**EWS-CREB1**: A Recurrent Variant Fusion in Clear Cell Sarcoma—Association with Gastrointestinal Location and Absence of Melanocytic Differentiation

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Abstract

**Purpose:** Clear cell sarcoma (CCS) usually arises in the lower extremities of young adults and is typically associated with a t(12;22) translocation resulting in the fusion of EWS (EWSR1) with *ATF1*, a gene encoding a member of the cyclic AMP–responsive element binding protein (CREB) family of transcription factors. CCS arising in the gastrointestinal tract is rare and its pathologic and molecular features are not well defined.

**Experimental Design:** We report a novel variant fusion of *EWS* to *CREB1*, a gene at 2q32 encoding another CREB family member highly related to *ATF1*, detected in three women with gastrointestinal CCS. All three cases contained an identical *EWS-CREB1* fusion transcript that was shown by reverse transcription-PCR. In two of the cases tested, *EWS* gene rearrangement was also confirmed by fluorescence in situ hybridization and the *EWS-CREB1* genomic junction fragments were isolated by long-range DNA PCR.

**Results:** Morphologically, all three tumors lacked melanin pigmentation. By immunohistochemistry, there was a strong and diffuse S100 protein reactivity, whereas all melanocytic markers were negative. Ultrastructurally, two of the cases lacked melanosomes. The melanocyte-specific transcript of *MITF* was absent in two cases, and only weakly expressed in the third case. The Affymetrix gene expression data available in one case showed lower expression of the melanocytic genes *MITF, TYR,* and *TYRP1*, compared with four *EWS-ATF1*-positive CCSs of non-gastrointestinal origin.

**Conclusions:** *EWS-CREB1* may define a novel subset of CCS that occurs preferentially in the gastrointestinal tract and shows little or no melanocytic differentiation. Thus, evidence of melanocytic lineage or differentiation is not a necessary feature of sarcomas with gene fusions involving *CREB* family members.

Materials and Methods

**Case history patient 1.** This 81-year-old woman underwent a hemicolectomy for a moderately differentiated adenocarcinoma of the transverse colon. The hemicolectomy specimen showed a synchronous CCS of the gastrointestinal tract in the ascending colon. Surgical
Hybridization of Affymetrix oligonucleotide chips. Adequate tumor tissue for RNA extraction was available in case no. 1 (CCS no. 1). RNA was isolated using RNAzol RNA isolation reagent (Ambion) and run through a column with Rnase-free DNase (Qiagen). Twenty-five nanograms of total RNA were tested for quality by RNA 6000 NanoAssay on a Bioanalyzer 2100 (Agilent, Palo Alto, CA). Two micrograms of high-quality total RNA (A260/280 ratio > 1.8) was then labeled according to the manufacturer's instructions. Ten micrograms of labeled and fragmented cRNA were then hybridized onto a Human Genome U133A expression array (Affymetrix, Santa Clara, CA). Posthybridization staining, washing, and scanning were done according to instructions from the manufacturer (Affymetrix). The raw expression data were derived using the Affymetrix Microarray Analysis 5.0 (MAS 5.0) software. The data were normalized using a scaling target intensity of 500 to account for differences in the global chip intensity. The expression values were transformed using the logarithm base two. Affymetrix U133A gene expression data were also available in four cases of non-gastrointestinal CCS, all four carrying an EWS-ATF1 fusion, as previously reported in a separate study (1).

