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Molecular Diagnosis of Clear Cell Sarcoma

Detection of EWS-ATF1 *and* MITF-M *Transcripts and Histopathological and Ultrastructural Analysis of 12 Cases*

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Clear cell sarcoma (CCS), also known as melanoma of soft parts, is an uncommon deep soft tissue tumor presenting typically in the lower extremities of young adults. Previous cytogenetic studies have established the specificity of the recurrent t(12;22)(q13;q12), resulting in a *EWS-ATF1* fusion, for CCS. The prevalence of the *EWS-ATF1* fusion in CCS remains unclear, since most genetically confirmed CCS have been reported as isolated cytogenetic or molecular diagnostic case reports. We therefore studied histologically confirmed CCS from 12 patients for the presence of *EWS-ATF1* by reverse-transcriptase polymerase chain reaction (RT-PCR), using RNA extracted from either frozen (four cases) or formalin-fixed paraffin-embedded (eight cases) material. All primary tumors were located in the deep soft tissues of the extremities. Histologically, 10 cases had a typical epithelioid nested appearance. Most or all cases showed immunostaining for HMB45 (12 of 12), S-100 protein (10 of 12), and MITF (12 of 12). Ultrastructural analysis showed melanosomes in six of seven cases. The presence of an *EWS-ATF1* fusion transcript was identified by RT-PCR in 11 of 12 cases (91%), all of which showed the same fusion transcript structure, namely the previously described in-frame fusion of *EWS* exon 8 to *ATF1* codon 65. RT-PCR analysis for the melanocyte-specific splice form of the *MITF* transcript was positive in all cases tested (4 of 4). These data confirm that *EWS-ATF1* detection can be used as a highly sensitive diagnostic test for CCS and that CCS expresses the melanocyte-specific form of the *MITF* transcript, further supporting its genuine melanocytic differentiation. *(J Mol Diag 2002, 4:44–52)*

Clear cell sarcoma (CCS) is an unusual tumor with a predilection for the deep soft tissues of the lower extremity, and close proximity to tendon, fascia or aponeuroses. CCS preferentially affects adolescents and young adults, and is associated with a high propensity for regional or distant metastases. The histogenesis of this tumor has long been controversial. Since its original description by Enzinger in 1965[1] as a novel type of soft tissue sarcoma designated CCS, several lines of evidence supporting melanocytic differentiation have been presented. In 1983, Chung and Enzinger[2] renamed it melanoma of soft parts, to connote a proposed origin from migrated neural crest cells with a capacity for melanin synthesis. The majority of CCS show immunoreactivity for melanoma markers, such as HMB45 and contain melanosomes as demonstrated ultrastructurally.[3–5] More recently, the expression of microphthalmia transcription factor (MITF) has been shown to be a sensitive marker for both cutaneous melanomas and CCS.[6–10] MITF is a transcription factor with several isoforms one of which (MITF-M) is critical in the differentiation of melanocytes, while other isoforms are similarly important in the biology of retinal pigment epithelium, mast cells, and osteoclasts.[11] Despite some overlap with melanoma, a significant number of clinical and biological features are consistent with the notion that CCS represents a unique entity, rather than simply a deep form or metastatic implant of malignant melanoma,[6] most notably its cytogenetic profile.

Unlike melanomas, most CCS are characterized cytogenetically by a recurrent chromosomal translocation, t(12;22)(q13;q12), resulting in fusion of the *EWS* gene on 22q12 with the *ATF1* gene on 12q13.[12] In the resulting chimeric protein, the C-terminal of EWS, which contains an RNA binding domain, is replaced by a functional bZIP DNA-binding and dimerization domain of ATF1, a transcription factor which is normally regulated by cAMP.[13,14] EWS-ATF1 binds to ATF sites in cAMP-responsive promoters through the bZIP domain derived from ATF1 and its function as a constitutive transcriptional activator is dependent on an activation domain within EWS containing repetitive elements.[15] EWS-ATF1 functions as a potent constitutive activator of several cAMP-inducible promoters when assayed by transfection in cells lacking the *EWS-ATF1* fusion.[13,14] However, the *in vivo* targets of

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EWS-ATF1 in CCS cells still remain unclear.[16] Nonetheless, it appears that the viability of CCS cells is dependent on the activity of EWS-ATF1, based on a study using intracellular anti-ATF1 antibody.[17] Recent data suggest that EWS-ATF1 may operate at least partly by interfering with P53 function, through competition with the latter for the transcriptional coactivator CBP.[18]

Since most of the genetically confirmed CCS have been studied by conventional karyotyping and published as isolated case reports in the cytogenetic or molecular diagnostic literature,[19–25] the exact prevalence of t(12; 22)-positive cases as confirmed by molecular techniques among cases histologically diagnosed as CCS remains uncertain. In this study, we assessed the prevalence of *EWS-ATF1* fusion as detected by reverse transcriptase polymerase chain reaction (RT-PCR) in a group of 12 pathologically confirmed cases of CCS. In addition, we present for the first time an RT-PCR assay for the detection of *EWS-ATF1* transcripts applicable to tumor RNA extracted from formalin-fixed paraffin-embedded material and we show that CCS expresses the melanocyte-specific form of the *MITF* transcription factor, further supporting its melanocytic differentiation.

