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ORIGINAL ARTICLE

Cutaneous carcinosarcoma: further insights into its mutational landscape through massive parallel genome sequencing

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Abstract Cutaneous carcinosarcoma (CCS) is an extraordinarily rare neoplasm with a biphasic morphological pattern exhibiting both epithelial and sarcomatoid components. Although its histogenesis and biological aspects remain poorly understood, previous studies have postulated that this tumor may arise from single cancer stem cells which subsequently differentiate into distinct tumor lineages. In this study, we explored a wide array of mutational hot spot regions, through high-depth next-generation sequencing of 47 cancer-associated genes in order to assess the mutational landscape of these tumors and investigate whether the epithelial and mesenchymal components shared the same genetic signatures. Results from this study confirm that despite their striking phenotypic differences, both elements of this infrequent tumor indeed share a common clonal origin. Additionally, CCS appears to embrace a heterogeneous spectrum with specific underlying molecular signatures correlating with the defining epithelial morphotype, with those carcinosarcomas exhibiting a squamous cell carcinoma epithelial component exhibiting diverse point mutations and deletions in the *TP53* gene, and those with a basal cell carcinoma morphotype revealing a more complex mutational landscape involving several genes. Also, the fact that our findings involve several targetable gene pathways suggests that the underlying molecular events driving the pathogenesis of CCS may represent future potential targets for personalized therapies.

Keywords Carcinosarcoma · Cutaneous · Mutations · Next-generation sequencing · Stem cells · Histogenesis

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Introduction

Originally described by Dawson in 1972, primary cutaneous carcinosarcomas are exceedingly rare skin tumors with around 70 cases reported to date in the English literature [1–3]. Cutaneous carcinosarcomas (CCSs) are biphenotypic tumors, which embrace a heterogenous spectrum of morphotypes characterized by an intimate admixture of epithelial and mesenchymal components with varying degrees of differentiation amongst both elements [4, 5]. The epithelial component can include squamous cell carcinoma, basal cell carcinoma, basal cell carcinoma with focal squamous differentiation, as well as malignant adnexal morphological features [5–8]. On the other hand, the sarcomatous component may be composed of spindle and pleomorphic cells with marked atypia as well as by heterologous elements with chondroblastic and osteoblastic differentiation [4, 5, 9–13]. Currently, literature concerning the molecular events underlying CCS is unavailable. Previous molecular analysis of these histological components in other organs has

revealed common genetic aberrations, suggesting that tumors may arise as a clonal population that de-differentiates to yield the biphasic phenotypic lineages [14–17]. Herein, we present a clinicopathological, immunohistochemical, and molecular study of a series of six cases of CCS. We aim to identify a morphological and molecular correlation amongst the different morphotypes of CCS in an attempt to improve their classification based on the harbored molecular derangements. Also, we report novel mutations in this tumor group that provide further insights into their histogenesis and help in identifying possible targeted therapy candidate genes.

Material and methods

Immunohistochemistry (IHC)

Slides were cut at 4 μm, and IHC was performed using a polyvalent horseradish peroxidase (HRP) 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; CA, USA); cytokeratin AE1-3 cocktail (AE1/AE3; 1:200 dilution; Covance; Princeton, NJ, USA); high molecular weight cytokeratin (K903) (34BE12; 1:50 dilution; Dako; CA, USA); c-kit (CD117) (polyclonal; 1:200 dilution; Dako Cytomation; Carpinteria, CA, USA); CD34 (QBEnd/10; RTU; Leica Biosystems); Bcl-2 (124; 1:80 dilution; Dako; CA, USA); factor XIIIa antigen (polyclonal; 1:500; CalBiochem; San Diego, CA, USA); vimentin (V9; '1:1.6 k dilution; Dako; CA, USA); p53 (DO-1, RTU, 1:50; Immunotech; Westbrook, ME, USA); p63 (monoclonal; 4A4; 1:100; Ventana Medical Systems, Inc.; Tucson, Arizona, USA); cytokeratin (1:20; Dako); E-cadherin (HECD-1, RTU, 1:400; Cell Marque; Rocklin, CA, USA); smooth muscle actin (SMA) (1A4; 1:250; Dako; CA, USA); beta-catenin (17C2; RTU; Leica Biosystems); 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.

Laser capture microdissection (LCM) and DNA extraction

LCM was performed using a Zeiss, LLC laser capture microdissection system. Both carcinomatous and sarcomatous components were microdissected separately from formalin-fixed paraffin embedded (FFPE) tumor sample slides (0.4 μM) using a hematoxylin and eosin (H&E) slide as a guide. DNA was extracted from the cells 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 quantity and quality were assessed using the Qubit DNA HS assay kit (Life Technologies, Carlsbad, CA).

Library preparation

In brief, 10 ng of purified genomic DNA was used to build the library using the Ion Torrent Ampliseq Kit 2.0 (Life Technologies, Carlsbad, CA) and the Ion Torrent Ampliseq cancer panel primers, with the amplicon library targeting mutational hot spot regions on the following 47 cancer-associated genes: *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*, *PTPN1*, and *AKT1*.

Next, target genomic regions to be sequenced were PCR amplified using the 191 primer pair pool. Bar-coded sequence adaptors were ligated to the amplicons using the Ion Xpress Barcode Adaptors Kit (Life Technologies). The obtained library was quantified by the Bioanalyzer high-sensitivity DNA chip (Agilent Technologies Inc., Santa Clara, CA).

