AGAACACCCACAGCAGCCTGAGTGACTCTGGGGGCACCTTCGTGATGCCCACGGTGAAAA
     CGGAGGCCTCGTCCCAAACCAGCTCCTGCAGCGGTCACGTGGAGACGCGGATCGAGTCCA
     CTTCCTCCCTCCACCTCATGCAGTTCCAGGCCAACTTCCAGGCCATGACGGCAGAAGGGG
     AGGTCACCATGGAGACCTCGCAGGCGGCGGAAGGGAGCGAGGTCCTGCTCAAGTCTGGGG
     AGCTGCAGGCTTGCAGCTCTGAGCACTACCTGCAGCCGGAGACCAACGGGGTAATCCGAA
     GCGCCGGCGGCGTCCCCATCCTCCCGGGCAACGTGGTGCAGGGACTCTACCCCGTGGCCC
     AGCCCAGCCTCGGCAACGCCTCCAACATGGAGCTCAGCCTGGACCACTTTGACATCTCCT
     TCAGCAACCAGTTCTCCGACCTGATCAACGACTTCATCTCCGTGGAGGGGGGCAGCAGCA
     CCATCTATGGGCACCAGCTGGTGTCGGGGGACAGCACGGCGCTCTCACAGTCAGAGGACG
     GGGCGCGGGCCCCCTTCACCCAGGCAGAGATGTGCCTCCCCTGCTGTAGCCCCCAGCAGG
     GTAGCCTGCAGCTGAGCAGCTCGGAGGGCGGGGCCAGCACCATGGCCTACATGCACGTCG
     CCGAGGTGGTCTCGGCCGCCTCGGCCCAGGGCACCCTAGGCATGCTGCAGCAGAGCGGAC
     GGGTGTTCATGGTGACCGACTACTCCCCAGAGTGGTCTTACCCAGAG
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Fig 6: Sequencing of three amplicons identified two molecular variants, with exon 3 (variant 1, upper panel) or exon 4(variant 2, lower panel) of WWTR1 being fused in-frame to exon 9 of CAMTA1.

Discussion

As with other vascular tumors, epithelioid hemangioendotelioma can be multifocal in up to 50% of cases (Deyrup and Montag, 2007; O'Connell et al., 2001). Because of its usual indolent clinical behaviour, this finding is commonly referred to as multifocal disease, and is often not accepted or recognised as a metastatic process (Gupta et al., 2006; O'Connell et al., 2001).

In this study, we examined the question of whether EHE is a multicentric or metastatic tumor. We hypothesized that molecular analysis could help elucidate this question.

A variety of molecular genetic molecular techniques can be utilized to determine the clonality of multifocal tumors. Monoclonal tumors should exhibit the identical initial genetic alteration in genes responsible for early tumor development. However, additional genetic changes will subsequently accumulate, leading to sub-clonal divergence and intratumoral heterogenicity (Hafner et al., 2002).

A frequent used method for evaluating clonality is based on X-chromosome inactivation. However, the reliability of X-chromosome inactivation analysis for clonality study in tumors has been challenged (Sieben et al., 2003). One problem is that tumors may show altered DNA methylation patterns. Furthermore, non-random X-chromosome inactivation in germline DNA of healthy and cancer-affected females may complicate the interpretation (Sieben et al., 2003).

In contrast to analysis of clonality by X-chromosome inactivation, loss of heterozygosity (LOH) is an irreversible genetic event acquired during tumorigenesis rather than an epigenetic phenomenon like methylation. The weakness of this approach is that in the absence of informative markers and the failure to detect LOH it is likely to underestimate the frequency of clonality (Sieben et al., 2003).

Another technique used to investigate clonality in multifocal cancer is comparative genomic hybridization (CGH). However, in contrast to LOH analysis, the alternate loss of paternal and maternal alleles, strongly indicating different clones, is not detectable with this method. Therefore, CGH and LOH analysis may be less sensitive methods for detecting genetic aberrations as compared to polymerase chain reaction (PCR) (Kros et al., 2002).

If the gene rearrangement is the initiating event of tumorigenesis, fusion product seems to be the most powerful idiotypic clonal marker (Antonescu et al., 2000; Melotti et al., 2010). In chromosomal translocations, the genomic breakpoints usually occur within introns. However, like in our study, the gene rearrangement can also occur within exons. Within introns or exons, the distribution of breakpoints from different cases seems essentially random. This provides formal support for the use of these rearrangements to establish clonal relationships in multifocal tumors characterized by specific chromosomal translocations (Antonescu et al., 2000).

To our knowledge, there are only a few reports that have been used gene rearrangements to prove the clonal origin of multifocal tumor (Antonescu et al., 2000; Melotti et al., 2010; Ohta K et al., 2008; Plaza JA et al., 2008; Shah ZH et al., 2009; Sugg et al., 1998). Most of them have investigated multifocal lymphoproliferative processes and the analysis of clonality by PCR has played an important diagnostic role (Melotti et al., 2010; Ohta K et al., 2008; Plaza JA et al., 2008; Shah ZH et al., 2009; Sugg et al., 1998). Ohta et al. reported a case in which B cell monoclonality was found in an intraocular lymphoma and a primary breast lymphoma. They showed an identical gene rearrangement in the vitreous and breast tumors. The same-sized band were detected in both samples and direct sequencing of the PCR products revealed an identical monoclonal rearrangments of the IgH gene.