Materials and Methods

Histological, Immunohistochemical, and Ultrastructural Analysis

A prospectively gathered adult soft tissue sarcoma database at Memorial Sloan-Kettering Cancer Center was searched for the diagnosis of CCS, made during the 18-year period, 1982–2000. Out of 4496 cases in this database, 16 (0.3%) were diagnosed as CCS and of these, 12 cases of CCS were identified in which both pathological material for reconfirming the histological diagnosis and also adequate tumor tissue for molecular analysis were available. Before inclusion in the sarcoma database, medical charts and clinical histories were reviewed in all cases to exclude an antecedent or concurrent diagnosis of primary cutaneus melanoma.

Criteria for the microscopic diagnosis of CCS included the presence of polygonal cells with abundant clear to pale eosinophilic cytoplasm, vesicular nuclei with prominent nucleoli, and arrangement in a nested pattern. The cases were assessed for the presence of melanin pigment, multinucleated giant cells, and also for the presence of an association with tendon or aponeurotic structures.

Immunohistochemical studies for S-100 protein [Biogenex, San Ramon, CA; 1:50,000; citrate buffer] and HMB45 [DAKO, Carpinteria, CA; 1:500; citrate buffer] were done in all cases. Immunostaining with D5 (Labvision, Fremont, CA), a monoclonal antibody generated against human MITF, was also performed in all 12 cases. The D5 antibody binds a portion of MITF encoded by both melanocyte-specific (*MITF-M*) and non-melanocyte-specific isoforms of *MITF* transcripts.[11,26]

Ultrastructural examination was performed on seven tumors. Representative fresh tumor tissue was fixed in 2% glutaraldehyde, postfixed in 1% osmium tetroxide, and embedded in epoxy resin using standard procedures.

Molecular Analysis

Frozen tissue, collected under an Institutional Review Board-approved protocol, was available in 4 of the 12 cases (CCS 1, 8–10), and represented primary (CCS 8 and 10) or metastatic (CCS 1 and 9) tumor samples. Tumors were snap-frozen and stored at −70°C. Total RNA was extracted using 1 ml of Trizol reagent (Gibco BRL Inc., Gaithersburg, MD). The extraction was carried out according to instructions supplied by the manufacturer.

In the remaining eight cases, RNA was extracted from a representative paraffin block containing formalin-fixed tumor tissue. In four cases (CCS 3, 4, 6, and 11) the block available for RNA extraction was from the primary tumor, in one case (CCS 5) from a local recurrence, and in three cases (CCS 2, 7, and 12) from a metastasis. The extraction was performed as directed by the manufacturer (Paraffin Block RNA Isolation Kit; Ambion, Inc., Austin, TX) with several alterations. In an effort to yield more RNA from the extraction, four 15-um sections were cut and proteinase K was used in twice the suggested volume (200 μl) for 25 minutes. To this, 2 μl of linear acrylamide were added followed by precipitation overnight at −20°C. Every effort was taken to prevent cross-contamination. For each case, a new microtome blade was used and the microtome was wiped with 10% bleach and 70% ethanol.

In all samples, RT-PCR was first performed with the outer pair of primers consisting of a sense primer located in exon 8 of *EWS* and an antisense primer in *ATF1* (codon 130). All primer sequences, annealing temperatures used, and expected product sizes are shown in Table 1. The reaction was carried out using the One-Step RT-PCR kit (Qiagen, Inc., Valencia, CA) at 50°C for 30 minutes, 95°C for 15 minutes, 30 cycles of 95°C for 30 seconds, 58°C for 30 seconds, 72°C 30 seconds, and a final extension at 72°C for 7 minutes. In parallel, each sample was amplified without reverse transcriptase in a reaction set up with the HotStartTaq DNA polymerase kit (Qiagen), according to package directions, as a "no RT" negative control. All reactions also included a no RNA negative control. The adequacy of the extracted RNA was assessed by RT-PCR, using primers for *PGK* (phosphoglycerate kinase) transcripts, as described previously.[27]

The eight tumors in which the RNA was obtained from paraffin-embedded material were subjected to a second PCR reaction (nested RT-PCR). One μL of each of the RT-PCR products (including "no RT" control products) was used in a second PCR with an inner pair of primers (Table 1). This reaction was set up using 2.5 units of AmpliTaq DNA polymerase (Perkin Elmer, Inc., Norwalk, CT), 1X PCR buffer, 1.5 mmol/L $MgCl_2$ (Perkin Elmer), 200 μmol/L