Emulsion PCR

Emulsion PCR (em-PCR), the process by which DNA is clonally amplified onto beads, was performed manually with the Ion Xpress™ Template kit (Life Technologies) in accordance to the manufacturer's guidelines. Samples were pooled and diluted in nuclease free water from the library stock to further generate a working library concentration of 20 pM. From this stock, IonSpheres™ (ISPs) were subsequently isolated by manual breaking of the emulsion, followed by enrichment to select DNA-binded ISPs through the automated Ion OneTouch ES System™, in order to maximize the number of sequencing reads generated by the Ion Torrent Personal Genome Machine (PGM) system. The quantity and quality of the obtained spheres was evaluated using the Qubit IonoSphere Quality control kit (Life Technologies). Sequencing was performed on the PGM system using the Ion Sequencing 2.0 kit (Life Technologies) as per manufacturer's protocol. For a sequencing sample to be considered successful, a cutoff of 300,000 reads with a quality score of AQ20 (1 misaligned base per 100 bases) was required. In addition for a sequence variant to be considered valid, a sequencing coverage of 250x and a variant frequency of at least 10 % (to wild-type background) were necessary. Amplicons failing to achieve a minimum coverage of 250x were recorded as "indeterminate."

Data analysis

PGM reads were aligned onto the reference human genome hg19 using the Ion Torrent Suite software V2.0.1 (Life Technologies). The IT Variant Caller Plugin, software V1.0 (Life Technologies) was used for calling variants from the PGM mapped reads, which were subsequently confirmed by

visualization via Integrative Genomics Viewer (IGV) [18] in order to check for probable strand biases and sequencing errors. An additional layer of filtering was applied using a customized software (OncoSeek) developed in-house to interface the data generated by Ion Torrent Variant Caller with the IGV [19]. This allowed visualizing the alignment and mutations detected, as well as to correctly annotate sequencing information, compare sequencing replicates, and filter out repeat errors due to nucleotide homopolymer regions.

Mutation confirmation by Sanger sequencing

To validate the presence of mutations detected by Ion Torrent next-generation sequencing, samples were analyzed by conventional Sanger sequencing. Mutation screening for exon 6 of the *TP53* gene was carried out using PCR conditions and × 2 bidirectional direct sequencing. Tumor DNA for exon 6 was amplified using the following M13-tagged primers: forward primer 5′-TGTAAAACGACGGCCAGTCAGGCCTCTGATTCCTCACT-3′ and reverse 5′-CAGGAAACAGCTATGACCGGTCAAATAAGCAGCAGGAGA-3′. Sequencing reactions were performed in both direct and reverse directions, and electropherograms were reviewed manually to detect any genetic alteration. All variants were confirmed by resequencing independent PCR products.

Results

Based on the epithelial morphotype, two subgroups of carcinosarcomas were recognized: a first group with a squamous cell epithelial component (SCC-derived CCS) and a second group with a basal cell epithelial component with the presence of heterologous elements (BCC-derived CCS).

Clinical-pathological findings

Our series included five males and one female patient. CCS was distributed between the scalp, back, axilla, and head and neck areas. Patients' age ranged from 54 to 92 years old. Patients presenting with SCC-derived CCS had a higher age range compared to those who presented with a BCC-derived CCS (Table 1). The BCC epithelial component showed areas arranged in an insular and organoid pattern. Within these areas, cells showed scant cytoplasm, focal palisading, and clefting (Fig. 1a). The SCC epithelial component showed cells with dense abundant eosinophilic cytoplasm and 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 (Fig. 1c). Pleomorphic spindle cells with dark bizarre-shaped nuclei were identified at the epithelial–stromal interface (Fig. 1a). Focal heterologous differentiation within the mesenchymal component was present in four cases (Table 1) including focal osteosarcomatous, leiomyosarcomatous, and rhabdomyosarcomatous features (Fig. 1d).

Immunohistochemical studies

On immunohistochemical studies (Table 2), the malignant epithelial cells (BCC- and SCC-derived CCS) were labeled with cytokeratin AE1/AE3, K903, and EpCAM, while the malignant mesenchymal cells were labeled with vimentin, factor XIIIa, and focally with SMA (Fig. 1e, f). *p53* was expressed on both epithelial and mesenchymal components in all cases except cases 2 and 4, in which epithelial expression was weak, and absent in the mesenchymal component. In addition, the epithelial component of all tumors was positive for *p63*, whereas the sarcomatous component was negative, confirming the diagnosis of primary cutaneous carcinosarcoma (Fig. 1a inset). The pleomorphic intermediate cells located at the epithelial–stromal interface labeled with the stem cell markers CD34, CD117, k19, bcl-2 (Fig. 2), and p63. Both carcinomatous and sarcomatous components as well as transitional tumor cells located at the interface labeled with the pan-epithelial differentiation antigen EpCAM. β-catenin and E-cadherin were expressed in the cytoplasmic membrane of the benign epithelium in all cases (Fig. 3a, d). Within the tumor cells, β-catenin showed a cytoplasmic and focal nuclear expression, while E-cadherin membranous expression was decreased in the same cell population (Fig. 3b, c, e, and f).