Our study shows similar result. In fact, we tested two multifocal liver EHEs with different rearrangments of WWTR1 and CAMTA1 genes. An identical monoclonal rearrangment was found in each lesion fron each patient, but not in tumors from different patients. The identical WWTR1-CAMTA1 rearrangment suggests that multifocal EHE resulted from metastasis of the same neoplastic clone rather than a simultaneous neoplastic formation of multiple EHE cell clones.

Our conclusions are supported by the results of a recent study that reported a series of patients with liver EHE. Sixsteen patients received liver transplant and 5 of them (31%) had recurrence of disease in the new liver (Lau et al., 2011).

This finding follows the “seed and soil” theory that Paget (1989) proposed in 1889, namely, “When a plant goes to seed, its seeds are carried in all directions; but they can only live and grow if they fall on congenial soil.”

Recently, many investigators have validated this metastatic theory (Kaplan et al., 2006; Gupta et al., 2006; Norton and Massagué, 2006). They defined the metastatic niche (soil) as a friendly site for the tumor cell (seed) to attach to and grow. In addition, Norton and Massagué (2006) proposed that cancer was a self-seeding disease and that the appearance of multifocality was conveyed by self-seeds returning to the primary tumor’s organ of origin but not attaching to the primary tumor mass.

Following these hypotheses, we can speculate that in both our cases the EHE cells were able to attach and grow only in the liver. Therefore, it seems that multifocal EHE is more likely a metastatic disease rather than manifestation of multicentricity.

Our data could have therapeutic implications. In fact, metastatic disease suggests an aggressive tumor that warrants further treatment; in contrast, tumors arising independently may simply reflect the propensity of an organ to develop occult tumors, which may or may not progress to clinically significant disease.

In summary, our present analysis of the genomic rearrangments of WWTR1-CAMTA1 genes in 2 patients with liver EHE confirms the monoclonal origin of multifocal EHE. This unusual clinical manifestation most likely represents an intrinsic property of this subset of EHE to re-seed in a congenial soil like the tissue of origin.

Chapter 3

Epithelioid Hemangioma of Bone and Soft Tissue: a benign tumor with metastatic potential?

Introduction

Vascular tumors encompass a wide histologic spectrum and include hemangioma, hemangioendothelioma, angiosarcoma, and their epithelioid variants (Wenger and Wold, 2000; O'Connell et al., 2001). The vast majority of both benign and malignant vascular tumors are readily diagnosed based on their characteristic histologic features, such as the formation of vascular spaces and the expression of endothelial markers. However, some vascular tumors have atypical histologic features, such as a solid growth pattern, epithelioid change, or spindle cell morphology, which complicates their diagnosis (Folpe et al., 2001).

For this rare subset of vascular tumors, there remains considerable controversy in regards to the terminology and the classification that should be used (O'Connell et al., 2001; Evans et al., 2003). For instance, epithelioid hemangioma (EH) continues to be confused with hemangioendothelioma (O'Connell et al., 2001). Evans et al. (2003) recently argued that EH is not a distinct clinicopathologic entity but rather a misdiagnosed hemangioendothelioma, a tumor that, unlike hemangioma, has malignant potential. In a series of 13 patients with so-called hemangioendothelioma reported by Evans et al. (2003), 3 of patients were treated with chemotherapy, and another 3 underwent amputation. Remarkably, none of the patients in their series died. However, in a "Letter to the Editor" in the *International Journal of Surgical Pathology*, Rosenberg argued that Evans et al.'s illustrations of the tumors showed characteristics of EH, a benign neoplasm (Floris et al., 2006). This example not only illustrates the current confusion surrounding the classification of this rare subset of vascular

tumors but also indicates the danger inherent in using poorly defined and inappropriate terminology to classify them.

Because the clinical behavior and, consequently, treatment and prognosis of vascular tumors can vary significantly, it is important to effectively and accurately distinguish them from each other. In this study, we examined the question of whether EH is a benign tumor with metastatic potential. We hypothesize that the clinical behavior of EH can help elucidate this question and establish if this rare tumor is a benign or malignant neoplasm.

Material and Methods

We performed a retrospective analysis of all cases of EH from the surgical pathology files of our institution. In each case, the diagnosis of EH was confirmed by reviewing available histologic slides (Fig. 7).

Available radiographic images were also reviewed, and treatment and follow-up information was obtained from the patient records. In addition, for each case, fluorescence in situ hybridization (FISH) was performed to exclude the presence of the specific chromosomal rearrangement t(1;3)(1p36.23;3q25.1), which has been shown to be characteristic of epithelioid hemangioendothelioma (EHE) (Errani et al., in press). BAC clones were selected according to the UCSC genome browser (http://genome.ucsc.edu) and were obtained from the BACPAC Resources Center of the Children's Hospital of Oakland Research Institute (CHORI) (Oakland, CA) (http://bacpac.chori.org). Probe preparation and FISH analysis were performed on paraffin-embedded, 4-μm-thick tissue sections, as previously described (Antonescu et al., 2010).

