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Case study

Primary cutaneous carcinosarcoma: insights into its clonal origin and mutational pattern expression analysis through next generation sequencing☆,☆☆

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Summary Primary cutaneous carcinosarcoma is a rare biphenotypic neoplasm exhibiting both epithelial and sarcomatous elements. Even though its origin and biological aspects remain poorly understood, it has been postulated that this tumor may arise from progenitor cells which subsequently differentiate into distinct tumor components. We have investigated the histological and immunohistochemical staining patterns of a cutaneous carcinosarcoma case, as well as its ultrastructural aspects. In addition, sarcomatous and epithelial tumor components were separated by laser capture microdissection and subjected to targeted, high-depth, Next-Generation Sequencing of a 46-cancer gene panel to asses the gene mutational pattern amongst both components. There were transitional cells at the epithelial/mesenchymal transition which labeled with putative progenitor cell markers (K 19, c-kit, CD34 and BCL-2). There was shared reactivity to antibodies directed against the progenitor cell marker EpCAM (epithelial cell adhesion molecule) in both components. Ultrastructurally, individual cells were demonstrated to have overlapping features of epithelial and mesenchymal differentiation. The mutational analysis revealed point mutations in exon 5 of *TP53* which were identical in both the epithelial and sarcomatous components, and which was concordant with p53 expression at a tissue level. The aforementioned histological, ultrastructural, immunohistochemical and mutational pattern is strongly suggestive of a common clonal origin to the distinct elements of this tumor.

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1. Introduction

Biphasic tumors of the skin are rare neoplasms [1], which are subject to a variety of descriptive terms based on their morphology, making an accurate assessment of case numbers from the literature very difficult [2]. Cutaneous carcinosarcoma (CCS) is a biphasic tumor composed of an intimate admixture of malignant epithelial and mesenchymal elements [3,4]. It has been reported to occur in a variety of anatomical sites, including the urogenital and gastrointestinal tracts, breast, lung, thymus, and thyroid [3,5]. To date, approximately 65 cases of CCS have been described in the literature, and, even though they are known to be aggressive tumors, with potential for local recurrence and metastasis [6], their prognosis remains unclear [3]. Recent studies suggested that stem/progenitor cells can play an important role in all tissues, not only during embryogenesis but also in adult tissue maintenance, repair and oncogenesis [7-9]. This fact supports the hypothesis that stem/progenitor cells can serve as common precursors for tumors of mixed phenotype such as squamo-melanocytic tumors [10] and perhaps carcinosarcomas. Herein we examine a case of primary cutaneous carcinosarcoma using immunohistochemical, ultrastructural, and molecular studies. Our goal is to test the divergent/monoclonal hypothesis postulating that these tumors derive from a common progenitor stem cell, by further analyzing the clonality of the different morphologic tumor components through next generation sequencing based mutation screening.

2. Material and methods

Tissue sections were fixed in 10% neutral-buffered formalin and embedded in paraffin. Sections measuring 4 mm were cut for hematoxylin and eosin (HE) staining and immunohistochemical examinations.

2.1. Immunohistochemistry

Immunohistochemistry was performed using a polyvalent horseradish peroxidase polymer detection system (Bond 111, Leica Microsystems, Wetzlar, Germany). The primary antibodies against the following antigens were used: keratin 19 (K19) (RCK108; 1:100 dilution; Dako, Carpinteria, CA); Cytokeratin AE1-3 Cocktail (AE1/AE3; 1:200 dilution; Covance, Princeton, NJ). High-molecular-weight cytokeratin (K903) (34BE12; 1:50 dilution; Dako, Carpinteria, CA); c-kit (CD117) (polyclonal; 1:200 dilution; Dako Cytomation, Carpinteria, CA); CD34 (QBEnd/10; RTU; Leica Biosystems); Bcl-2 (124; 1:80 dilution; Dako; CA, USA); Vimentin (V9; ’1:1.6k dilution; Dako; CA, USA); p53 (DO-1, RTU, 1:50; Immunotech; Westbrook, ME) and epithelial cell adhesion molecule (EpCAM) (VU-1D9; RTU; Leica Biosystems). Proper antigen retrieval was carried out for each antibody according to each of the manufacturer’s instructions.