Table 1. PCR Primers

Primer pair	Primer name	Primer sequence	T_A (°C)*	Product size
EWS-ATF1 Type 1 or 2 outer pair	EWSex8-F1 ATF1-R1	GAGGCATGAGCAGAGGTGG GAAGTCCCTGTACTCCATCTGTG	58°	Type 1: 246 bp Type 2: 183 bp
EWS-ATF1 Type 1 or 2 inner pair	EWSex8-F2 ATF1-R2	GAGGAGGACGCGGTGGAATG CTGTAAGGCTCCATTTGGGGC	64°	Type 1: 185 bp Type 2: 122 bp
EWS-ATF1 Type 3 outer pair	EWSex7-F1† ATF1-R1	TCCTACAGCCAAGCTCCAAGTC See above	58°	Type 3: 124 bp
EWS-ATF1 Type 3 inner pair	EWSex7-F2 ATF1-R2	TATAGCCAACAGAGCAGCAG See above	55°	Type 3: 63 bp
MITF‡ consensus	MITF-C-F MITF-C-R	ACAACCTGATTGAACGAAG AATCTGGAGAGCAGAGACC	48°	303 bp
MITF melanocyte-specific	MITF-M-F MITF-M-R	TTATAGTACCTTCTCTTTGCC GCTTGCTGTATGTGGTACTTG	48°	188 bp

All primer sequences are 5′ to 3′.
*T_A (°C) = annealing temperature.
† corresponds to primer EWS22.3 (see Ref. 29).
‡ correspond to primers from Ref. 10.

PCR nucleotide mix (Boehringer Mannheim, Corp., Indianapolis, IN) and 0.6 μmol/L each of the forward and reverse primers. Initial denaturation at 95°C for 2 minutes was followed by 95°C for 30 seconds; 64°C for 30 seconds; 72°C for 30 seconds for 25 cycles with a 7 minutes final extension at 72°C. PCR products were electrophoresed on a 2% agarose gel and bands were cut and purified with the Concert rapid gel extraction system (Gibco BRL). Products were routinely sequenced on automated sequencer.

The nested and non-nested *EWS-ATF1* RT-PCR assays were designed to detect the most common type of *EWS-ATF-1* fusion transcript, namely the *EWS* exon 8 to *ATF1* codon 65 fusion,[12] which we designate type 1 and one of the molecular variants, namely the *EWS* exon 10 to *ATF1* codon 110 fusion,[28] which we designate type 2. The only negative case for these primers was also subjected to a nested RT-PCR using nested *EWS* exon 7 forward primers[29] in combination with the nested *ATF1* primers (Table 1) to exclude the *EWS* exon 7 to *ATF1* codon 110 fusion described by Pellin et al,[30] which we designate type 3. The nested RT-PCR protocol used was exactly as above, except for the different annealing temperatures (Table 1).

RT-PCR analysis for *MITF* mRNA expression was performed using two sets of primers (Table 1). The MITF-C consensus primers[10] amplify a portion of the *MITF* transcript shared by all *MITF* splice forms. The MITF-M primers are designed to amplify only the melanocyte-specific isoform of *MITF*, which differs from other forms by the usage of an alternative exon 1 (exon 1M).[31] RT-PCR analysis for *MITF* transcripts was performed without nesting and therefore was only considered appropriate in cases with good quality RNA. This included three of four cases with RNA obtained from frozen tissue (CCS 9 had insufficient RNA) and one case (CCS 3) in which good quality RNA was extracted from the archival paraffin-embedded sample. The target amplicons of both pairs of *MITF* primers included exon boundaries, to avoid amplification of trace amounts of genomic DNA in the RNA preparations. RNA from the SK-MEL-19 melanoma cell line was used a positive control for both *MITF* RT-PCR assays.

Results

Clinical Data

There were seven males and five females, with a mean age of 32 (range, 20 to 69 years). All cases were located in the deep soft tissues of the extremities (see Table 2 for demographics and exact locations). All tumors were deep and were excised by a wide-local resection. In seven cases the surgical margins were microscopically free. The tumors in 10 cases were localized at the time of diagnosis, while in two cases there were lung metastases at presentation as detected by staging chest CT-scans. In 11 of 12 cases follow-up information was available and the follow-up ranged from 5 to 204 months (mean of 44 months for survivors). Seven (63%) patients developed local recurrences. All five tumors with positive surgical margins eventually recurred locally. Seven of 11 (63%) patients developed regional lymph node metastases, diagnosed at intervals of 0 to 47 months (mean of 21 months) from the primary diagnosis. Distant metastases were identified in nine cases (82%), at time intervals after original diagnosis ranging from 0 (three cases) to 132 months, with a mean of 42 months. In all cases, the site of distant spread was the lung, while additional sites such as brain, bone, adrenal gland, and spleen were noted in one case each. Adjuvant chemotherapy information is listed in Table 2. At last follow-up, there were three patients with no evidence of disease (NED), one patient alive with disease (AWD), and seven patients dead of their disease (DOD). Only one of the three NED patients did not develop any regional or distant recurrences; this after a 22-month follow-up period. The other two patients remained NED after resection of their regional (CCS 2) or distant (CCS 1) metastasis, 9 and 136 months later. All patients alive with or dead of disease had had distant metastases.