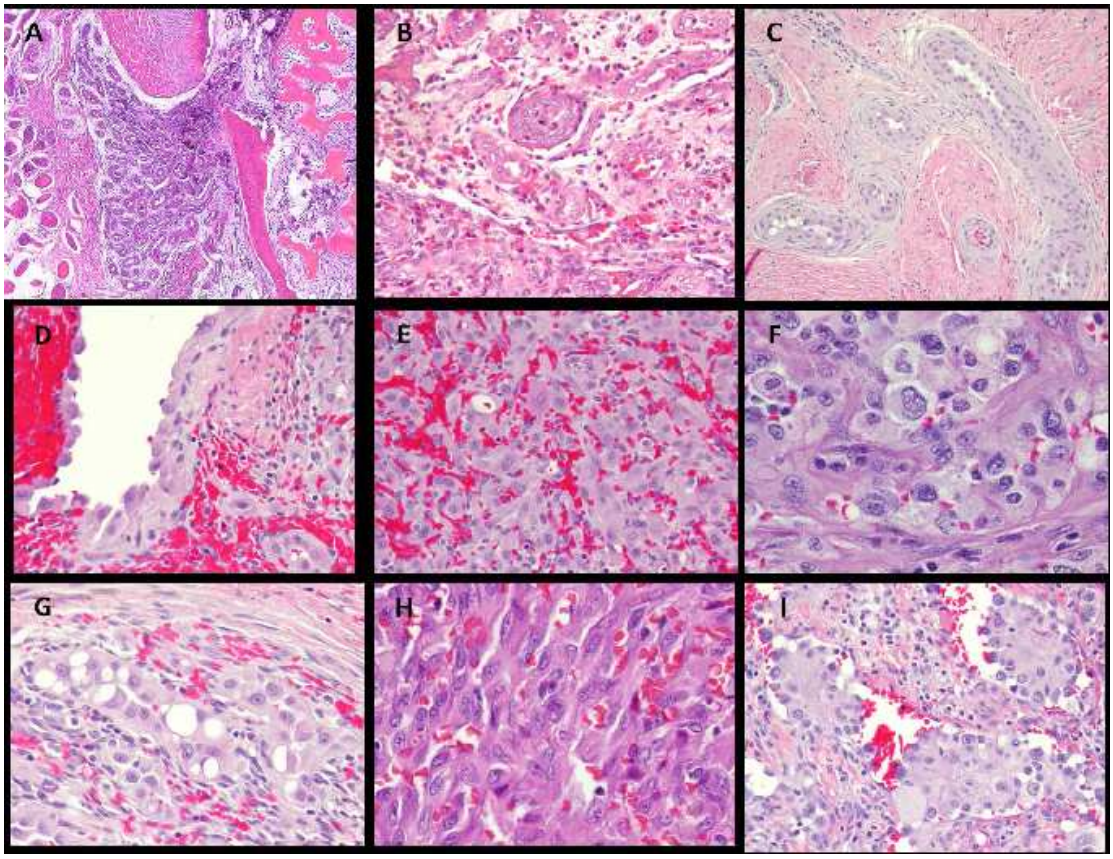

Fig. 7: Histologic analysis showed (A) a lobular growth pattern of the lesion with extension outside the cortex in adjacent soft tissue; (B) mature vascular lumen formation with eosinophilis infiltrating the adjacent stroma; (C) vasoformative properties at the periphery of the lesion, with larger calibre vessels lined by epithelioid cells; (D) hobnailed endothelial cells protruding in the lumen in a characteristic tomstone appearance; (E) the central portion of the lesion typically had more solid growth with sheets of epithelioid cells with densely eosinophilic cytoplasm and lacked obvious vessel formation. Occasionally abundant erythrocyte extravasation was seen; (F) epithelioid cells with a more foamy, vacuolated cytoplasm and focal, moderate pleomorphism and pseudonuclear inclusions; (G) intracytoplasmic vacuoles but typically these were not a predominant feature; (H)occasional areas of bland spindle cell component; and (I) vascular ivasion in one patient who had lymphonode spread.

At least 200 successive non-overlapping nuclei were examined using a fluorescence microscope. A case was considered to have a specific genetic rearrangement if ≥ 20% of the nuclei examined showed a break-apart signal pattern using its respective BAC probes (Fig. 8).