Table 1 Summary of clinical and histological features of CCS

Case	Age	Gender	Localization	Clinical diagnosis	Epithelial component	Mesenchymal component	Heterologous elements
1	92	F	Left axilla	BCC	SCC	Spindle cell, sarcomatous	Focal rhabdomyosarcomatous
2	90	M	Scalp	SCC	SCC	Spindle cell, sarcomatous	None
3	83	M	Forehead	SCC	SCC	Spindle cell, sarcomatous	None
4	54	M	Back	BCC	BCC	Spindle cell, sarcomatous	Osteosarcomatous
5	73	M	Back	BCC	BCC	Spindle cell, sarcomatous	Osteosarcomatous
6	59	M	Scalp	BCC	BCC	Spindle cell, sarcomatous	High grade leiomyosarcoma

Molecular findings

Mutational analysis revealed a point mutation affecting the *TP53* gene with a resulting encoded amino acid change from cysteine to tyrosine (p.Cys135Tyr) in both epithelial and stromal tumor components of one of the cases of SCC-derived CCS (case 1). The two other SCC-derived CCSs exhibited an identical 11-bp deletion in exon 6 of the *TP*53 gene amongst both components of these tumors (cases 2 and 3). However, these 11-bp deletions in exon 6 were not recognized by the IT variant caller software, thus requiring subsequent Sanger confirmation (Fig. 4). The fact that this deletion was detected in the sequence information on both of the aforementioned cases but was not by the IT variant caller software is not surprising. In fact, the mutation-calling algorithm for the IT-PGM platform is intended to detect single nucleotide mutations within gene hot spots rather than large insertions/deletions [18].

Interestingly, BCC-derived CCS (cases 4, 5, and 6) showed more complex mutations affecting numerous genes including *PIK3CA* (E545K GAA > AAA/D350N GAC > AAC), *PDGFRα* (E556K GAA > AAA), *CDKN2A* (p.r58* GCC > ACC or CGG > TGG), *APC* (p.P1361L CCC > CTC), *KDR* (p.R961W GCC > ACC or CGG > TGG), and *SMARCB1* (c.489_490delinsTA p.F164I) (Fig. 5). Furthermore, these cases harbored mutations affecting the *TP53* gene but different from those identified in case 1 (p.R196* GCT > ACT or CGA > TGA and P278L CCT > CTT) (Table 3). When tissue laser