Table 2. Summary of Clinicopathological Data, Management, and Follow-up

CCS	Age	Sex	Site	Size (cm)	Stage at diagnosis	Surg Mg	Radiotherapy	Chemotherapy	Local recurrence (mo)	Reg LN metastasis site	Reg LN (mo)	Distant metastasis sites	Distant metastasis (mo)	Follow-up (mo)	Status
1	25	M	Arm	4	1°	Neg	Preop: 4897cGy Postop: 1600cGy	After mets: Cis DTIC, IFN	30	Distal arm	36	Lung	120	136	NED
2	31	M	Knee	3.5	1°	Neg	Postop ERT	—	—	Popliteal	8	—	—	9	NED
3	25	F	Foot	<5	1°	Neg	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
4	23	M	Buttock	13.5	1°	Pos	—	—	3	Inguinal Iliac	1	Lung, Brain Bone, Adrn	5	5	DOD
5	36	M	Wrist	<5	1°	Pos	Postop ERT	Cis limb-perfusion	8	Mid-forearm	54	Lung	85	85	DOD
6	36	F	Thigh/ pelvis	>10	1°	Pos	Preop: 5200cGy Postop: 200cGy		5	Inguinal	0	Lung	12	12	DOD
7	26	M	Hand	4.6	1°	Neg	—	—	—	—	—	Lung, Med LN	32	39	DOD
8	21	M	Thigh	2.5	1°	Neg	—	—	—	—	—	—	—	22	NED
9	25	M	Arm	7	1°	Neg	Preop: 5000cGy Postop: Brachy	Preop/Postop: Cis DTIC,Adr,Ifos	47	Cubital	47	Lung	38	42	DOD
10	46	F	Hip/ buttock	15.7	Mets	Pos	Postop: ERT for residual disease	Preop:Cis, Vinbl, IFN, Dacarbazine Postop: Docetaxel, Gem	—	Sacral-tuberous ligament	3	Lung, LN, spleen	0	10	AWD
11	69	F	Thigh	6.2	Mets	Neg	—	—	—	—	—	Lung	0	17	DOD
12	20	F	Foot	<5	1°	Pos	—	—	12	—	—	Lung	132	204	DOD

Cis, cisplatin; IFN, interferon; Adr, adriamycin; Ifos, ifosfamide; Vinbl, vinblastine; Gem, gemcitabine; NED, no evidence of disease; DOD, dead of disease; AWD, alive with disease; N/A, not available; ERT, external radiation; mets, metastasis; Surg Mg, surgical margins; Adrn, adrenal gland; LN, lymph node; Med, mediastinal.

Histopathological Data

On histological review, 10 cases (all but CCS 6 and 10) had a classic microscopic appearance, with a distinct compartmentalization of the tumor cells by thin fibrous septa. The ovoid tumor cells showed clear to pale eosinophilic cytoplasm, and centrally located nuclei, with minimal pleomorphism but prominent nucleoli. In 7 of these 10 cases, areas of spindling were at least focally noted. Only one case (CCS 3) showed a predominantly spindle cell morphology, arranged in nests or short fascicles. Multinucleated giant cells were also identified in 5 cases (Figure 1). In 8 cases definite evidence of tumor invasion of tendon or aponeurotic structures was identified microscopically. Cases CCS 6 and CCS 10 showed a distinct microscopic appearance, with epithelioid cells, a higher degree of nuclear pleomorphism, anaplasia, and an alveolar pattern of growth, due to a striking loss of cell cohesion (Figure 2). In some areas the tumor cells had a

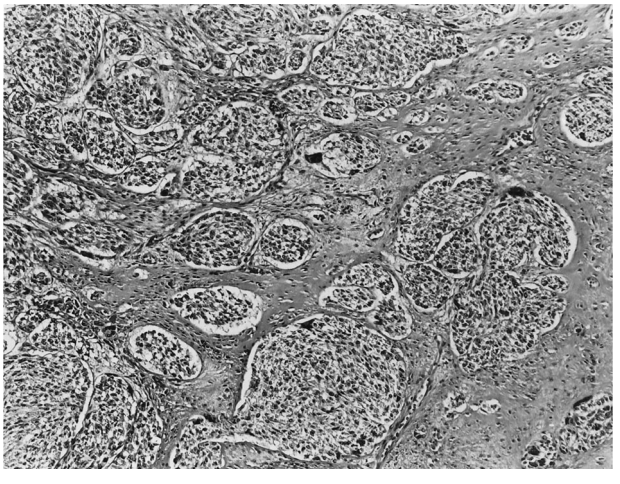

Figure 1. Microscopic appearance of nested epithelioid cells with clear cytoplasm and prominent nucleoli, separated by fibrous bands and showing scattered multinucleated giant cells (hematoxylin & eosin; ×10).