Results

Morphology, immunohistochemical findings, and ultrastructure. Grossly, the lesions had a white-tan cut surface with infiltrative borders. Areas of hemorrhage and necrosis were also seen in some tumors. The mean tumor size was 5.7 cm and ranged from 3.7 to 7.5 cm. Microscopically, the tumors had a multinodular and infiltrative growth pattern, being centered in the submucosa and muscularis propria, but focally extending into the mucosa, as well as into subserosal fat. At low power, the tumors showed a variable growth pattern, with areas of solid, nested, single-file, and pseudo-papillary growth noted even within the same tumor (Fig. 1A and B). Morphologically, the tumors displayed predominantly uniform small epithelioid cells, with limited variation in the nuclear size and shape. However, ovoid cell morphology and even spindling was also noted. The nuclei showed a smooth nuclear contour, with open and fine chromatin, and prominent nucleoli. Although the main pattern included epithelioid cells with a minimal amount of cytoplasm arranged in solid nests, focal areas of more abundant cytoplasm, either clear or granular-eosinophilic, were noted in two cases (Fig. 1C). Particularly striking was the pseudo-papillary pattern with preservation of the tumor cells around blood vessels, mimicking an epithelial neoplasm with papillary architecture (Fig. 1B). No melanin pigment was identified. Scattered multinucleated, osteoclast-type giant cells were identified in one case (case no. 2, Fig. 1D). Mitotic activity was high in all three cases, with a mean of eight mitotic figures per 10 high-power fields.

The immunohistochemistry findings showed a consistent pattern, with diffuse and strong reactivity for S100 protein in the overwhelming majority of the tumor cells (Fig. 2A). All three cases showed staining for neural markers, such as NSE, CD56 and synaptophysin, either in a diffuse (case nos. 2 and 3) or focal (case no. 1) manner (Fig. 2B). Vimentin was only focally positive. The tumors were completely negative for all melanoma markers, such as HMB45, A103, MITF, and tyrosinase. All the other markers tested, including CD117, CD34, cytokeratins, muscle markers, and chromogranin were negative.

Ultrastructurally, the tumors examined (case nos. 1 and 3) showed moderate amounts of cytoplasm, rich in organelles...
such as mitochondria, polyribosomes, and lysosomes. A significant number of electron-dense granules of variable sizes and shapes were identified, but no diagnostic melanosomes or dense-core neurosecretory-type granules were identified (Fig. 2C). A number of primitive or simple cell junctions were seen. Pertinent negative findings included lack of basement membrane material, glycogen, myofilaments, and tonofilaments.

**Detection of novel EWS-CREB1 fusion.** Fluorescence in situ hybridization for EWS rearrangement was done in case nos. 1 and 2 and showed multiple nuclei with split signals indicative of EWS rearrangement in both (Fig. 3A). RT-PCR for the EWS-ATF1 fusion was attempted in case no. 1, using the forward primer EWSEX7-F1 and the reverse primer ATF1-R1. This showed a weak but distinct product that differed in size from the product obtained from the EWS-ATF1-positive control cell line SU-CCS-1 (data not shown). Direct sequencing of the product in case no. 1 showed a chimeric transcript consisting of a junction between EWS exon 7 and a nucleotide sequence similar to ATF1 exon 7, which BLAST analysis revealed to be a perfect match with exon 7 of CREB1 (Fig. 3B). The novel EWS-CREB1 fusion transcript was confirmed by RT-PCR using EWSEX7-F1 with a specific reverse primer CREB1-Ex7bR (Fig. 3C). Using the same respective primer pairs as above, case nos. 2 and 3 were negative by RT-PCR for the EWS-ATF1 fusion, but were positive for EWS-CREB1 showing the same fusion structure as case no. 1 (Fig. 3C and D). Thus, these three patients provide evidence for the existence of a recurrent variant chromosomal translocation of EWS (22q12) and CREB1 (2q32.3), presumably a (2;22)(q32.3;q12), in CCS. Alignment of the EWS-CREB1 fusion product with native ATF1 highlights the extensive similarity in CREB1 and ATF1 (Fig. 4).