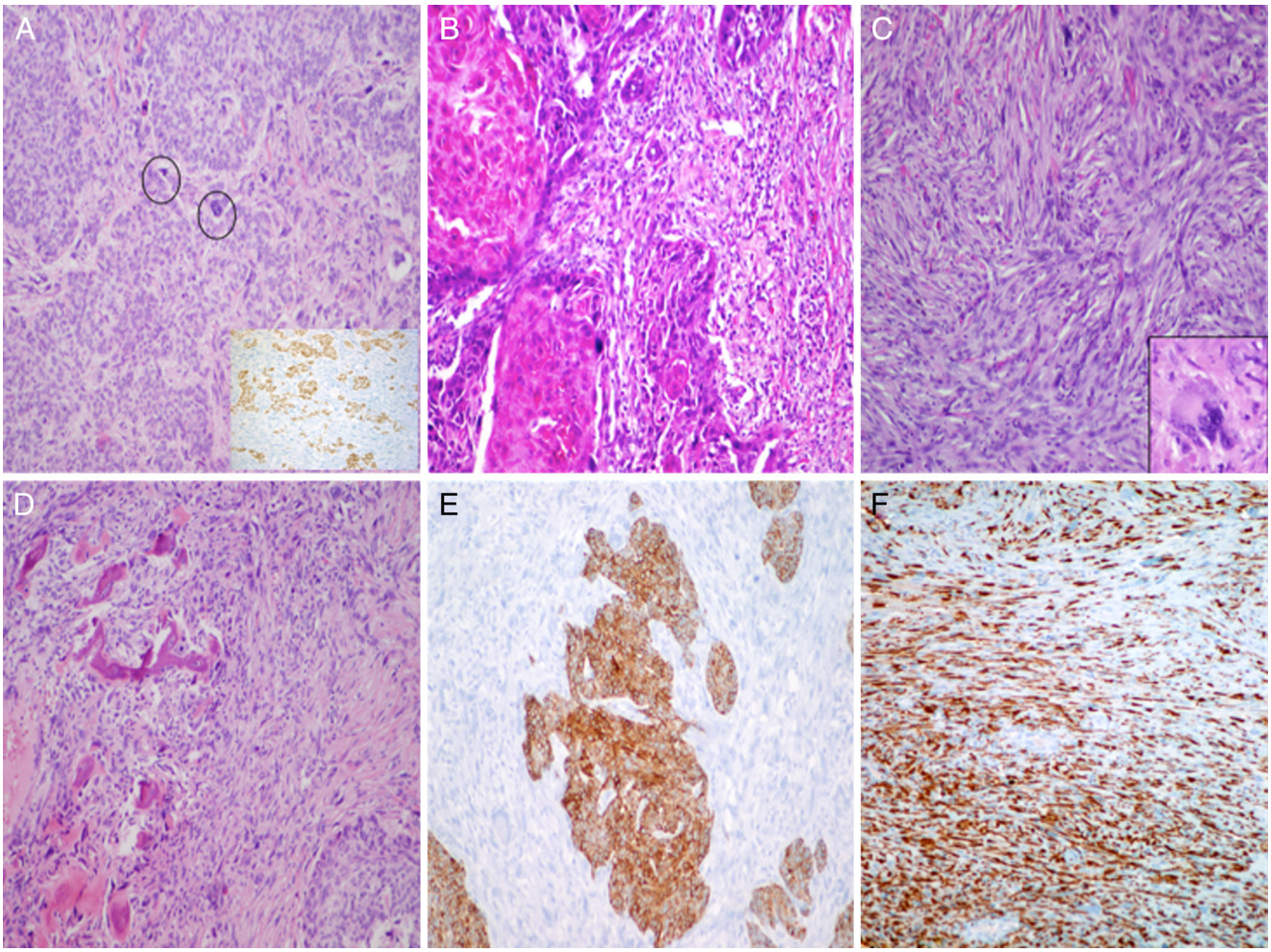

Fig. 1 **a**–**f** Hematoxylin-eosin stained sections. **a**, **b** 10X showing malignant epithelial islands consisting of basal cell carcinoma and squamous cell carcinoma, respectively, with intermediate cells showing dark bizarre-shaped nuclei at the epithelial–stromal interface (*circles*); *inset* demonstrates p63 expression within the epithelial component and transitional cells but not in the mesenchymal component. **c** 10X showing malignant stromal component with atypical spindle cells and osteoclast-like cells (*inset*). **d** 10X, highlighting the heterologous osteosarcomatous areas identified in both cases 4 and 5. **e** epithelial markers, 10X showing strong diffuse membranous and cytoplasmic reactivity with pankeratin in the malignant epithelial component. **f** mesenchymal/stromal markers, 10 X showing diffuse membranous and cytoplasmic immunoreactivity with vimentin in the mesenchymal component

Table 2 Summary of immunohistochemical features of CCS

Case	Epithelial component	Mesenchymal component	Intermediate/interface cell
1	k903+, panK+, EpCAM+, p53+, p63+	Vimentin+, factor XIIIa+, SMA + weak, EpCAM+, p53+ weak, p63−	CD34+, CD117+, bcl2+, k19+, p53−, p63+
2	k903+, panK+, EpCAM+, p53+ weak, p63+	Vimentin+, factor XIIIa+, SMA + weak, EpCAM+, p53−, p63−	CD34+, CD117+, bcl2+, k19+, p53−, p63+
3	k903+, panK+, EpCAM+, p53+ weak, p63+	Vimentin+, factor XIIIa+, SMA + weak, EpCAM+, p53−, p63−	CD34+, CD117+, bcl2+, k19+, p53−, p63+
4	k903+, panK+, EpCAM+, p53+, p63+	Vimentin+, factor XIIIa+, SMA + weak, EpCAM+, p53+ weak, p63−	CD34+, CD117+, bcl2+, k19+, p53−, p63+
5	k903+, panK+, EpCAM+, p53+, p63+	Vimentin+, factor XIIIa+, SMA + weak, EpCAM+, p53−, p63−	CD34+, CD117+, bcl2+, k19+, p53−, p63+
6	k903+, panK+, EpCAM+, p53+, p63+	Vimentin+, factor XIIIa+, SMA + weak, EpCAM+, p53+ weak, p63−	CD34+, CD117+, bcl2+, k19+, p53−, p63+

microdissection was possible (cases 1, 2, 3, and 6), all identified mutations were consistently identical in between the epithelial, stromal, and whole tumor samples subjected to next-generation sequencing. Cases 4 and 5 were small punch biopsies with very intimately admixed epithelial and mesenchymal components rendering microdissection of the different components impossible.

Discussion

Carcinosarcomas are a group of biphenotypic tumors which simultaneously express both epithelial and mesenchymal elements and which have been described to occur in a variety of anatomical sites, such as the urogenital and gastrointestinal tracts, breast, lung, thymus, and thyroid [14–16, 20–24].