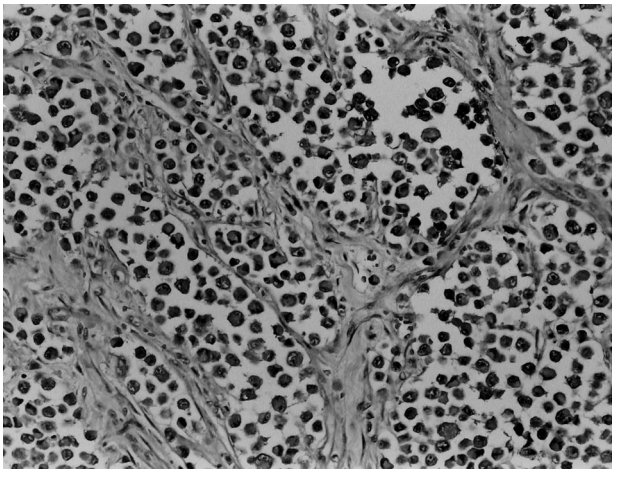

Figure 2. Case CCS 6 showed a distinct microscopic appearance, with epithelioid cells, showing a higher degree of nuclear pleomorphism and anaplasia, arranged in an alveolar pattern of growth, due to a striking loss of cohesion (hematoxylin & eosin; ×20).

Table 3. Summary of Immunohistochemical, Ultrastructural, and Molecular Data

	IHC findings			EM findings				RT-PCR		
									MITF	
CCS	S-100	HMB45	MITF	Melanosomes	Glycogen pools	PCJ	BM	*EWS-ATF1*	C	M
1	+	+	+	Stage I and II	+	−	−	+	+	+
2	+	+	+	Stage II and III	−	−	−	+	ND	ND
3	−	+	+	ND	ND	ND	ND	+	+	+
4	+	+	+	Stage II possible Stage III	+	+	−	+	ND	ND
5	+	+	+	Stage II possible Stage III	−	+	+	+	ND	ND
6	+	+	+	ND	ND	ND	ND	+	ND	ND
7	+	+	+	ND	ND	ND	ND	+	ND	ND
8	+	+	+	ND	ND	ND	ND	+	+	+
9	+	+	+	Stage II	−	−	+	+	ND	ND
10	+	+	+	−*	+	−	+	+	+	+
11	−	+	+	Stage II and III	+	+	+	−	ND	ND
12	+	+	+	ND	ND	ND	ND	+	ND	ND

*Rare cytoplasmic granules having the shape and size of melanosomes, but lacking an internal structure and therefore not-diagnostic for melanosomes.

PCJ, primitive cell junctions; BM, basement membrane; ND, not done; C, *MITF*-consensus RT-PCR; M, RT-PCR for melanocyte-specific *MITF* transcript.

rhabdoid appearance, with bright eosinophilic cytoplasm and an eccentrically located nucleus. Case CCS 10 showed in addition scattered multinucleated giant cells and evidence of infiltration into a tendon structure.

Immunohistochemical Data

These results are summarized in Table 3. All cases tested showed strong and diffuse immunoreactivity for HMB45 (cytoplasmic) and MITF (nuclear) (Figure 3). Two cases were negative for S-100 protein (CCS 3 and 11), but the remaining cases were strongly and diffusely positive.

Ultrastructural Data

Electron microscopy revealed scattered stage II unpigmented melanosomes and rare pigmented stage III melanosomes in six of the seven cases studied (Table 3) (Figure 4). In case CCS 10, rare cytoplasmic granules of the size and shape of melanosomes but lacking an internal structure were noted. In addition, electron-dense myelin figure-like structures indistinguishable from lysosomes were focally present in some cells. There were external lamina and rudimentary cell junctions in four and three cases, respectively. Numerous mitochondriae were identified in most cases. The presence of glycogen pools was a consistent finding only in four cases.

Molecular Results

In all four cases in which RNA was extracted from frozen tissue (cases CCS 1, 8–10) a 246-bp product was identified by RT-PCR (Figure 5) using the outer pair of primers for the *EWS-ATF1* type 1 or 2 fusion products (Table 1). Of the eight cases analyzed using paraffin-extracted RNA, four showed a faint 246-bp amplified product using the outer primers (Figure 6). After the nested PCR step using the inner primers (Table 1), seven of eight cases showed an amplification product of 185 bp (Figure 6).