**Analysis of genomic structure of EWS-CREB1 fusion.** Detailed genomic analyses of EWS breakpoints, available only for the EWS-FLI1 fusions seen in Ewing’s sarcomas, have shown that all fusions involving EWS exon 7 as the 5' partner arise from genomic rearrangements within 4.2 kb downstream of EWS.
exon 7 (10). Likewise, it is likely that fusions involving CREB1 exon 7 as the 3' partner arise from genomic rearrangements of CREB1 intron 6, which measures 4.95 kb. To characterize the genomic fusion point of the EWS-CREB1 chimeric gene, we did PCR amplification of genomic DNA from case no. 1 with the EWSEx7-F1 and CREB1-ex7REVb primers. This revealed a strong discrete product of about 2 kb (data not shown). Stepwise direct sequencing of this fragment was done. PCR amplification and DNA sequencing with intronic forward primer EWS/IVS7F and the intronic reverse primer CREB1/IVS6R localized the genomic fusion point to nucleotide position g20493 of EWS intron 7 and nucleotide position g44998 of CREB1 intron 6 (Supplementary Fig. S1A). The EWS-CREB1 fusion gene in case no. 1 retained 1,052 bp from the 5' end of EWS intron 7 and 913 bp from the 3' end of CREB1 intron 6 resulting in the formation a chimeric intron of 1,965 bp, which corresponded with the size of the initial long-range DNA PCR product.

In case nos. 2 and 3, only paraffin-embedded tissue was available for DNA extraction. PCR amplification of genomic DNA extracted from paraffin-embedded tissue was attempted in case no. 2 only. Long-range PCR using primers EWSEx7-F1 and

Fig. 3. A, fluorescence in situ hybridization for EWS rearrangement. The probe centromeric to EWS (red); the probe on the telomeric side of EWS (green). Four nuclei with split signals indicative of EWS rearrangement (case no. 1). B, electrophoretogram of direct sequencing of junction point of EWS-CREB1 fusion transcript with in-frame fusion (case no. 1). C and D, agarose gels of EWS-CREB1 RT-PCR products in case nos. 1 and 2 using primers EWSEx7F1 and CREB1ex7REVc and in case no. 3 using the same forward primer but with reverse primer CREB1ex7REVa (consensus primer for CREB1 and ATFI—see Fig. 4). Lane C is the SU-CCS-1 positive control cell line containing EWS-ATFI. No R.T. designates the negative controls lacking reverse transcriptase.

Fig. 4. Partial alignment of EWS-CREB1 fusion with native ATFI. Exons are indicated by alternating upper and lower case letters. The positions of reverse PCR primers in CREB1 and ATFI are shown in italics. CREB1ex7REVC is a CREB1-specific primer, whereas CREB1ex7REVA is a consensus primer for CREB1 and ATFI.
We also examined the transcript levels of expression of melanocytic proteins by immunohistochemistry. substantial differences. This was consistent with its poor TYR expression of the key melanocytic genes involved in melanogenesis. As shown in Fig. 5A, case no. 1 expressed lower levels of the MITF, TYR, and TYRP1, but SOX10 transcript levels were not substantially different. This was consistent with its poor expression of melanocytic proteins by immunohistochemistry. We also examined the transcript levels of CREB1 and ATF1 (probe set 222103_at). The CREB1 transcript level was higher in case no. 1 than in the four EWS-ATF1 cases, possibly reflecting the detection of the EWS-CREB1 transcript by this probe set. However, ATF1 transcript levels did not differ between case nos. 1 and the 4 CCS with the EWS-ATF1 fusion (data not shown).

There are several alternative forms of the MITF transcript, reflecting different promoters and transcription start sites, only one of which, MITF-M, is highly specific for the melanocytic lineage (5). We have previously shown that classic soft tissue CCS with EWS-ATF1 express the melanocyte-specific MITF-M transcript, further supporting their genuine melanocytic differentiation (5). In contrast, RT-PCR analysis for the MITF-M transcript in our three EWS-CREB1 cases was negative in two cases and only weakly positive in one case (Fig. 5B), the latter being case no. 1, which also expressed the highest level of the consensus MITF transcript among these three cases (Fig. 5B). Together with the above immunohistochemical and ultrastructural studies, these gene expression data confirm that evidence of melanocytic differentiation in CCS with EWS-CREB1 is either absent or much less than in classic CCS with EWS-ATF1. This, in turn, strengthens the notion that melanocytic features are not a necessary finding in this type of sarcoma.