2.2. Electron microscopy

Wet tissues retrieved from formalin were transferred to glutaraldehyde and postfixed in 1% phosphate-buffered osmium tetroxide. Osmicated tissues were embedded in epoxy resin in standard fashion. Prior to ultrathin sectioning, approximately 1- to 2-mm epoxy sections were tolluidine stained for light microscopic orientation. Ultrathin (around 80 nm) sections were collected on collodion-coated open slot grids for unobstructed evaluation and stained in uranyl acetate and lead citrate. Thin sections were evaluated on a Zeiss EM 900 electron microscope from 150 to 50.000×. Images were captured with an Optronics digital camera utilizing Microfire software.

2.3. Laser capture microdissection and DNA extraction

DNA was extracted from formalin-fixed, paraffin embedded tumor samples as follows: unstained tissue sections of 0.4 μmol/L thick were stained with hematoxylin and eosin for accurate localization of tumor components. Both the carcinoma and sarcoma components were microdissected separately using a hematoxylin and eosin–stained slide from the same block as a guide, with a laser capture microscope (Zeiss, LLC). Cells were subjected to DNA extraction using the Pico Pure DNA extraction Kit (Arcturus, Mountain View, CA), and later purified with the AMPureXP kit (Agentcourt Biosciences, Beverly, MA) magnetic bead purification method. DNA concentration and purity were assessed using the Qubit DNA HS assay kit (Life Technologies, Carlsbad, CA).

2.4. Library preparation

The amplicon library preparation and sequencing were performed as described earlier [11], using the Ion Torrent Ampliseq Kit 2.0 (Life Technologies, Carlsbad, CA) and the Ion Torrent Ampliseq cancer panel primers (Life Technologies). In brief, 10ng of DNA was used as template to generate an amplicon library aimed to sequence hotspot mutations in 46 target genes. The gene panel included the following: AKT1, BRAF, FGFR1, GNAS, IDH1, FGFR2, KRAS, NRAS, PIK3CA, MET, RET, EGFR, JAK2, MPL, PDGFRA, PTEN, TP53, FGFR3, FLT3, KIT, ERBB2, ABL1, HNF1A, HRAS, ATM, RB1,CDH1, SMAD4, STK11, ALK, SRC, SMARCB1, VHL, MLH1, CTNNB1, KDR, FBXW7,APC, CSF1R, NPM1, SMO, ERBB4, CDKN2A, NOTCH1, JAK3, PTPN11, as well as a customized primer (Life Technologies) to interrogate potential mutational hotspots on the *AKT1* gene. For sequencing, genomic target regions were polymerase chain reaction–amplified using the 191-primer pair pool.

2.5. Emulsion polymerase chain reaction

The emulsion polymerase chain reaction was carried out manually using the Ion Xpress Template kit (Life Technologies) following the manufacturer's guidelines. From the library stock, samples were pooled and diluted to further generate a working library concentration of 20 pM. IonSpheres, which were then isolated by manual breaking of the emulsion following the manufacturer's instructions with subsequent enrichment of template IonSpheres using the automated Ion One Touch ES System. Quality and quantity of the enriched spheres were assessed using the Qubit Ion Sphere Quality control kit (Life Technologies). Sequencing of the amplicon libraries was carried out on the Ion Torrent Personal Genome Machine system using the Ion Sequencing 2.0 kit (Life Technologies) following the manufacturer's protocol. Successful sequencing of a sample was considered when a cutoff of 300,000 reads with a quality score of AQ20 (1 misaligned base per 100 bases) was obtained. In order to consider a sequence variant authentic, a minimum sequencing coverage of 250 sequencing reads and a variant frequency of at least 10% in the background of wild type had to be achieved.

2.6. Data analysis

Base calling and alignment to hg19 reference genome were performed by the Ion Torrent Suite software V2.0.1 (Life Technologies). Variant calling was facilitated using the IT Variant Caller Plugin, software V1.0 (Life Technologies) and confirmed by visualization via Integrative Genomics Viewer [11] to check for possible strand biases and sequencing errors. In addition, to visualize the alignment and mutation detected, as well as to correctly annotate sequencing information, compare sequencing replicates and filter-out repeat errors due to nucleotide homopolymer regions, we used customized in-house developed software (OncoSeek) to interface the data generated by Ion Torrent Variant Caller with the Integrative Genomics Viewer [12].

2.7. Mutation confirmation

The presence of mutation detected by Ion Torrent next generation sequencing was confirmed by Sanger sequencing.