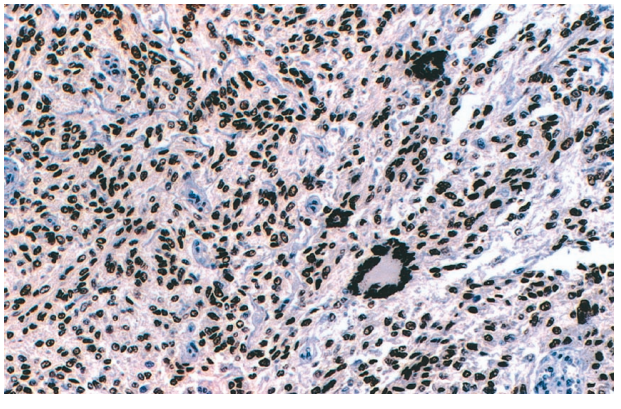

Figure 3. Immunohistochemical study with D5 monoclonal antibody showing strong and diffuse nuclear labeling in the tumor cells and also in the multinucleated giant cells (×20).

Figure 4. Ultrastructural appearance of case CCS 4 with few stage II melanosomes (**arrows**) and stage III pigmented melanosomes (**arrowheads**) (×29,400).

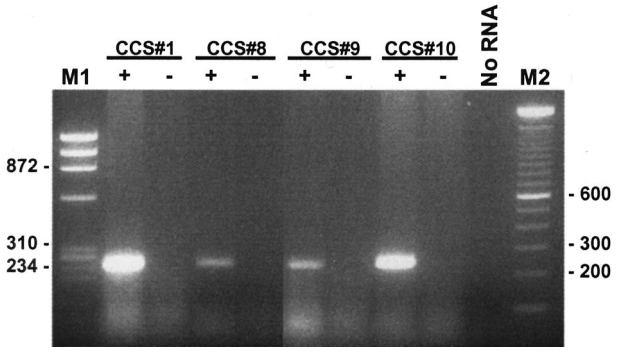

Figure 5. Detection of *EWS-ATF1* transcripts in CCS by RT-PCR using RNA extracted from frozen tissues. **M1:** Size marker (*Hae*III digest of ΦX174); **M2:** 100-bp DNA ladder; ±, reverse transcriptase added/not added (the latter representing the "no RT" negative control).

Thus, in all, 11 of 12 cases (91%) contained *EWS-ATF1* fusion transcripts by RT-PCR (Table 3). Controls lacking RT were appropriately negative in all 11 *EWS-ATF1*-positive cases. Paraffin block case CCS 11 was negative despite repeated assays on two RNA samples extracted from separate paraffin blocks, and despite satisfactory results with the control RT-PCR assay for *PGK* transcripts. Direct sequencing was performed in 11 of the 11 *EWS-ATF1* positive cases and showed the same in-frame junction of exon 8 of *EWS* to *ATF1* codon 65 in all cases. No variant fusion structure was identified. RT-PCR assays using the two pairs of primers for the *MITF* transcripts showed that all 4 CCS analyzed were positive with the consensus *MITF* primers, as well as for the melanocyte-specific *MITF-M* transcript (Figure 7).

Discussion

Despite an often prolonged clinical course, CCS is associated with a poor prognosis. A high incidence of metastases was identified in our *EWS-ATF1* positive group; 8 of 10 (80%) patients with available clinical follow-up developed either regional lymph node or distant metastases, and seven (70%) were either alive with or dead of disease. This outcome is similar to that reported in larger

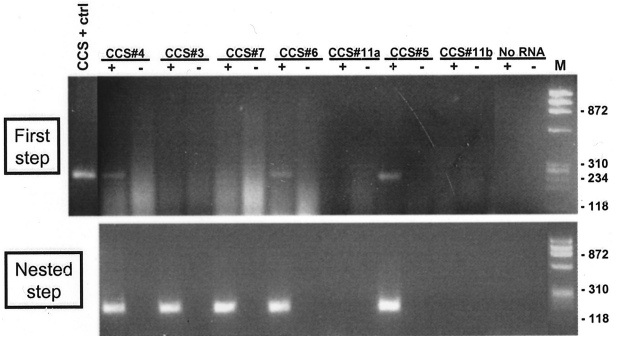

Figure 6. Detection of *EWS-ATF1* transcripts in CCS by nested RT-PCR using RNA extracted from paraffin-embedded tissues. **Top panel** shows products of RT-PCR (first step); **bottom panel** shows products of nested PCR performed on products of first step. **M:** *Hae*III digest of ΦX174. CCS + ctrl: RNA extracted from frozen tissue in CCS 1, used as positive control. CCS 11, a and b: different paraffin blocks used for RNA extraction. ±, reverse transcriptase added/not added (the latter representing the "no RT" negative control).