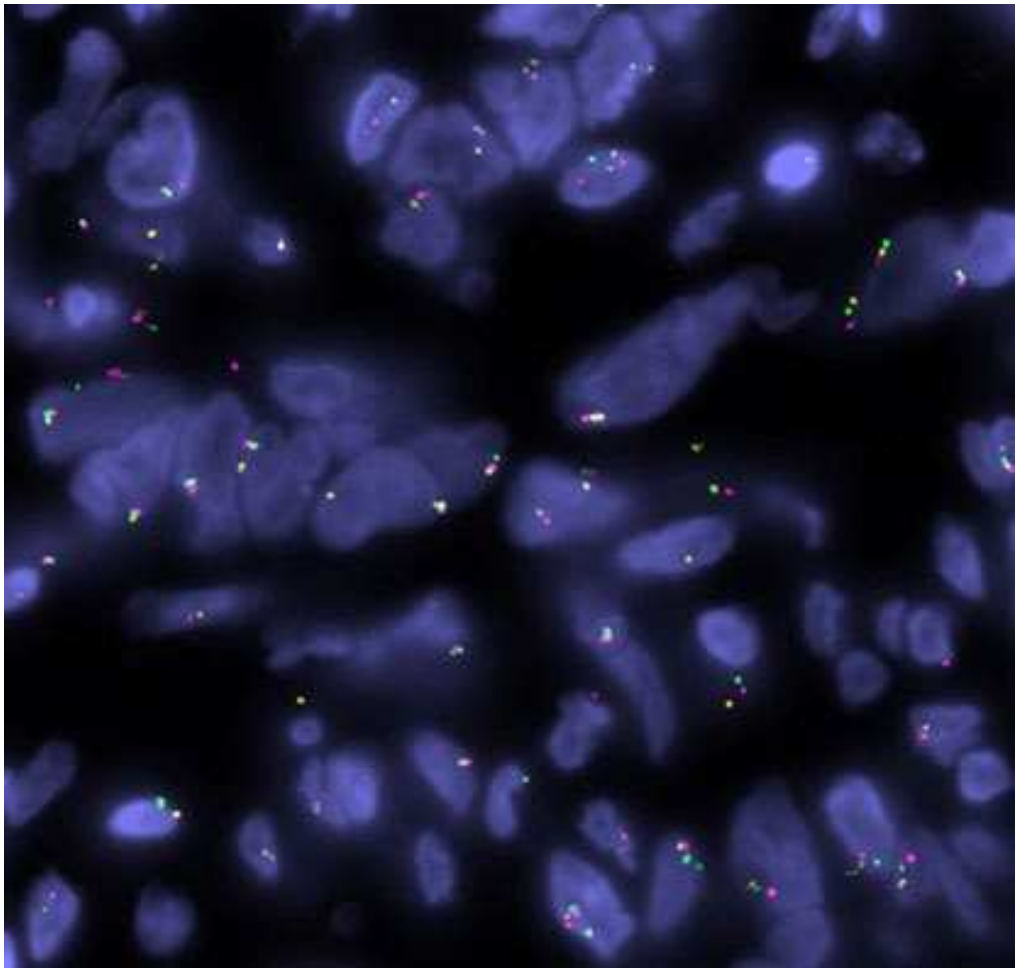

Fig. 8: Three-color fluorescence in situ hybridization (FISH) shows no break-apart (split signal) in the region of 3q25.1-25.3.

Results

We identified 16 patients with tissue available for molecular analysis: 4 women and 12 men, with a mean age at presentation of 44 years (range, 18 to 81 years). Additional demographic data for these patients are shown in the Table.

In each case, the diagnosis of EH was first confirmed by reviewing available histologic slides. All tumors were positive for the CD31 endothelial marker. Morphologically, EHs were defined as either lobulated or well-circumscribed lesions, which had clear vasoformative properties, forming “mature” vessels with open lumina. The lesional

cells occasionally had the so-called tombstone appearance and consistently showed an abundant, glassy, eosinophilic cytoplasm.

The correct diagnosis of all our EH cases based on immunophenotype alone was confirmed thanks to lack of the specific genetic rearrangement *[t(1;3)(1p36.23;3q25.1)]* characteristic of epithelioid hemangioendothelioma that we showed in a previously report (Errani et al., 2011).

The anatomic distribution of EH was as follows: 9 cases in bone, 5 in soft tissue and 2 in both bone and soft tissue. Four patients had an unusual multifocal presentation of EH in the hand, wrist, foot, head and neck respectively.

On conventional x-rays, the bone lesions were usually lucent with well-defined margins (Fig. 9).

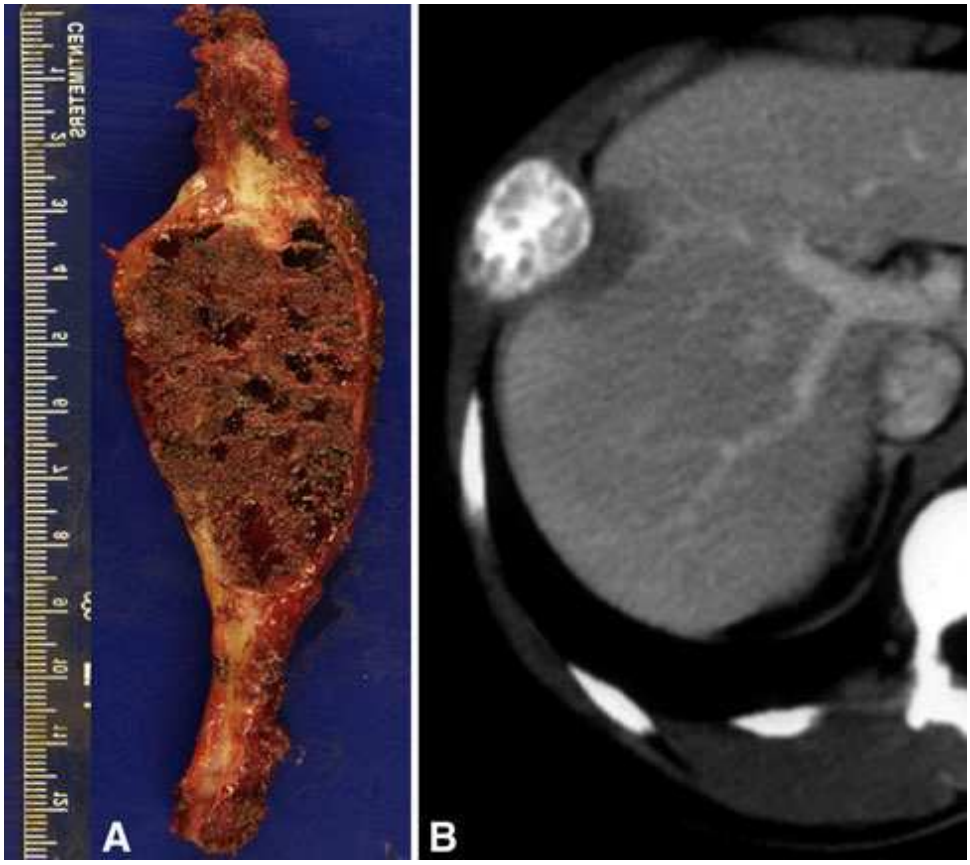

Fig 9: (A) The en bloc resection specimen shows a diffusely hemorrhagic cut surface lesion expanding the rib. (B) A contrast-enhanced CT image of the same patient shows a multiseptated, expansile lytic lesion in the anterior portion of the right ninth rib, indenting and causing low-attenuation presumed to be reactive edema in the subjacent liver.