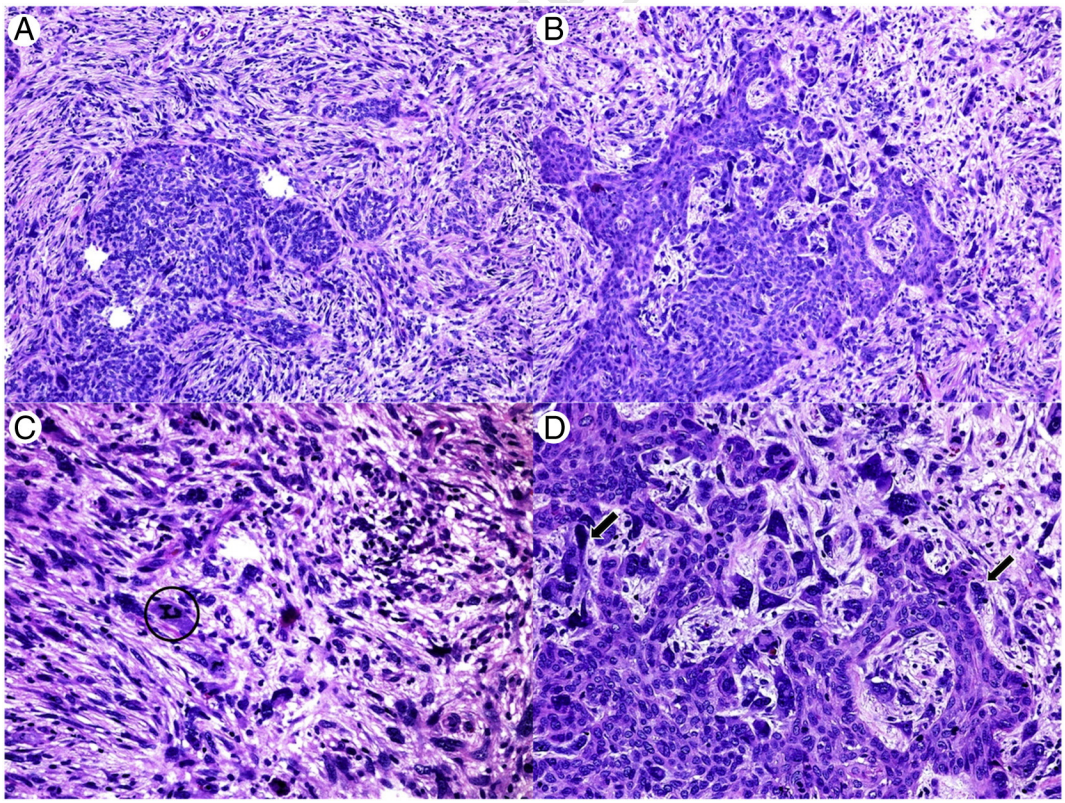

Fig. 1 Hematoxylin-eosin stained sections. A and B, Malignant epithelial islands consisting of basal cell carcinoma and high grade carcinoma with focal squamous differentiation respectively (original magnification 10). C, Malignant stromal component with atypical mitotic figures (circle) and atypical spindle cells (original magnification ×20). D, The osteoclast-like giant cells as well as the pleomorphic spindle cells at the epithelial/stromal interface (arrow) (original magnification ×20).

3. Results

Microscopically, the lesion showed a biphasic pattern with both malignant epithelial and mesenchymal components in close juxtaposition the one to the other. The epithelial component comprised areas of typical basal cell carcinoma arranged in an insular and organoid pattern merging with areas of high grade carcinoma with focal squamous differentiation (Fig. 1A and B). In areas with classic basal cell carcinoma morphology, the epithelial cells showed scant cytoplasm, palisading and clefting (Fig. 1A). In the high grade carcinomatous areas, cells showed intracellular bridges focally and increased mitotic activity (Fig. 1B). The mesenchymal component consisted of fascicles of large atypical spindle cells as well as numerous osteoclast-like giant cells. Brisk mitotic activity and atypical mitoses were readily identified within the stromal component (Fig. 1C and D). Pleomorphic spindle cells with dark bizarre shaped nuclei were identified at the epithelial-mesenchymal transitions in the vicinity of the aforementioned giant cells (Fig. 1D).

