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Monoclonality of Multifocal Epithelioid Hemangioendothelioma of the Liver by Analysis of *WWTR1-CAMTA1* Breakpoints

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Abstract

Like other vascular tumors, epithelioid hemangioendothelioma (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 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.

Keywords

vascular tumor; epithelioid hemangioendothelioma; *WWTR1-CAMTA1*; metastasis; multifocality; monoclonality

INTRODUCTION

Like other vascular tumors, epithelioid hemangioendothelioma (EHE) have a multifocal presentation in approximately 50% of cases, but it remains unclear whether these separate, segmental lesions represent multifocal disease or metastases (1–3). Multifocality is generally defined as the presence of two or more lesions in the same organ separated by normal tissue

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(4). Because the clinical course of EHE is often indolent, the prevalent concept is that separate lesions are independent primary tumors, rather than metastatic disease (3, 5). By contrast, monoclonal lesions are defined as descendants of a single malignant transformed cell, which proliferates and spread through the vascular system (6).

In this study, we investigated the question of whether regional distribution of separate EHE nodules is a metastatic or multifocal disease. Our recent identification of a *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 establish the monoclonal origin of multifocal EHE (7). In fact, as expected, in our previous study the genomic breakpoints of the t(1;3)(p36;q25) translocation varied among different patients. Thus, we hypothesized that demonstration of identical *WWTR1-CAMTA1* breakpoints in different EHE lesions from the same patient supports the monoclonal origin of EHE. To test our hypothesis, we undertook a molecular analysis of two multicentric EHE of the liver, with separate tumor samples from each patient.

MATERIAL AND METHODS

Patients and Tumor Characteristics

We retrieved two patients with multifocal EHE from the surgical pathology files of our institution with frozen tissue available for molecular analysis from different tumor nodules (IRB-protocol 02-060). In each case, the diagnosis and histologic grade were confirmed by reviewing the hematoxylin-eosin-stained slides. Both cases were positive for CD31 and ERG endothelial markers. The tumors were assessed morphologically for growth pattern, vasoformative nature, cellular pleomorphism, mitotic activity, and necrosis. Both patients presented with multifocal EHE of the liver; one patient had two lesions and the other had five lesions detected clinically and removed surgically (Fig. 1).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from the frozen tissue collected from each of the seven nodules (TRIzol Reagent; Invitrogen Corporation, Carlsbad, CA). RNA quality was determined as adequate in six of the nodules by Eukaryote Total RNA Nano assay as well as being tested by RT-PCR for PGK housekeeping gene, as previously described. Subsequently, a two-step RT-PCR was applied for detection of *WWTR1-CAMTA1* fusion transcript from each nodule, with oligo(dT)20 primer under SuperScript[®] III system (Invitrogen Corporation) used for first-strand cDNA synthesis, followed by a second-step PCR, using the HotStar Taq Master Mix (QIAGEN Inc., Valencia, CA). The *WWTR1* and *CAMTA1* primers used for the RT-PCR are the following: *WWTR1* exon 3 Forward: 5'-ATGTCCGCTCGACTCGTCGC-3' and *CAMTA1* exon 9 Reverse 3'-GAACCTTGACCCCGACTGTTTCCTTA-5'. The RT-PCR products were analyzed by electrophoresis and individually sequenced by the Sanger method.

RESULTS

Patient and Tumor Characteristics

The first patient (EHE#1) was a 42 year-old female diagnosed with hepatic EHE on a core biopsy of one of the two nodules located in the segment 7 of the liver. Subsequently, she underwent partial hepatectomy of the segment 7 and resection of the two lesions (each measuring 2.7 cm in largest dimension grossly) with negative margins. On light microscopy, the tumors were composed of relatively bland epithelioid cells with variable number of intra-cytoplasmic vacuoles, arranged in cords and single cells with an infiltrative pattern within the liver parenchyma. There was only minimal cytologic atypia and a mitotic activity

of 2 MF/50HPFs; in keeping with a diagnosis of 'classic' EHE. Intra-vascular growth was also appreciated (Fig. 2C,D).

The second patient (EHE#2) was a 44 year-old man diagnosed with EHE in the liver by core biopsy. Subsequently, four different wedge resections and partial hepatectomy were performed to remove the five different lesions in segments III, V, VII and VIII, ranging from 0.5 cm to 3.8 cm grossly. The tumor cells showed moderate nuclear atypia, a mitotic activity of 3MF/10HPFs and focal necrosis, consistent with a diagnosis of 'malignant' EHE (Fig. 2A,B). Intra-vascular tumor growth was appreciated involving medium sized vessels with complete obstruction, as well as hepatic sinusoids and small vascular spaces away from the grossly visible tumor, within normal liver parenchyma (Fig. 2E,F). The lesions were removed with negative margins.