**Discussion**

The classic cytogenetic hallmark of CCS, first described in cases arising in somatic soft tissues, is a recurrent t(12;22)(q13;q12) translocation, resulting in the EWS-ATF1 fusion (3, 5, 6), an alteration which was, until recently (see below), not observed in any other tumor type. Despite this distinctive gene fusion in CCS (absent in melanoma; refs. 4, 11) and the absence of the BRAF mutations that are so common in melanoma (2), immunohistochemistry and ultrastructural studies of CCS suggest that it is, like melanoma, a neuroectodermal tumor with melanocytic differentiation. Further evidence of the genuine melanocytic differentiation in CCS has been provided by a microarray-based gene expression profiling study which showed that unsupervised clustering grouped CCS with melanomas rather than with other high-grade sarcomas (1). This study also confirmed the up-regulation in CCS of a number of genes involved in melanocytic differentiation (such as MITF and SOX10), relative to other soft tissue sarcoma types.

We have identified a novel recurrent variant fusion in CCS, EWS-CREB1. CREB1 maps to 2q34, and like EWS, is oriented with its 3’ end telomeric, suggesting that both of our cases may have contained a simple t(2;22)(q34;q12) at the cytogenetic
level, but no karyotypes were available. Our finding that all three CCS cases with this novel fusion arose in the gastrointestinal tract suggests that this fusion may be preferentially associated with a gastrointestinal location, whereas non-gastrointestinal CCS show the EWS-ATF1 fusion in at least 90% of the cases (5, 6).

ATF1, cyclic AMP (cAMP)–responsive element binding protein (CREB1), and cAMP response element modulatory protein constitute a subfamily of the basic leucine zipper superfamily of transcription factors and have been implicated in cAMP and Ca2+-induced transcriptional activation. CREB1 is a nuclear protein that binds cAMP response elements as a homodimer or heterodimer (with members of the ATF and AP1 transcription factor families). Overexpression of CREB transcription factors contributes to the acquisition of the metastatic potential in human melanoma cells and is also oncogenic in the myeloid lineage (12, 13). Genome-wide screens for promoters bound by CREB reveal a very large number of potential target genes (~4,000) and a critical role for tissue-specific coactivators in determining target gene activation (14).

Like other members of the basic leucine zipper superfamily, CREB1 has a modular structure consisting of a carboxyl terminal basic leucine zipper domain mediating DNA binding and dimerization, and an amino terminal transactivation domain that contains a kinase-inducible domain mediating interactions with CBP and p300 (Fig. 6). The predicted protein structure of EWS-CREB1 thus parallels that of EWS-ATF1 (Fig. 6). Specifically, all CCS cases with EWS-ATF1 contain fusion transcripts in which the basic leucine zipper domain is retained, and this is also the case for EWS-CREB1. The kinase-inducible domain, which is either excluded or truncated in different forms of EWS-ATF1, is not part of EWS-CREB1 in the three current cases (Fig. 6). Thus, the structure of EWS-ATF1 and the predicted structure of EWS-CREB1 indicates that mediating cAMP-inducible transcription through PKA-mediated phosphorylation is not a necessary feature of either fusion protein. Interestingly, whereas the MITF-M promoter is cAMP-inducible in the presence of SOX10 in melanoma cells through binding of its cAMP response elements by CREB1 (15), EWS-ATF1 does not seem to transactivate the MITF-M promoter in CCS cells, at least in exogenous constructs (16). Thus, the expression of the MITF-M transcript in CCS may represent a feature of the precursor cell in which the translocation occurs. Indeed, a chromatin immunoprecipitation–based screen for EWS-ATF1 target genes in a CCS cell line did not detect MITF (17). In fact, none of the nine putative target genes isolated in that study were related to the melanocytic lineage. Another line of evidence that EWS-ATF1 and MITF-M expression are not tightly linked comes from data in angiomatoid fibrous histiocytoma. Some angiomatoid fibrous histiocytomas seem to contain a novel FUS-ATF1 fusion (18, 19) but there is a recent report of a case with an EWS-ATF1 fusion, and that case lacked expression of the melanocytic splice form of MITF (20).