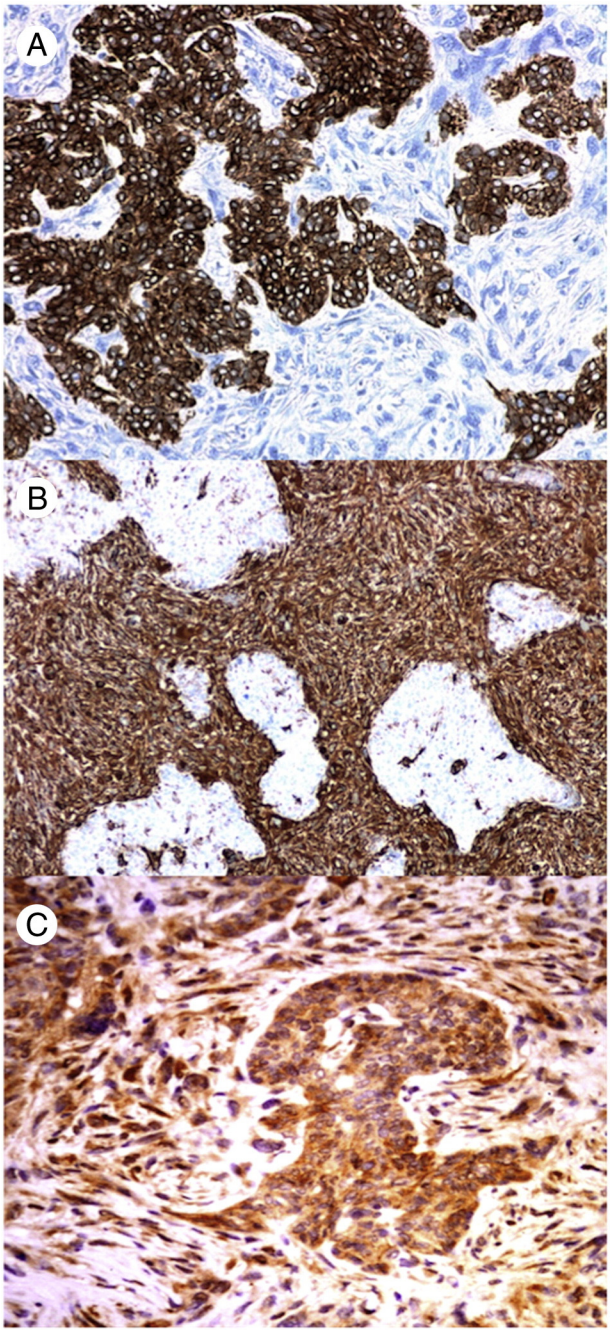

Fig. 2 Immunohistochemical studies. A, Showing strong diffuse membranous and cytoplasmic reactivity with Pan Keratin in the malignant epithelial component (original magnification ×10). B, Strong cytoplasmic reactivity with vimentin in the stromal spindle cell component (10 ×). C, showing diffuse membranous and cytoplasmic immunoreactivity with EpCam (Anti Ber-EP4) immunostain in the epithelial and stromal components (original magnification ×40).

On immunohistochemical studies, the carcinomatous component (approximately 60% of the examined tumor area) labeled with cytokeratin AE1/AE3 and K903 (Fig. 2A), while the sarcomatous component was positive for vimentin (Fig. 2B) and negative for all other markers. Both histological components as well as the transitional tumor cells showed positive immunoreactivity with EpCAM (Fig. 2C). Intermediate cells located at the epithelial-mesenchymal transition also showed immunoreactivity for the putative stem cell markers CD117, CD34, bcl-2, and k19 (Fig. 3A-D).

Ultrastructural analysis from the merging areas revealed transitional cells which showed chimerical features, with thin 5-nm actin-sized cytoplasmic filaments with focal densities (Fig. 4A) and dilated rough endoplasmic reticulum (Fig. 4B) characteristic of mesenchymal differentiation. Also, mucin-filled cytoplasmic vacuoles (Fig. 4C) and cytoplasmic tonofilaments with well-developed desmosomal attachments (Fig. 4D) typical of epithelial differentiation were identified within these same cells, supporting mixed biphenotypic features at the individual cell level.