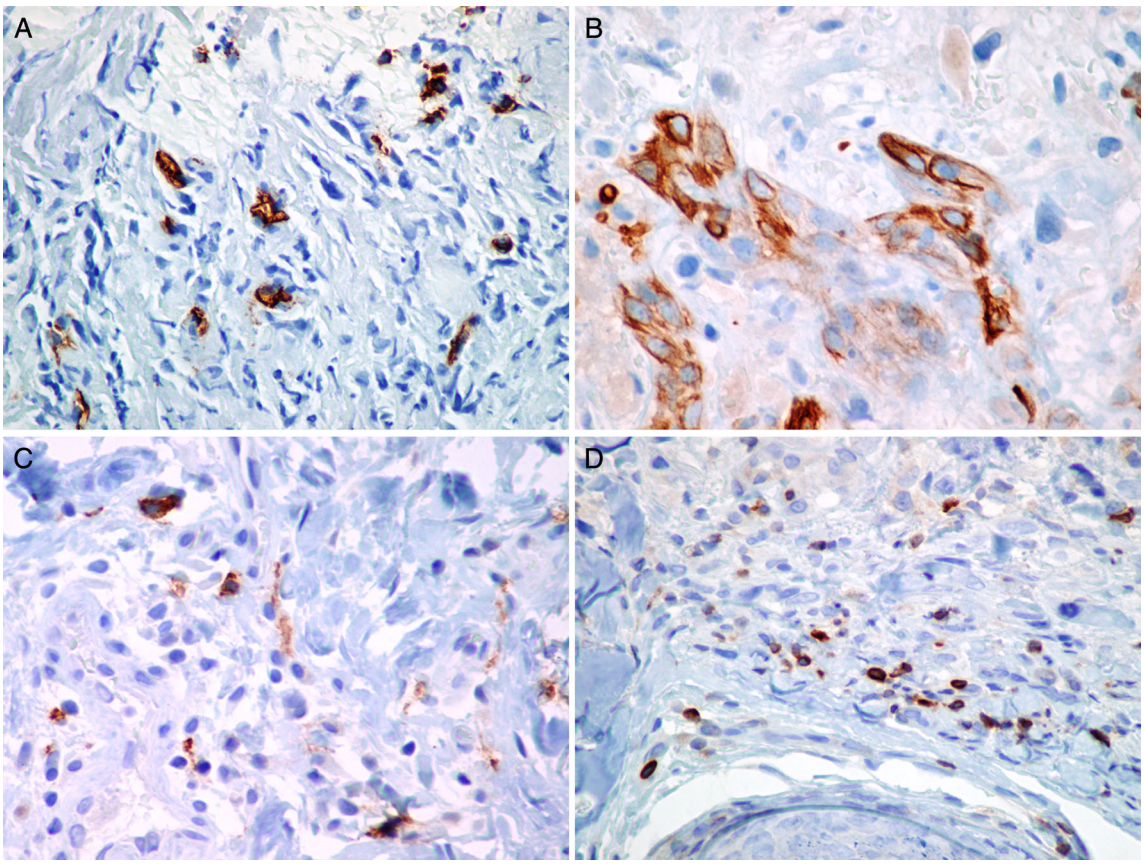

Fig. 2 **a**–**d** Putative stem cell markers. **a** 40X showing strong diffuse membranous reactivity with CD34 immunostain in the atypical intermediate cells at the epithelial/stromal interface. **b** 40X showing strong membranous and cytoplasmic reactivity in the same cell population as in (**a**) with K19 immunostain. **c** 40X showing strong membranous immunoreactivity with CD 117/C-kit in the same cell population as in (**a**). **d** 40X highlighting strong nuclear reactivity with BCL2 immunostain in the same cell population as in (**a**)