The occurrence of CCS in the gastrointestinal tract is exceptionally rare. Data from eight genetically confirmed cases along with the three current EWS-CREB1 cases and two additional unpublished cases from our center are summarized in Supplementary Table S1 (7–9, 21–23). Based on these 13 cases, CCS of the gastrointestinal tract seems slightly more prevalent in females, with a wide age distribution, 15 to 85 years, but with eight cases (62%) occurring between 30 and 51. The most common location is the small bowel (69%) followed by colon and stomach. A common characteristic of these tumors is the transmural involvement of the bowel wall, often with mucosal ulceration and spreading to regional lymph nodes. Microscopically, the predominant pattern is of nested or solid growth of small epithelioid cells with amphophilic or clear cytoplasm and uniform nuclei with conspicuous nucleoli. However, certain cases contain somewhat distinctive morphologic features, due to a component of admixed reactive, KP1-positive, and osteoclast-type giant cells (9, 23). One of our cases showed a similar, although very minor, component of osteoclast-type giant cells. These cells are most likely reactive histiocytic cells, and are different morphologically and immunohistochemically from the multinucleated tumor cells seen in conventional CCS. This latter type of giant tumor cell was not seen in most cases of primary gastrointestinal CCS. Due to its unusual visceral presentation, coupled with the inconsistent expression of melanocytic markers and variant EWS fusion partners, CCS of the gastrointestinal tract can often be misdiagnosed. This is evident in our experience, in which the original or second-opinion consultation diagnoses included: poorly differentiated carcinoma, most likely metastatic from the other synchronous colonic primary adenocarcinoma (case no. 1); carcinoid tumor, gastrointestinal stromal tumor, and undifferentiated neoplasm, possibly neurogenic in origin (case no. 2); and metastatic melanoma and carcinoid tumor (case no. 3).

Interestingly, whereas non-gastrointestinal CCS shows the expression of melanocytic markers (HMB45, A103, MITF, etc.) in the overwhelming majority of cases (5), the reverse seems to be the case in the gastrointestinal tract, where most tumors (69%) are negative for these markers (Supplementary Table S1). In spite of the negativity for melanocytic markers, the morphologic appearance coupled with the t(12;22) translocation supports a diagnosis of CCS in such cases. Although all three of our EWS-CREB1-positive CCS lacked melanocytic expression by immunohistochemistry, this was also noted in...
four of eight previously reported EWS-ATF1-positive CCS of the gastrointestinal tract (9, 21, 23, 24), suggesting that gastrointestinal location rather than the fusion transcript type might determine the lack of melanocytic differentiation. Furthermore, two additional gastrointestinal CCS from our files (not previously reported, see Supplementary Table S1), showing either EWS-ATF1 by RT-PCR or EWS rearrangement by FISH lacked evidence of melanocytic differentiation by immunohistochemistry. The consistent expression of neuroectodermal markers, such as S100, NSE, CD56, and synaptophysin, in our cases, raises the possibility of a novel gastrointestinal neuroectodermal tumor carrying an EWS-CREB1 fusion and lacking melanocytic differentiation. However, the detection of EWS-ATF1 fusion in a number of gastrointestinal tract CCS also lacking expression of melanocytic markers, argue in favor of a common histogenesis, possibly from a gastrointestinal neuroectodermal precursor cell which has lost or does not have potential to differentiate along the melanocytic lineage, in contrast to the putative neuroectodermal precursor cell of non-gastrointestinal CCS.

Finally, we note that the clinical behavior of gastrointestinal CCS seems to be aggressive, regardless of the fusion type, with a high incidence of both distant and regional metastases. Most patients developed liver metastases, but intraperitoneal spread was noted in a few cases as well. The time to distant recurrence was variable, ranging from 9 months to 5 years.

Acknowledgments

The authors thank Tao Zheng for expert technical assistance with RT-PCR and Dr. Agnes Viale and the staff of the Memorial Sloan-Kettering Cancer Center Genomics Core Laboratory for assistance with microarray studies.

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