Mutational analysis revealed the same (TGC>TAC) point mutations in exon 5 of *TP53*, at codon 135, with identical G to A substitutions resulting in an encoded amino acid change from cysteine to tyrosine (p.Cys135Tyr) in both tumor components (Fig. 5A-D). In the laser-micro dissected carcinomatous component, a variant frequency of 30. 6% was obtained at a coverage depth of 600×; while, the sarcomatous component exhibited a 27.0% variant frequency at a 916× coverage depth. In addition, a whole specimen, including both components consistently demonstrated the mutation with a 20.9% variant frequency at a 736× coverage depth. The presence of this *TP53* mutation in all of the specimens was confirmed by a clinically validated Sanger sequencing assay (Fig. 5E-F). Concomitantly, both the sarcomatous and epithelial components exhibited p53 protein over expression (Fig. 5G). Furthermore, we found consistent silent and missense mutations in two additional genes, *MET* and *KDR* (respectively) on both components of the tumor as well as the whole specimen. The *MET* gene

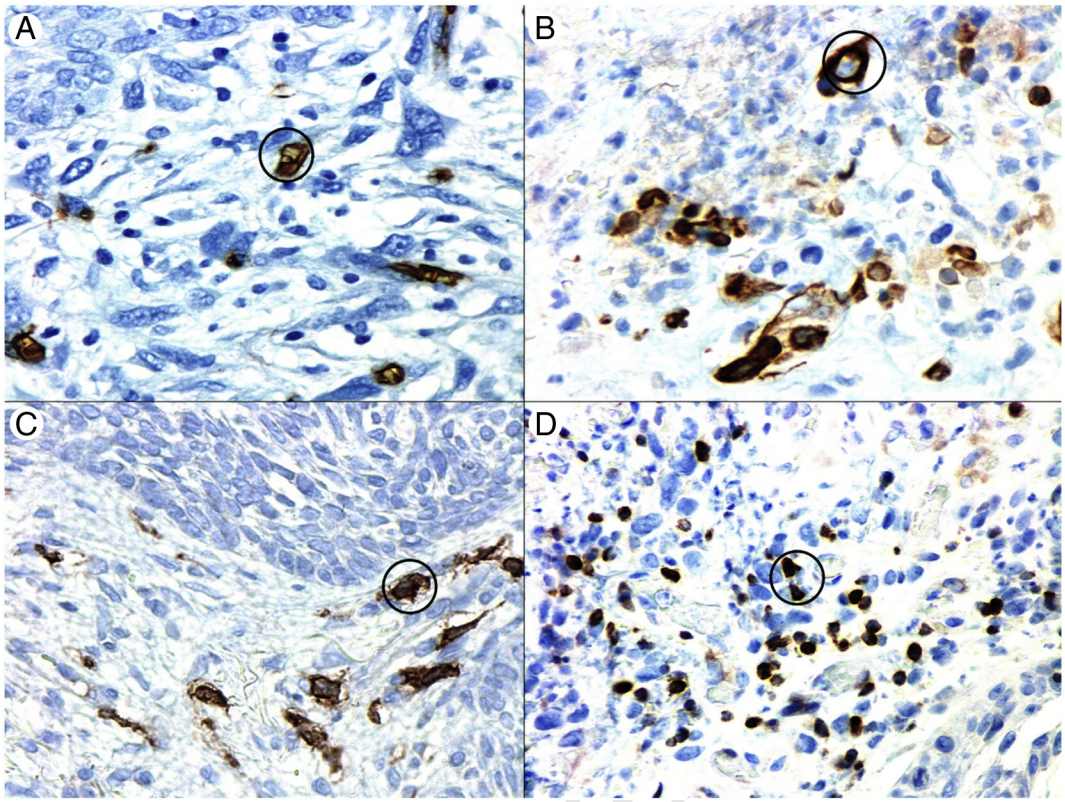

Fig. 3 Immunohistochemical studies. A, Strong diffuse membranous reactivity with CD34 immunostain in the atypical spindled cells at the epithelial/stromal interface (circle) (original magnification ×40). B, Strong membranous and cytoplasmic reactivity in the same cell population as in (A) with K19 immunostain (circle) (original magnification ×40). C, Showing strong membranous immunoreactivity with CD 117/C-kit in the same cell population as in (A) (circle) (original magnification ×40). D, Strong nuclear reactivity with BCL2 immunostain in the same cell population as in (A) (circle) (original magnification ×40).