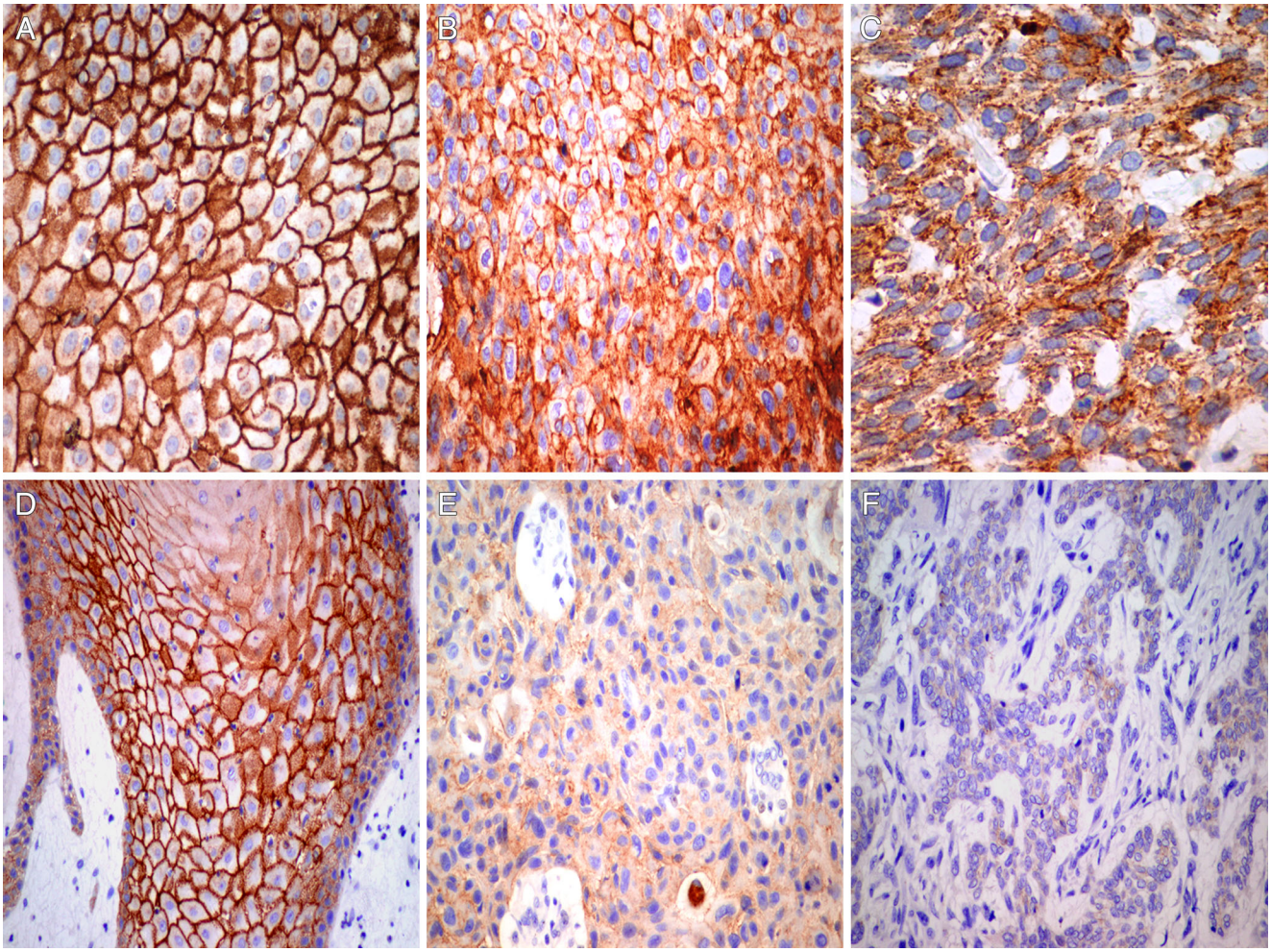

Fig. 3 **a**–**f** Epithelial–mesenchymal transition markers. **a** 20X expression of membranous β-catenin was noted in normal epithelium. **b**, **c** 20X increased expression of cytoplasmic β-catenin was seen in the malignant cells in SCC- and BCC-derived cases, respectively. **d** 20X expression of membranous E-cadherin was seen in normal epithelium. **e**, **f** 20X showing decreased membranous expression of E-cadherin in the malignant cells in both SCC- and BCC-derived cases, respectively (case 2 and case 4 are shown here)

Primary CCSs are extremely rare, and their nomenclature has varied through time under a variety of descriptive terms relating the heterogeneous morphology of these tumors. Metaplastic carcinoma, sarcomatous carcinoma, and pseudosarcoma or biphasic sarcomatoid carcinomas are among the many names used to describe CCS [6, 25]. The general morphological features show an admixture of carcinomatous and sarcomatous components [6, 26], which may be accompanied by a diversity of heterologous features such as osteoblastic, chondroblastic, myofibroblastic, angiosarcomatous, and fibrosarcomatous elements amongst others [2, 6, 14]. Cutaneous carcinosarcomas are broadly classified as adnexal-derived or epithelial-derived carcinosarcomas [5, 7, 27, 28], with adnexal-derived tumors depicting features of porocarcinomas [4], matrical carcinomas [29], spiradenocarcinoma [30–32], and proliferating tricholemmal cystic carcinoma [33–35], and with epithelial-derived tumors showing features of squamous or basal cell carcinoma [6]. While adnexal-derived tumors exhibit a poor 5-year disease-free survival rate, epidermal-derived tumors appear to have a disease-free survival rate near 70 % [5]. Nevertheless, overall recurrence and metastasis rate for these tumors are around 22 % with a mortality rate of 11 % [5].

To date, very little is known about the biology of these tumors, and the mechanisms involved in the progression of this complex malignancy remain yet to be elucidated. Many theories have emerged in an attempt to explain the histogenesis of CCS [2, 4]. The collision theory in which two synchronously occurring distinct tumors collide has been proposed by some authors, and while possible for some cases, it does not explain those cases rich in a variety of heterologous elements [2, 4, 6]. A second theory sustains that such tumors may arise from de-differentiation of an established malignancy [2, 4]. A third theory raises the possibility that the mesenchymal component observed in these tumors is nothing else but a reactive "pseudosarcomatous" stromal change to the malignant epithelial transformation; however, the reported