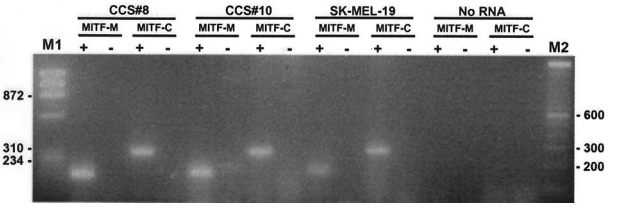

Figure 7. Detection of melanocyte-specific (M) and consensus (C) *MITF* transcripts in CCS by RT-PCR. RNA from the SK-MEL-19 melanoma cell line provided a positive control. **M1:** Size marker (*Hae*III digest of ΦX174); **M2:** 100-bp DNA ladder; ±, reverse transcriptase added/not added (the latter representing the "no RT" negative control).

clinicopathologic studies without confirmatory molecular data.[32–34]

The differential diagnosis of CCS includes a variety of epithelial and mesenchymal malignancies, but CCS can be particularly difficult to distinguish from the epithelioid variant of malignant peripheral nerve sheath tumor (MPNST) or from a metastatic implant from an occult cutaneous melanoma.[35] A diagnosis of epithelioid MPNST is favored in the presence of a significant myxoid stroma and tumor cells in a cord-like arrangement. Epithelioid MPNST typically lacks HMB45 immunoreactivity or the presence of melanosomes on electron microscopic examination. Clinical presentation of a malignant melanoma as a soft tissue mass generally occurs in patients with a known history of cutaneous melanoma. Soft tissue is not a common site for metastatic melanoma, yet neither is it exceedingly rare. Histologically, malignant melanoma usually displays a much greater degree of cellular anaplasia than CCS and often shows intranuclear inclusions. However, unlike the distinction between epithelioid MPNST and CCS, the distinction between CCS and metastatic melanoma cannot be made in all problematic cases based on pathological grounds alone, and therefore may often require genetic confirmation. At the genetic level, malignant melanomas demonstrate a broad range of genetic alterations, most commonly involving chromosomes 1 and 5, as well as deletions of 6q.[36,37] By DNA flow cytometry, most melanomas are markedly aneuploid, while most CCS are diploid or only mildly aneuploid.[38] The cytogenetic hallmark of CCS is the presence of a recurrent t(12;22)(q13;q12), resulting in the *EWS-ATF1* fusion, often found as the only chromosomal abnormality. Confirming existing data in the cytogenetic literature, molecular testing shows that *EWS-ATF1* is detected in almost all cases of CCS, as shown in the present study, and in no cases of cutaneous melanoma, as shown by van Roggen et al.[36] In aggregate, previous cytogenetic studies and the data of van Roggen et al[36] have established the specificity of the t(12;22)(q13;q12), and its resulting *EWS-ATF1* fusion transcript, for CCS.

Despite this fundamental genetic difference, immunohistochemical and ultrastructural findings indicate that, like melanoma, CCS is a neuroectodermal tumor with melanocytic differentiation.[3–5] Similar to previous reports, we find that the majority of CCS tumors containing the *EWS-ATF1* fusion also are immunoreactive for melanoma markers, such as HMB45, and contain melanosomes by ultrastructural examination. More recently, immunoreac-

Table 4. Distribution of *EWS-ATF1* Transcript Types Reported to Date

		EWS-ATF1 fusion type*				
Reference	Year	Type 1	Type 2	Type 3	Fusion structure not specified	No fusion detected
12	1993	3				
28	1997		1			
30	1998			1		
44	1999			1		
45	1999				1	
46	2000	1				
43	2000	1				
42	2001	2				2†
12, 13, 45	CCS cell lines	SU-CCS-1, DTC1			HS-MM	
Present study	2002	11				1
Totals	*N* = 28	20	1	2	2	3

*Type 1, *EWS* ex.8 (codon 325)–*ATF1* codon 65; Type 2, *EWS* ex.10 (codon 349)–*ATF1* codon 110; Type 3, *EWS* ex.7 (codon 265)–*ATF1* codon 110.
†Assay was not designed to detect type 3 fusion.

tivity for MITF has been used as a novel marker of melanocytic lineage.[7–10] The MITF transcription factor is known to regulate the differentiation of melanocytes and other pigmented cells.[11] MITF immunoreactivity has been previously reported in a total of 19 out of 26 CCS (73%),[6,7,10] but none of these CCS were studied for *EWS-ATF1*. In the present study, all CCS cases tested showed diffuse and strong nuclear labeling for MITF, suggesting that the prevalence of MITF positivity may be higher in series consisting of genetically confirmed CCS.