exhibited a (AGC>AGT) (dbSNP rs3577572) point mutation in exon 2, codon 178 with identical G to A (Ser→Ser) substitutions at variant frequencies of 48.1%, 46.7%, and 51.3% for the carcinomatous component, sarcomatous component and whole specimen respectively; the *KDR* gene the mutation in exon 11 (CAA>CAT) revealed identical A to T substitutions resulting in an encoded amino acid change from glutamine to histidine (p.Gln472H) at variant frequencies of 56.1%, 51.9%, and 50.1% for the carcinomatous component, sarcomatous component and whole specimen respectively. The variant frequency of around 50%, as well as reference to the literature and dbSNP database suggest the *MET* and *KDR* mutations to be germline polymorphisms in contrast to the *TP53* somatic mutation which was observed at lower frequencies.

4. Discussion

Originally described by Dawson in 1972 [13], CCS is a biphenotypic tumor exhibiting both malignant epithelial and mesenchymal differentiation [14]. The most common epithelial features represented are those of basal cell and squamous cell carcinomas [14], while the mesenchymal component shows features of atypical fibroxanthoma, leimyosarcoma or undifferentiated sarcoma [6,14]. The histopathogenesis of these tumors remains poorly understood [3,15]; although several theories have been proposed. Three distinct precursor pathways seem to be involved in CCS tumorigenesis; a first pathway following the occurrence and merging of 2 synchronous unrelated tumors (a collision phenomenon) [6,15], a second pathway in which the epithelial and sarcomatoid components undergo differentiation/metaplastic transformation from two or more stem cells (the "convergence" or multiclonal hypothesis), and a third pathway in which a single totipotent cell undergoes divergent differentiation into different cell lineages (the "divergence" or monoclonal hypothesis) [6,15]. In our case, the presence of transitional chimeric cells at the epithelial-mesenchymal interface suggests the possibility of a common precursor cell origin for CCS. These cells labeled intensely with putative stem cell markers: c-kit (CD117), CD34, K19, bcl2, and EpCAM; thus, sustaining the possibility that the tumor could have originated from these interface stem cells to differentiate simultaneously into an epithelial component (highlighted by the strong pancytokeratin and K903 expression) and into a mesenchymal component