In a few cases of EH of bone, the bone was expanded and focally destroyed with tumor extending into adjacent soft tissue. By contrast, in cases of EH of soft tissue, the lesions had less-defined margins on the magnetic resonance images. The size of the tumors was known in 12 patients, ranging from 1 cm to 8.5 cm in diameter.

Treatment varied widely, ranging from biopsy to segmental resection. Most patients with EH of bone were treated with intralesional curettage. Three patients, 2 with EH in the rib and 1 with EH in the carpus, underwent segmental resection. By contrast, all patients with EH of soft tissue underwent excision with marginal or wide margins, except for 1 patient who only underwent biopsy. Two patients were also treated with radiation therapy, and one of these patients with systemic therapy.

Follow-up information was available for all 16 patients; the mean follow-up time was 64.5 months (range, 6 to 162 months). None of the patients died of disease, including the 4 patients with a multifocal presentation of EH, and only 2 patients developed a local recurrence (Table 2).

A few cases, because of their unusual clinical features, are described in detail. The first patient was a 56-year-old woman with numerous lesions of the right foot (Fig 10). He was treated with trans-tarsal amputation. The patient is alive and well free of disease 66 months later. The second patient was an 18-year-old man with an EH of the right carpus involving the scaphoid and trapezium bones. He underwent segmental resection with wide margins, and he is currently alive with no evidence of disease 156 months after surgery. The third patient was a 49-year-old man who presented with numerous bone and soft tissue EHs in the index and middle fingers.

EHCase no.	Age (y)	Sex	Location	Multifocal vs Solitary	Treatment	Outcome (months)
1	63	M	Soft Tissue (arm)	Solitary	Biopsy	AWD (64)
2	38	M	Soft Tissue (arm)	Solitary	Surgery (M)	NED (58)
3	63	F	Soft Tissue (hand)	Solitary	Surgery (M)	NED (67)
4	38	F	Soft Tissue (axilla)	Solitary	Surgery (W)	NED (162)
5	31	M	Bone (metatarsus)	Solitary	Surgery (I)	NED (51)
6	20	M	Bone (metatarsus)	Solitary	Surgery (I)	NED (31)
7	40	M	Bone (metatarsus)	Solitary	Surgery (I)	NED1 (22)
7	59	M	Bone (cuneiform)	Solitary	Surgery (I)	NED (9)
8	23	M	Bone (rib)	Solitary	Surgery (W)	NED (68)
9	41	M	Bone (rib)	Solitary	Surgery (W)	NED1 (67)
10	81	M	Bone (clavicle)	Solitary	Surgery (I)	NED (6)
11	50	M	Bone (vertebra)	Solitary	Surgery (I) and RXT	DOO (16)
12	34	F	Bone (tibia)	Solitary	Surgery (I)	NED1 (114)
13	18	M	Bone (carpus)	Multifocal (scaphoid and trapezium)	Surgery (W)	NED (156)
15	49	M	Bone and Soft Tissue (hand)	Multifocal (index and middle fingers)	Surgery (I)	NED (48)
16	56	F	Bone and Soft Tissue (foot)	Multifocal (midfoot and forefoot)	Surgery (W)	NED (66)
17	35	M	Soft Tissue (head and neck)	Multifocal (bone and parotid)	Surgery (M), RXT and CHT	NED1 (240)

Table II: EH, epithelioid hemangioma; M, male; F, female; NED, no evidence of disease; NED1, no evidence of disease after local or distant recurrence; AWD, alive with disease; DOO, dead of other causes.

Several lesions were excised, and the remainder was treated with laser therapy. This patient is alive and disease-free 48 months after treatment. Another patient was a 35-year-old man, who presented with a facial mass in 1991. He was treated with chemotherapy (Adriamycin and Edatrexate) without significance response. Therefore, he received 3000cGy in 10 fractions to the whole brain with an excellent response. He developed a local recurrence involving lymphoid tissue adjacent to the salivary gland in 2002.

Thus, the patient underwent marginal excision of the lesion followed by radiation therapy (4500cGy). He did well until 2005, when he presented left sphenoid and orbital roof metastases. These lesions were excised only in 2011 and the presence of EH was subsequently confirmed. He is alive 20 years later the first appearance of the disease.