RT-PCR confirms identical *WWTR1-CAMTA1* transcripts in different nodules of each patient

The RT-PCR identified an amplified product in each case, but of different sizes (Fig. 3). However, the product size was identical among the different tumors from each patient (Fig. 3). The sequenced PCR products confirmed two different *WWTR1-CAMTA1* transcript variants from the two patients. In EHE#1, the transcript sequence showed the fusion of the entire *WWTR1* exon 3 to the last 464 bp of exon 9 of *CAMTA1* in both nodules (Fig. 4A, S1). In addition, two missense substitutions were detected in *CAMTA1* exon 9 close to the exonic breakpoint in all four nodules tested, L336P and Q338R. In EHE#2, the *WWTR1* exon 4 was fused to the last 262 bp *CAMTA1* exon 9 (Fig. 4B, S1), in all four nodules with good quality RNA that were sequenced.

DISCUSSION

Because of its often indolent clinical course, EHE with multiple foci of segmental involvement is commonly classified as multifocal, rather than metastatic disease (3, 5). This assumption is also supported by the multifocal presentation of other vascular tumors, such as epithelioid hemangioma. In this study, we examined two patients with multifocal liver EHE and found an identical *WWTR1-CAMTA1* fusion transcript in each lesion from the same patient, but not between the two patients. These findings suggest that multifocal EHE is the result of a peculiar pattern of vascular dissemination often confined within the same organ or loco-regional area, which is quite distinct from the metastatic pattern of most other sarcomas. As chromosomal translocations are thought to represent initiating events in sarcomagenesis, the presence of identical genomic breakpoints among different tumor nodules bring unequivocal proof for the monoclonal origin of multifocal EHE, rather than a field-effect resulting in simultaneous formation of multiple neoplastic clones.

A variety of molecular genetic techniques have been utilized to determine the clonality of multifocal tumors, such as X-chromosome inactivation, LOH analysis at multiple marker loci, mutations in oncogenes such as *KRAS* or in tumor-suppressor genes like *TP53* were the most often used. However, the most definitive molecular analyses investigate the similarities among the initial genetic alteration responsible for early tumor development, which will then be represented in all stages of tumor progression (6). An illustrative example is that of multifocal urothelial carcinoma of bladder and upper urinary tract, where the frequent recurrence and multifocal presentation raise the question of the clonal nature of the spatially distinct urothelial tumors. Although most studies have focused on advanced invasive carcinoma, there is overwhelming support for a monoclonal origin of the multiple tumors, arising from intra-luminal seeding as well as intra-epithelial migration(6). However, a minority of the multifocal tumors do not have a common origin and most likely occur as few independent clones within a field-effect (i.e. 'oligoclonality'), which subsequently may

evolve as a dominant clone and spread throughout the urothelium as secondary monoclonal tumors (i.e. 'pseudo-monoclonality') (6).

Among tumors characterized by recurrent chromosomal translocations, gene rearrangement is the most likely initiating event of tumorigenesis and the resulting fusion product represents the definitive idiotypic clonal marker (8, 9). There are only a few clonality studies comparing the rearrangement breakpoints, most of them investigating multifocal lymphoproliferative processes, where PCR analysis of clonality was used as an important diagnostic tool (9–13). Ohta et al. (2008) demonstrated the B cell monoclonality in a patient with intraocular and breast manifestations of lymphoma, by confirming identical gene rearrangements of the IgH gene by direct sequencing of PCR products.

In chromosomal translocations, the genomic breakpoints usually occur within introns. Although certain introns within a gene are more prevalent than others for rearrangements, with predilection for the longer introns, the region of break within a specific intron is random among different patients. Using the Southern blotting technique we have previously shown the monoclonal origin of multifocal myxoid liposarcoma, by confirming the identical intronic breaks at the genomic level (8). Subsequent transcription, the gene fusion brings together the full coding sequence of each of the exons neighboring the intronic break. However, in rare occasions, the breakpoint can also occur within exons, wherein only part of the exon is transcribed in the fusion product. This was in fact the case of both multifocal EHEs investigated here, where the breakpoints within *CAMTA1* occurred at different positions within exon 9, while the *WWTR1* breaks were in intron 3 and 4, respectively. This is in keeping with the transcript variants recently described by Tanas et al (14). Thus, in EHE#1 the rearrangement resulted in the fusion of the last 464 bp of *CAMTA1* exon 9 to exon 3 *WWTR1* in the two lesions analyzed, while in EHE#2, the last 262 bp of exon 9 of *CAMTA1* was fused to exon 4 of *WWTR1* in all four of the different nodules tested. In contrast, these fusion transcripts were different from our previously reported *WWTR1-CAMTA1* variants in three soft tissue EHE cases, where the genomic breaks occurred in the intronic region of both genes (*WWTR1* exon 4 was fused either with exon 8 or exon 9 of *CAMTA1*) (7).