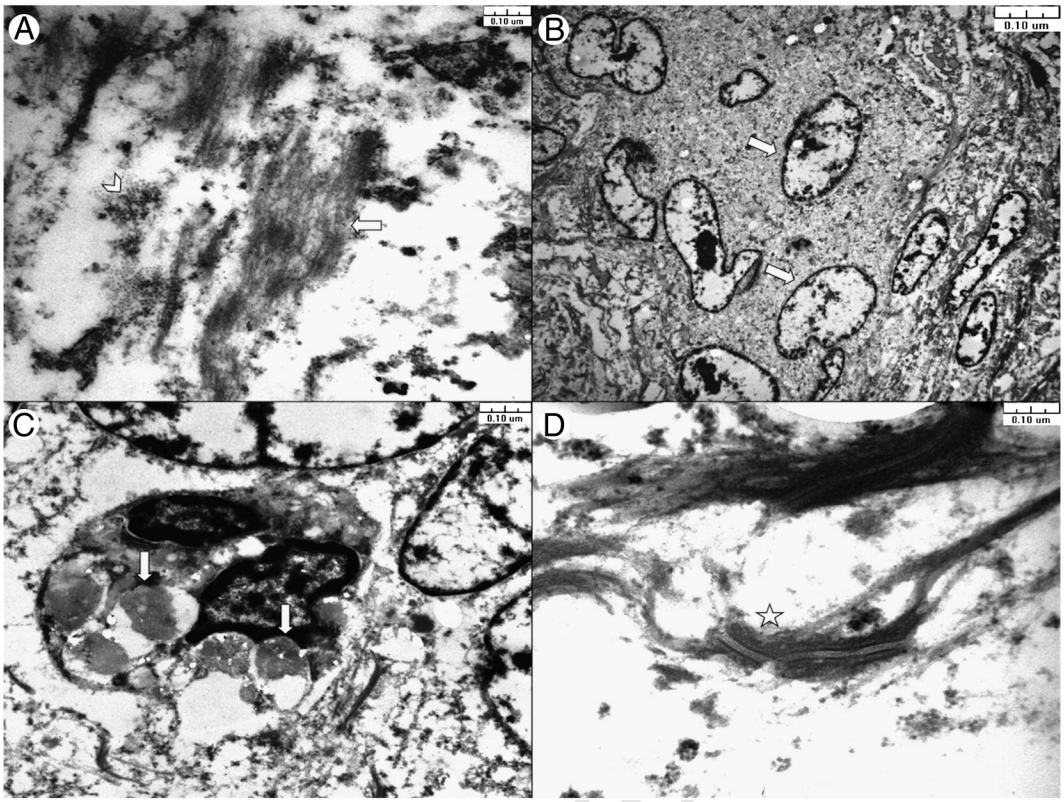

Fig. 4 Ultrastructural Studies of the spindle interface cell. A and B, High power magnification showing thin 5 nm "actin-sized" cytoplasmic filaments (arrow) with focal densities (arrowhead) and dilated rough endoplasmic reticulum measuring >60 nm in diameter (white arrows); both findings are characteristic of mesenchymal differentiation. C and D, High power magnification shows focal mucin vacuoles (white arrows) and cytoplasmic tonofilaments with well developed desmosomal attachments (star), both findings are characteristic of epithelial differentiation.

(highlighted by vimentin expression). Such observations are further supported by the ultrastructural findings that showed simultaneous evidence of epithelial and mesenchymal differentiation within the same cells (Fig. 4), strongly suggesting that the divergent monoclonal theory could be behind the development of CCS. Furthermore, the mutational expression pattern [5(*TP53*): c.404G>A] was identical in separately microdissected epithelial and sarcomatoid components, revealing a monoclonal origin for both. Our findings are in line with the cancer stem cell hypothesis [16], which sustains that epithelial stem cells may undergo a chain of oncogenic events leading to an uncontrolled expansion with aberrant differentiation and formation of tumors with heterogeneous phenotypes [16,17]. Also, the identification in the epithelial-mesenchymal transition zone of intermediate cells labeling with putative stem cell markers (Fig. 3) recapitulates the behavior of cancer-initiating stem cells. These cells are usually located in the core of the tumor to generate the dedifferentiating progeny that expands from the epithelial to the mesenchymal state [16]. Recent studies have linked the epithelial-mesenchymal transition not only with the acquisition of stem cell attributes but also with metastatic progression of cancer [18,19], and cell phenotype conversion [16] to acquire mesenchymal-like features as observed in this case. Furthermore, the mutational pattern exhibited suggests a clonal origin for the epithelial and mesenchymal elements of the tumor.

TP53 somatic mutation seems to be an early event in tumorigenesis that is maintained although progression of the stem cell progeny while differentiating into distinct tumor components. Among other upstream stimuli, DNA damage is a potent activator of p53 function, and p53 is required for DNA damage-induced G1 arrest and apoptosis in many cell types [20]. Given these functions, mutation of p53 would be expected to lead to genomic instability and inadequate cell longevity [20]. Since CCS appears to derive from early established stem cell epithelial-nested precursors which may harbor *TP53* mutant cell clones (as in this case), it is possible that conversion to the mesenchymal component is also driven by selection of tumor cells containing mutations and which confer a clonal advantage towards malignant differentiation. Numerous p53 mutations have been described in a large number of human non-melanoma skin cancers [21,22]. Yet, to date, this particular point mutation in the *TP53* exon 5 has not been described in CCS. Furthermore, the shared p53 over expression amongst both components of the tumor supports the