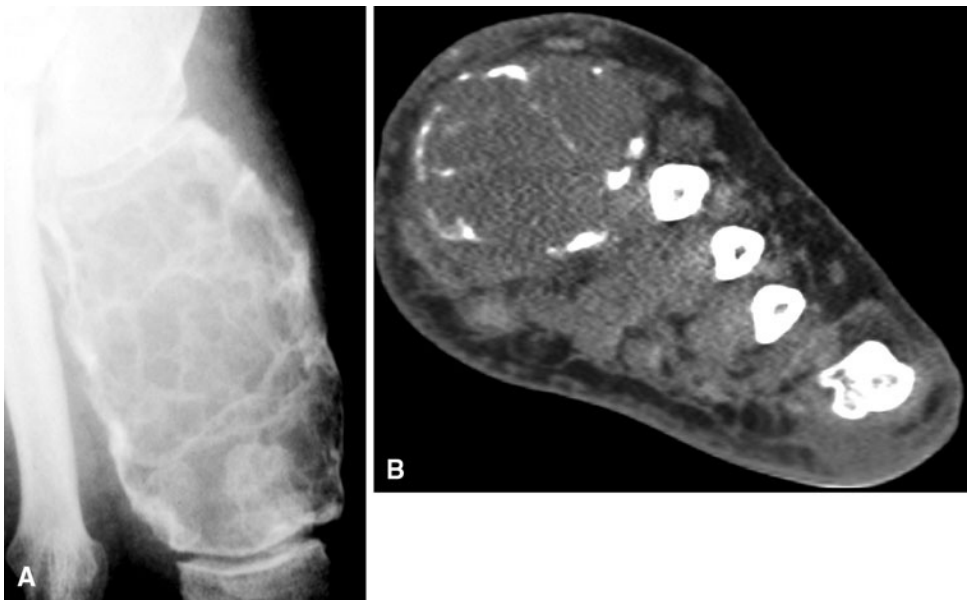

Fig 10: (A) A radiograph shows the first metatarsal has been replaced and expanded by a multiseptated lytic lesion. No gross calcified matrix is evident in the lesion. (B) A coronal non contrast CT image through the forefoot of the same patient shows marked expansion of the first metatarsal with extensive cortical destruction and several thin intralesional septa. The attenuation of the tumor is slightly lower than that of muscle.

Finally, the last interesting case was that of a 63-year-old man diagnosed with a soft tissue EH in the arm (Fig 11). He was treated with biopsy alone and did not show any disease progression at follow-up 64 month after treatment.

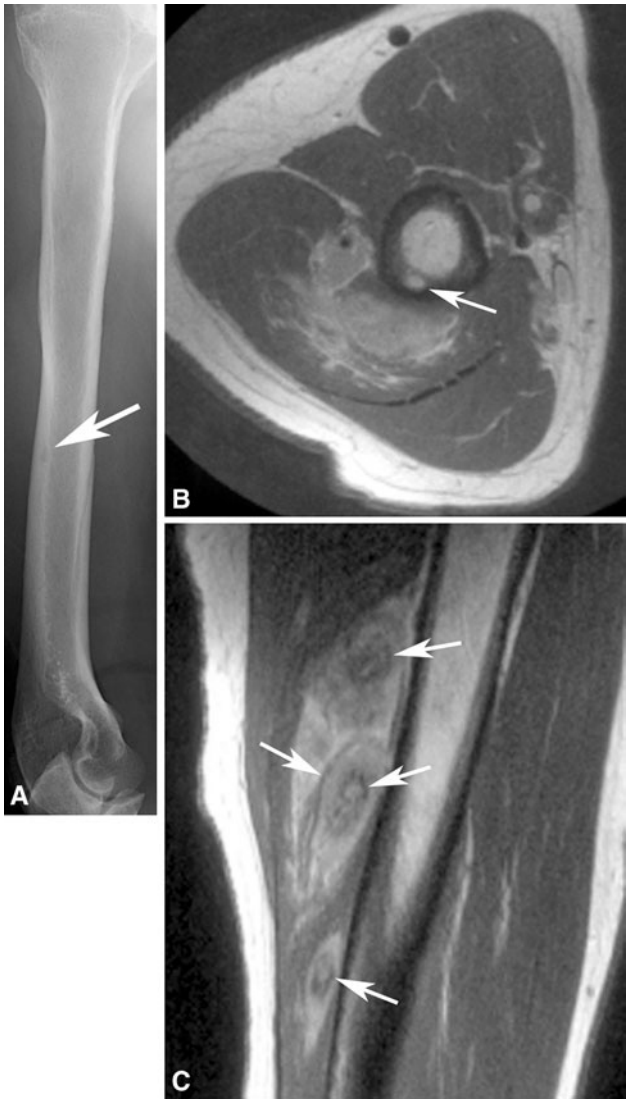

Fig 11: (A) The radiograph shows a tiny permeative, lytic focus in the posterior cortex of the midhumeral shaft (arrow). No calcifications are evident in nearby soft tissues. (B) An axial proton density MR image shows heterogeneous tumor deposits in the triceps muscle and an intracortical tumor deposit (arrow). (C) A sagittal proton density MR image shows multinodular tumor deposits with low-signal intensity inner rings (arrows), possibly related to hemosiderin deposition.

Discussion

Epithelioid vascular tumors remain controversial because of their unusual morphology, poorly understood histogenesis, and unpredictable biologic behavior (Keel et al., 1999). In fact, there is much debate involving certain vascular tumors that show an epithelioid phenotype and that share many of the same histologic features. This has resulted in the frequent misdiagnosis and inappropriate treatment of EH (Nielsen et al., 2009).

Although imaging is extremely helpful in the diagnosis of hemangioma and usually excludes the need for biopsy, it cannot be used effectively in the diagnosis of EH and other vascular tumors because these entities lack characteristic radiologic features (Gupta et al., 2006; Shah et al., 2005). In fact, the presence of multifocal lesions may be the only clue indicating a diagnosis of a vascular tumor (Wenger and Wold, 2000).