Most immunohistochemical studies of MITF, including the present, have used the D5 and/or C5 monoclonal antibodies (Labvision, Fremont, CA). The precise epitopes bound by these antibodies are unknown but appear to lie in a portion of MITF encoded by both melanocyte-specific (*MITF-M*) and non-melanocyte-specific isoforms of *MITF* transcripts.[11,26] Indeed, some recent immunohistochemical studies have noted reactivity of D5 antibody with a variety of non-melanocytic normal and neoplastic cells.[7,10,39] Therefore, we also performed RT-PCR for two portions of the *MITF* transcripts, a portion specific to the *MITF-M* isoform expressed only in melanocytes,[11] and a portion shared by all isoforms of *MITF*. The latter primer pair had been previously used to demonstrate by RT-PCR the ubiquitous expression of *MITF* in a variety of normal tissues.[10] We found that all four CCS analyzed were positive by RT-PCR with the consensus *MITF* primers, as expected from their immunoreactivity for MITF. Furthermore, we were able to confirm using the RT-PCR assay for the *MITF-M* isoform, that they expressed the melanocyte-specific form of *MITF*. MITF induces the expression of key melanocytic genes such as tyrosinase, *TRP-1* and *TRP-2*, and appears sufficient to convert fibroblasts to melanocyte-like cells.[40] Hypothetically, the aberrant expression of MITF, possibly induced by EWS-ATF1-mediated transcriptional deregulation in the mesenchymal precursor cell of CCS, may be sufficient to trigger an ectopic melanocytic differentiation cascade. Indeed, there is recent evidence for direct transactivation of the *MITF-M* promoter by EWS-ATF1 (David E. Fisher, unpublished data).

Detection of specific fusion transcripts in sarcomas can be particularly useful not only to distinguish CCS from other morphological mimics but also to confirm the diagnosis in the setting of an unusual histology or uncommon primary site.[41] Two cases in the present study had an unusual alveolar growth pattern and rhabdoid cells with significant nuclear pleomorphism. In these cases, the detection of the *EWS-ATF1* fusion transcript was useful in confirming the diagnosis.

In CCS, three hybrid transcript variants have been described so far in the literature[12,13,28,30,42–46] (Table 4). The most common fusion product consists of EWS amino acid residues 1 to 325, followed by ATF1 residues 65 to 271, seen in 87% of positive cases (20 of 23) with fusion structure data. In the present study, all *EWS-ATF1* positive cases had this same fusion structure, as originally described by Zucman et al,[12] which we have designated the type 1 fusion. The other two *EWS-ATF1* variant fusions are rare and were previously described as single case reports.[28,30,44] A fusion mRNA with a junction between *EWS* exon 10 and *ATF-1* codon 110 was identified by Speleman et al[28] and we now designate this as the type 2 fusion. The *EWS* exon 7–*ATF1* codon 110 fusion, designated here as the type 3 fusion, was recently described in two separate case reports.[30,44] There have been too few cases of CCS with non-type 1 *EWS-ATF1* fusions to begin to comment on possible clinical or pathological correlates. However, some of the differences in the structure of the two variant fusion proteins could be functionally significant. The absence of codons 65 to 109 of ATF1 in the type 2 and type 3 fusion products would exclude the remainder of a putative activation domain in ATF1,[14] which could hypothetically affect overall transactivation by EWS-ATF1. The absence of the portion encoded by *EWS* exon 8 in the type 3 fusion would remove the EWS IQ domain whose phosphorylation appears to enhance EWS-ATF1 function.[47]

RT-PCR assays in one CCS sample in the present series (CCS 10) were negative for *EWS-ATF1*. This tumor showed a typical histological appearance, was positive for MITF1 and HMB45, but negative for S-100, and

showed melanosomes by electron microscopy. Although we cannot entirely rule out a soft tissue metastasis of a cutaneous melanoma in this case, the lack of a clinical history of cutaneous melanoma and the histological appearance favored the diagnosis of CCS. The interpretation of this lone negative *EWS-ATF1* RT-PCR result is complicated by the possibility that the limited quality of the RNA extracted from the archival paraffin-embedded material in this case may have been insufficient to detect a novel molecular variant of *EWS-ATF1* producing a larger than expected fusion product. Another possibility is that this case contains a variant translocation, replacing one of the two usual translocation partners by a related gene, as seen in several other sarcomas.[41] Finally, it is also possible that rare CCS have a different molecular pathogenesis.

In conclusion, the present study shows that the *EWS-ATF1* gene fusion is found in the majority of CCS cases (91%) and thus provides a sensitive molecular diagnostic marker for CCS. Conventional RT-PCR methodology can be applied with good results for *EWS-ATF1* detection using RNA extracted from frozen tissues. When using archival material nested RT-PCR can yield similar results, in the presence of adequate RNA. The expression of the melanocyte-specific *MITF-M* transcript is further evidence of the *bona fide* melanocytic differentiation of CCS.

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