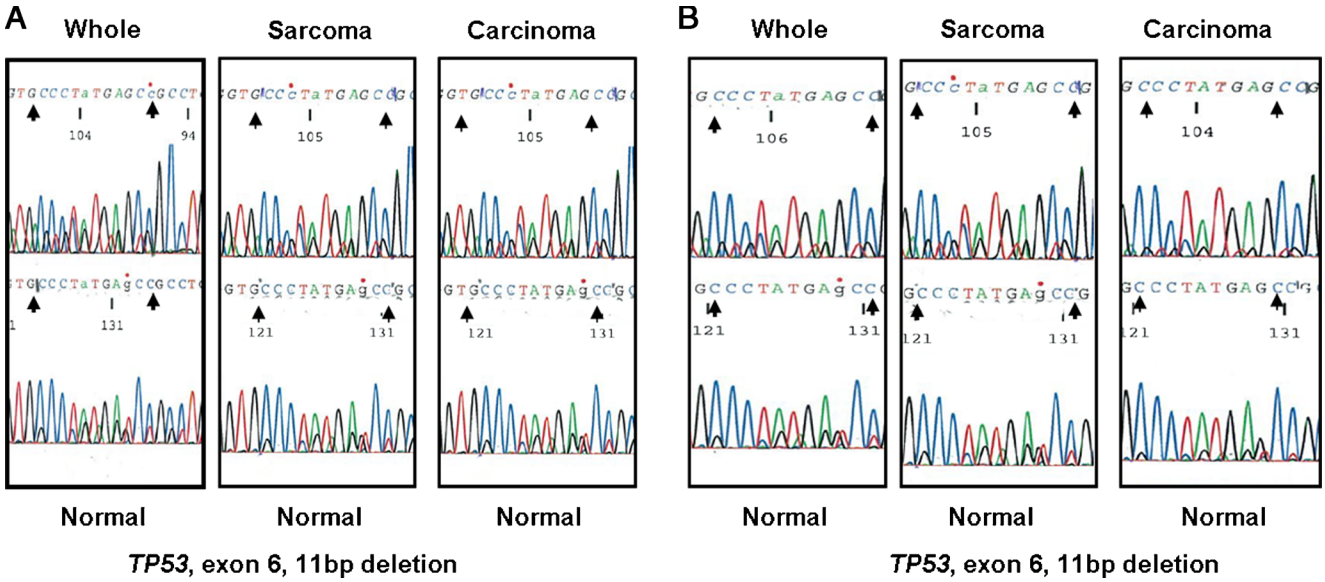

Fig. 4 Summary of the Sanger sequencing in case 2 (**a**) and 3 (**b**) showing the 11 base pair deletion in exon 6 of the *TP53* gene. The same deletion is noted in the whole tumor, microdissected sarcoma and carcinoma, respectively. *Upper arrows* point to the sequence position in the tumoral tissue in comparison with the position of the sequence in control normal tissue (*lower arrows*)

capacity to metastasize for the mesenchymal component argues against this theory [2, 4]. The fourth theory sustains that both the epithelial and mesenchymal components arise from a common progenitor cells which then undergoes a biphenotypic differentiation [2, 4, 6]. This aforementioned stem cell theory is further divided into two plausible pathways based on whether differentiation occurs from two or more stem cells (the "convergence" or multiclonal hypothesis) or from a single totipotential cell undergoing divergent differentiation to different cell lineages (the "divergence" or monoclonal hypothesis) [2, 4]. There is increasing histological and molecular evidence that extracutaneous carcinosarcomas elsewhere than the skin are monoclonal in origin [16, 36]. Such is the case for example, of metaplastic carcinoma of the breast in which the epithelial- and mesenchymal-derived elements were noticed to share the same *TP53* mutation, suggesting its possible origin from a single totipotent cell [37, 38]. Similar results were revealed in a study performed by Armstrong et al. in which the authors were able to demonstrate identical *TP53* mutations on both the epithelial and sarcomatoid components of a series of cases of urothelial carcinosarcomas [16]. Moreover, in a recent study performed by our group, an analysis of the distinct laser capture-microdissected tumor components from a case of primary cutaneous carcinosarcoma also revealed point mutations of *TP53* which were identical in both the epithelial and sarcomatous components, with concordantly aberrant p53 protein overexpression on immunohistochemical studies [39]. This finding along with the shared immunoreactivity with putative stem cell markers in a population of intermediate cells at the epithelial–mesenchymal interface, as well as the presence of chimeric cells (cells with evidence of both epithelial and mesenchymal differentiation by ultrastructural studies) strongly suggested a monoclonal origin of the tumor [39].

In our study, we performed next-generation sequencing of 47 target genes which revealed differing mutational landscapes for the two main epithelial-derived carcinosarcoma morphotypes (squamous cell and basal cell). While all carcinosarcomas exhibiting an SCC epithelial component exhibited diverse point mutations and deletions in the *TP53* gene, those with a BCC morphotype revealed a more complex mutational landscape involving several genes (Table 3). In our series, finding *TP53* variants in all squamous cell-derived carcinosarcomas (cases 1, 2, and 3) is not a surprising fact. Indeed, p53 is mutated in most keratinocyte-derived carcinomas especially in SCC (90 % of SCC identified in the USA contain at least one p53 mutation throughout the tumor) [40, 41]. The underlying ultraviolet (UV) mutational signature of *TP53* along with the clinical profile (elderly individuals, sun-exposed areas of head and neck) correlate with the pathogenesis of SCC [40, 41], in which a subset of cells that could later have undergone clonal expansion and differentiation to the sarcomatous component. On the other hand, the heterogeneous mutational pattern seen in BCC-derived CCS (cases 4, 5, and 6) correlates with the presence of heterologous elements within these tumors [12]. Interestingly, both BCC-derived CCS cases with osteosarcomatous differentiation showed missense mutations involving PDGFR-α gene [42]. Previously, PDGFR-α has been shown to participate in matrix metalloproteinase-13 (MMP-13) expression when induced by mechanical strain in the cells [43, 44]. These findings suggest that these activating