A recent internet registry study, capturing a large cohort of 206 EHE patients from the International HE, EHE, and Related Vascular Disorders (HEARD) Support Group, investigated clinical patterns with prognostic significance in EHE (2). A major drawback was the fact that the study lacked a central pathology review, as well as included patients with the diagnosis of so-called 'hemangioendothelioma, NOS', in 10% of cases. Thus, the study does not provide information on histologic factors associated with outcome (mitotic activity, etc)(15). Their data analysis revealed that 'pattern B' of disease defined as 'uncontained tumor growth' (i.e. ascites, pleural effusion), hemoptysis or the presence of 3 dis-contiguous bone lesions correlate with poor survival. Patients with other types of clinical presentations were classified as 'pattern A'. More importantly, there was no difference in survival based on the presence of metastatic disease, or single organ (liver, lung) versus multiple organ involvement (i.e. liver/lung, liver/bone, liver/lung/bone) (2). Some of these findings are difficult to reconcile and require further investigations, using strict pathologic and molecular diagnosis confirmation and histologic grading. In our previous study, including only EHE patients with *WWTR1-CAMTA1*-positive tumors, four of the six patients with multifocal/metastatic EHE with more than one year follow-up died of disease (range 24–82, median 37 months) (7).

Also included in the HEARD registry were 16 patients who received liver transplant for their multifocal hepatic involvement, three of them having already involvement of another organ, two of them being considered as 'uncontained'. Five of them (31%) developed

recurrence of disease in the new liver, but there was no survival difference between patients who were transplanted versus not in the ‘pattern A’ of disease (2).

The loco-regional confinement of intra-vascular spread noted in a subset of EHEs, is reminiscent of the so-called ‘seed and soil’ theory proposed by Paget, referring to the secondary growths in breast cancer: ‘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’ (16). Later investigations have endorsed this metastatic theory, by defining the importance of a ‘metastatic niche’ and its cellular preconditioning by bone-marrow cells contributing to the organ-specific metastasis of individual types of malignancies. (17–19). Norton and Massagué (2006) proposed that cancer was a self-seeding disease, possibly related to the inappropriate cell movement, mechanism already implicated in invasion and metastasis. In their view, self-seeds may return to the organ of origin and may not attach to the primary tumor, giving the appearance of multifocality. Following these examples, one can speculate that in a subset of EHE patients, presenting with multifocal disease restricted to one organ (i.e. liver, lung) or anatomic region, the tumor cells have the capability to spread intravascularly and seed only to this defined area, but lack additional properties to seed distantly. Additional secondary genetic events may be required to trigger tumor progression and thus enable to a ‘more disseminated state’ of metastases. This pattern may represent in fact one of the few examples in human neoplasia where the propensity for metastasis does not translate into an unfavorable outcome. Therefore, further genomic approaches are needed to investigate the molecular events involved in this unusual pattern of metastasis.

In summary, our present molecular analysis supports the monoclonal origin of multifocal EHE by demonstrating identical breakpoint rearrangements of *WWTR1-CAMTA1* fusion genes in two patients with liver EHE. This unusual clinical manifestation most likely represents an intrinsic property of this subset of EHE to re-seed in a congenial soil, similar to the tissue of origin.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. MRI imaging of multifocal hepatic EHE, showing (A) a subcapsular 2.0 cm heterogeneous lesion in the segment 7 and (B) a 2.0 cm intra-parenchymal, more homogenous lesion also located in segment 7 (EHE#1, axial T2 fat-saturated).

Fig. 2. Morphologic appearance of a malignant EHE, showing moderate nuclear pleomorphism (A, 200 $\times$, EHE#2) and areas of necrosis (B, 200 $\times$, EHE#2); intra-vascular growth with partial obstruction of medium-sized blood vessel, highlighted by ERG immunostaining (C,D, 100 $\times$, EHE#1), as well as small tumor emboli within hepatic sinusoids and positive for ERG endothelial marker (E,F, 200 $\times$, EHE#2).

Fig. 3. RT-PCR using the WWTR1 and CAMTA1 primers showed amplified products of two different sizes in the two multifocal hepatic EHE#1 and EHE#2; however the two nodules from EHE#1 as well as the four nodules from EHE#2 showed an identical size band of 645 bp and 580bp, respectively.

Fig. 4.

ABI sequencing of the fusion transcripts showed (A) that the entire *WWTR1* exon 3 was fused to the last 464 bp of *CAMTA1* exon 9 in both nodules tested of EHE#1. In addition two missense substitutions were detected in *CAMTA1* exon 9 close to the exonic breakpoint in all four nodules tested, L336P and Q338R; while in (B) EHE#2 all four nodules tested had an identical fusion structure, of *WWTR1* exon 4 fused to the last 262 bp of *CAMTA1* exon 9.