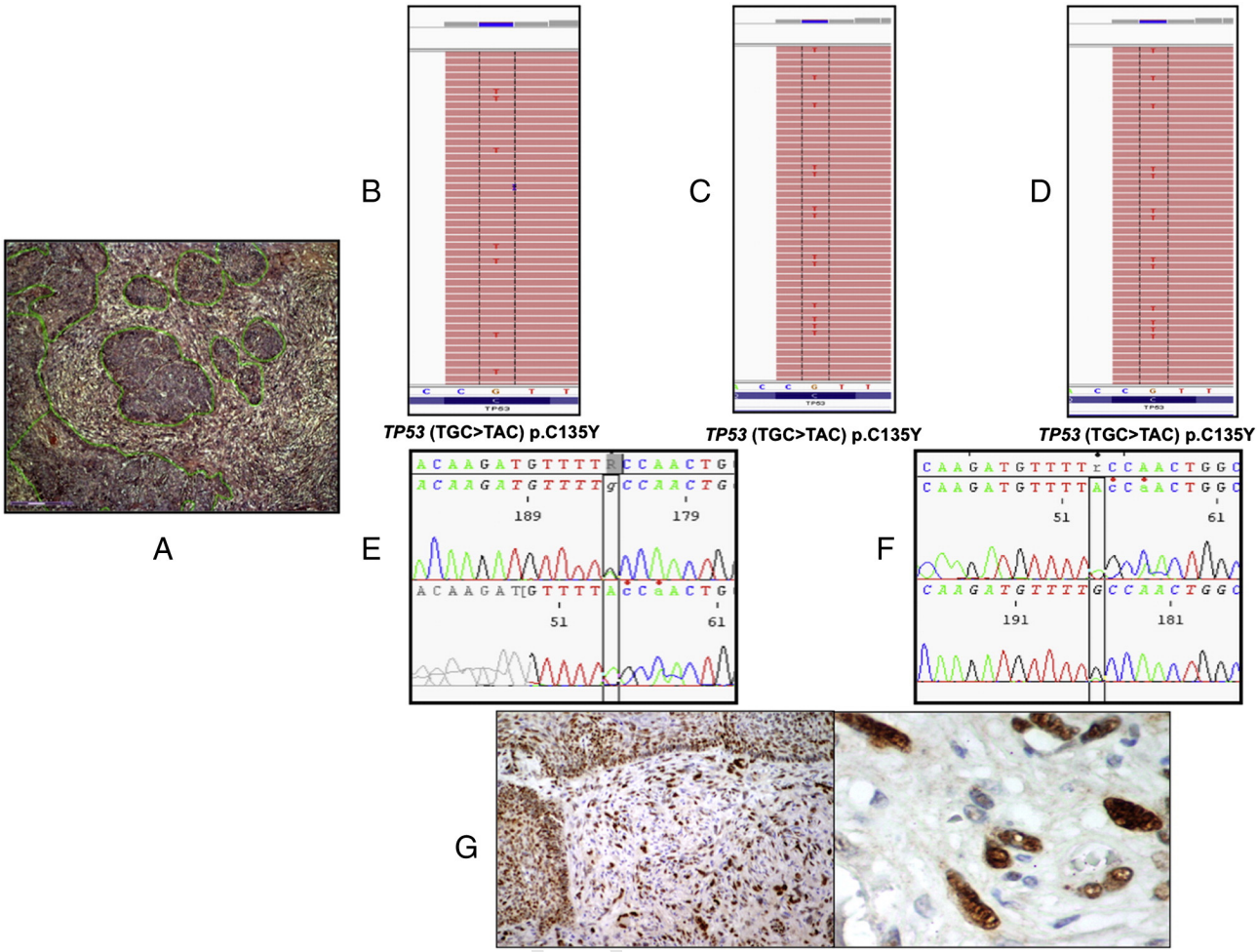

Fig. 5 Molecular studies. A, Laser micro capture microdissection (LCM) image showing the epithelial islands delineated in green. The left out areas represent the stromal component of the tumor. B-D, Next generation sequencing of the whole tumoral tissue, the epithelial component, and the stromal component reveals identical point mutations in *p53* gene at exon 5,codon 135, with similar G to A substitutions resulting in an encoded amino acid change from cistern to tyrosine (p.Cys135Tyr). E and F, Sequencing using the SANGER method reveals the same point mutations as identified in next generation sequencing method in the whole tumoral tissue and the stromal component confirming the aforementioned results. G, Diffuse nuclear overexpression of p53 antigen in both the epithelial and stromal components correlating with the point mutation identified in *TP53* gene (original magnification ×20 and ×40).

monoclonal origin of this entity. EpCAM is a pan-epithelial differentiation antigen which also serves as a marker for stem/progenitor cells [23-25]. EpCAM is an oncogenic signaling molecule whose expression is regulated by Wnt/b-catenin signaling pathway and has recently been linked to tumorigenic capabilities [26]. In line with these findings, our case showed shared immunoreactivity for EpCAM in both the mesenchymal and epithelial components as well as the stem cells. This over expression may provide a potential target for anti-EpCAM antibodies in the treatment of these tumors.

To the best of our knowledge, this case is the first to provide convincing immunohistochemical, ultrastructural and molecular data concerning CCS histopathogenesis. Yet, solid conclusions cannot be drawn based on a single case. Further similar studies including additional cases are underway in order to validate our findings.

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