Morphologic and immunohistochemical features thus remain the cornerstone of diagnosis of vascular tumors and their epithelioid variants. The differential diagnosis of EH includes EHE and epithelioid angiosarcoma. Because of their epitheloid appearance, epithelioid vascular neoplasms may also be misdiagnosed as metastatic carcinoma. However, antibodies against certain vascular and endothelial antigens have been shown to be helpful in differentiating vascular tumors from metastatic carcinomas (Kleer et al., 1996). Furthermore, features that distinguish EH from epithelioid angiosarcoma include the absence of significant cytologic atypia, brisk mitotic activity, and necrosis and the presence of well-formed vessels (Deyrup et al., 2007). The more difficult distinction between EH and EHE could be made on the basis of our recent discovery of a novel genetic rearrangement that is specific to EHE, [t(1;3)(1p36.23;3q25.1)] (Errani et al., in press), which was not present in all cases of EH analyzed in the current study. The correct differential diagnosis between these 2 entities is critical because EHE exhibits a more aggressive clinical course than EH. It is also more frequently multifocal when occurring in bone (O'Connell et al., 1993). By contrast, the vast majority of bone EHs are solitary. However, up to 25% of bone EHs can affect the skeleton in a multifocal fashion (Sung et al., 2000; O'Connell et al., 2001; Deshpande et al., 2003). Moreover, Floris et al. (2006) reported a case of EH of the 2nd toe with secondary involvement of the ipsilateral inguinal, iliac, and paraortic lymph nodes. The groin lymph nodes were excised, and the presence of EH was subsequently confirmed.

This 2006 article by Floris et al. sparked a controversy reflected in an exchange of opinions in the form of "Letters to the Editor" in the *International Journal of Surgical Pathology* (Floris et al., 2006). In his letter, Evans reiterated his opinion that EH is not a distinct clinicopathologic entity but rather a misdiagnosed hemangioendothelioma, a tumor with malignant potential. However, in his own letter, Rosenberg argued that these neoplasms are histologically and biologically different from one another. In a series of 13 patients with so-called hemangioendothelioma reported by Evans et al. (2003), none of the patients died of disease and, in Rosenberg's opinion, Evans et al.'s illustrations of the tumors show characteristics of EH. Of these 13 patients, 3 were treated with aggressive chemotherapy, and 3 underwent a forequarter amputation, hip disarticulation, and internal hemipelvectomy, for what Rosenberg considers a benign neoplasm. Clearly, the classification of epithelioid vascular tumors remains a topic of considerable controversy as EH continues to be confused with EHE or some other type of vascular sarcomas.

Crucial to the significance of this controversy is what effect, if any, the classification of these vascular tumors has on their treatment and prognosis (O'Connell et al., 2001). In a recent study, Nielsen et al. (2009) analyzed 50 cases of EH of bone. In their series, most patients presented with a single lesion, but 9 patients (18%) presented with lesions involving more than 1 bone. Two of the patients with multifocal EHs had discontinuous lesions of bone, skin, artery, and lymph node, but none of these patients with an unusual multifocal presentation of EH experienced an adverse outcome. Therefore, the nonaggressive behavior of EH reported in the literature (Evans et al., 2003; Floris et al., 2006; Nielsen et al., 2009) supports the hypothesis that this tumor is indeed benign.

Despite the fact that our series is relatively small, our findings confirm that EH does not behave aggressively and thus is a benign tumor. In fact, although most patients received conservative treatment, including only biopsy in 1 case, their long-term prognosis was

excellent, and none of them died of disease. By contrast, as we previously reported, EHE is also associated with good prognosis, but it can metastasize in some cases and produce a fatal outcome (Errani et al., in press).

In the current study, we found that 4 cases of EH had a multifocal appearance. Although we cannot disprove a multicentric origin for EH, we favor the theory of metastatic spread of the tumor from bone and soft tissue, given the intimate relationship that vascular tumors typically have with non-neoplastic vessels (Bollinger et al., 1994). However, we do not believe that metastatic potential necessarily means malignancy. In 1889, Paget (1989) originally proposed the "seed and soil" theory, namely, "When a plant goes to seed, its seeds are carried in all directions; but they can only live and grow if they fall on congenital soil." Recently, many investigators have validated the metastatic theory (Kaplan et al., 2006; Gupta et al., 2006; Norton and Massagué, 2006). They defined the metastatic niche (soil) as a friendly site for the tumor cell (seed) to attach to and grow. In Kaplan et al.'s and Gupta et al.'s hypotheses, the metastatic niche is prepared by a substance secreted by the primary tumor. The metastatic niche contains precursor cells or bone marrow-derived stem cells. Subsequently, the invading metastatic cell must exhibit the proper features to effectively colonize the new site. Their data suggest that differences in tumor-secreted humoral factors promote metastatic spread to specific distant organs, and, as expected, the genes that mediate these different site-specific metastatic activities are largely distinct. In addition, Norton and Massagué (2006) proposed that cancer was a self-seeding disease and that the appearance of multifocality was conveyed by self-seeds returning to the primary tumor's organ of origin but not attaching to the primary tumor mass.

Building upon these hypotheses, Mihm and Nelson (2010) proposed that the metastatic niche theory can elucidate infantile hemangioma development. They reported that infantile hemangiomas may be metastases from the fetal component of placenta. In fact,

certain aspects of the biology of infantile hemangioma cells suggest a relationship to the placenta as a possible site of origin for hemangioma precursor cells. First, distinct immunohistochemical markers are uniquely co-expressed by fetal microvessels of the human placenta and juvenile hemangiomas (North et al., 2001). Second, the genome-wide gene expression profiles of the placenta and hemangiomas exhibit a higher degree of global similarity relative to other tissues (Barnes et al., 2005). Finally, the natural progression of infantile hemangiomas is similar to that of the placenta (rapid proliferation followed by subsequent stabilization). Thus, they hypothesized that the site where hemangioma forms is prepared by humoral factors that determine the site of infantile hemangioma development, in the same way that malignant tumor cells prepare a site for tumor metastases (Mihm and Nelson, 2010). Taken together, these findings suggest that hemangioma precursor cell arise from the placenta as a "benign metastasis."

The possible existence of benign metastasis is further supported by the behavior of giant cell tumors, another type of benign bone tumor that can metastasize without producing a fatal outcome. At the Rizzoli Institute, the overall metastatic rate of 349 giant cell tumors of the extremity was 4%, and all tumors were associated with good long-term prognosis (Errani et al., 2010). Similarly, Klenke et al. (2011) found the same rate of pulmonary metastases in 118 patients with giant cell tumors, and none of the patients died of disease. However, we ultimately agree with Rosenberg, who pointed out, "Currently, it seems that we are limited to our subjective interpretations and that we must wait for molecular analysis of vascular tumors before a more definitive and objective answer becomes apparent" (Floris et al., 2006).

In summary, our findings confirm that EH does not behave aggressively and support the contention that EH is a benign tumor. Based on our experience, EH of bone can be effectively treated with curettage and EH of soft tissue with marginal/wide excision; EH is thus associated with an excellent prognosis. Like other vascular tumors, however, EH may

present with multifocal involvement. Therefore, we conclude that EH is a benign tumor with metastatic potential. It is important to distinguish EH from other epithelioid vascular tumors because of the significant differences in their management and clinical outcome.

Conclusions

The classification of epithelioid vascular tumors remains challenging with considerable morphologic overlap spanning across benign to malignant categories. A prior t(1;3)(p36.3;q25) was identified in 2 cases of EHE, however no follow-up studies have been performed to identify the gene fusion or to assess its prevalence in a larger cohort of patients. We undertook a systematic molecular analysis of 17 EHE, characterized by classic morphologic and immunophenotypic features, from various anatomic locations and with different malignant potential. Also included for comparison was a group of epithelioid hemangioma and epithelioid angiosarcoma. FISH positional cloning strategy, spanning the cytogenetically defined regions on chromosomes 1p36.3 and 3q25, confirmed rearrangements in two candidate genes from these loci in all EHE cases tested. Subsequent RT-PCR confirmed the *CAMTA1-WWTR1* fusion product in 3 cases. None of the other benign or malignant epithelioid vascular tumors examined showed these abnormalities. *CAMTA1* and *WWTR1* genes have been previously shown to play important roles in oncogenesis. Our results demonstrate the presence of *CAMTA1-WWTR1* fusion in all EHE tested from bone, soft tissue and visceral location (liver, lung) in keeping with a single tumor entity. Thus FISH or RT-PCR analysis for this fusion can serve as a useful molecular diagnostic tool in challenging diagnoses.

Like other vascular tumors, EHE can have multifocal presentation in up to 50% of cases. However, whether multifocal EHE represents an unusual pattern of metastasis or multiple

separate primary tumors remains to be elucidated. Our recent identification of *WWTR1-CAMTA1* fusion as the genetic hallmark of EHE irrespective of anatomic location was used to clarify this question by comparing the similarity of translocation breakpoints. In our previous study, we found variability of the fusion transcripts of the t(1;3)(p36;q25) translocation among different patients with EHE. Thus, we undertook a molecular analysis of six samples from two patients with multicentric hepatic EHE to test our hypothesis that the presence of identical breakpoints in *WWTR1* and *CAMTA1* support the monoclonal nature of multifocal EHE. Using FISH, RT-PCR and subsequent sequencing we confirmed an identical *WWTR1-CAMTA1* fusion transcript product from different nodules in each patient. Our results confirm that multifocal EHE are monoclonal and thus representing metastatic implants of the same neoplastic clone rather than a 'field-effect' or synchronous occurrence of multiple neoplastic clones.

The controversy surrounding EH diagnosis, particularly when arising in skeletal locations, stems not only from its overlapping features with other malignant vascular neoplasms, but also from its somewhat aggressive clinical characteristics, including multifocal presentation and occasional lymph node metastases. Specifically, the distinction from EHE has been considerably controversial. The recurrent t(1;3)(p36;q25) chromosomal translocation, resulting in *WWTR1-CAMTA1* fusion, recently identified in EHE of various anatomic sites, but not in EH or other epithelioid vascular neoplasms, suggests distinct pathogeneses. Thus, we investigated the clinicopathologic and radiographic characteristics of bone and soft tissue EHs in patients treated at our Institution with available tissue for molecular testing. Seventeen patients were selected after confirming the pathologic diagnosis and FISH analysis for the *WWTR1* and/or *CAMTA1* rearrangements. Four patients had multifocal presentation, including one with locoregional lymph node metastases. Most patients with EH of bone were treated by intralesional curettings, while patients with EH of soft tissue underwent excision

with marginal or wide margins. None of the patients died of disease and only four patients developed a local recurrence. Our results, using molecular testing to support the pathologic diagnosis of EH, reinforce prior data that EH is a benign lesion, characterized by an indolent clinical course, with occasional multifocal presentation and rare metastatic potential to locoregional lymph nodes. These findings highlight the importance of distinguishing EH from other malignant epithelioid vascular tumors due to their difference in management and clinical outcome.

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