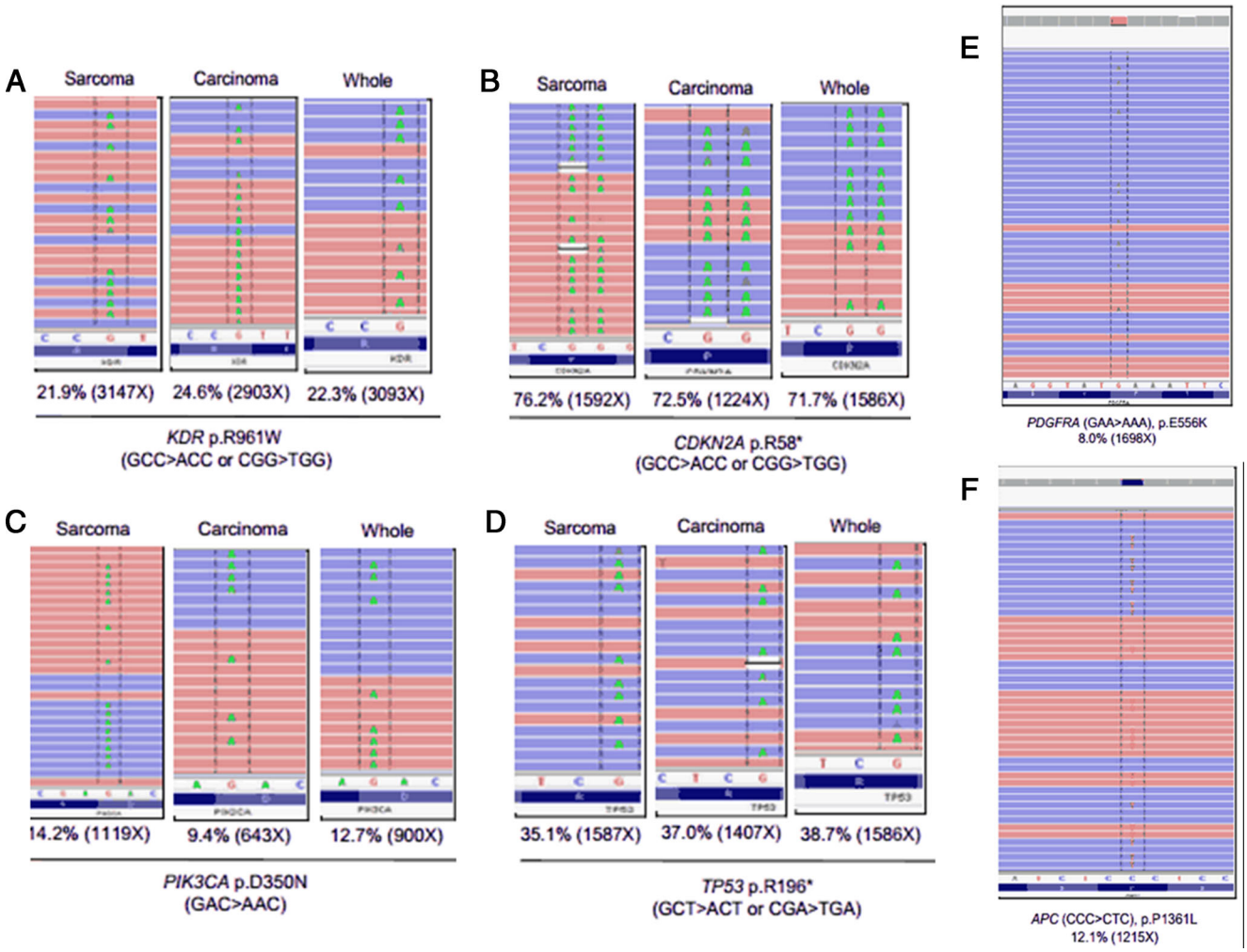

Fig. 5 **a**–**e** Capture of the IGV screen highlighting selected point mutations detected in different cases. Each *column* represents a separate microdissected component (cases 4 and 5 have one *column* only as no microdissection was performed). The frequency of the detected particular mutation in the background of the reference genome is listed below the *column*. The depth of coverage is given in brackets next to it. **a** Point mutation in the *KDR* gene leading to a substitution of arginine to tryptophan residue at position 961 (p.R961W) (case 6). **b** Another missense mutation in the *CDKN2A* gene leading to a stop codon at position 58 (p.R58*) and unstable mRNA with protein decay (case 6). **c** A point mutation in the *PIK3CA* gene leading to a substitution of aspartic acid to asaparagin residue at position 350 (p.D350N) (case 6). Another missense mutation in the *TP53* gene leading to a stop codon at position 196 (p.R196*) and unstable mRNA with protein decay (case 6). **d** A point mutation in the *PDGFRA* gene leading to a substitution of glutamic acid to lysine residue at position 556 (p.E556K) (case 4). **f** A point mutation in the *APC* gene leading to a substitution of proline to leucine residue at position 1361 (p.P1361L) (case 5)

PDGFR-α mutations may have a role in the bone matrix deposition leading to osteosarcomatous heterologous differentiation. When microdissection was possible (cases 1, 2, 3, and 6), identical mutations were constantly identified in all tumor components (Table 3). These findings strongly suggest a monoclonal origin for these tumors. This is further supported by the positive immunoreactivity with putative stem cell markers (CD34+, CD117+, bcl2+, k19+) within the group of intermediate cells located in the epithelial mesenchymal interface.

The presence or absence of overlapping mutations amongst the different components (as seen in case 6 with *SMARCB1*) could be interpreted as a sign of further progression and divergence, in which one component of the tumor can independently acquire subsequent alterations during the course of tumorigenesis. However, because the majority of the mutations in all studied cases were evenly distributed across both histological elements, our results are most consistent with the divergent theory, in which carcinosarcomas arise from a monoclonal stem cell population to undergo subsequent differentiation into different cell lineages.

Amongst those mutations observed in the more complex landscape of BCC-derived CCS, two (*PDGFR*-α and *PIK3CA*) are of particular interest due to their pivotal role within cellular pathways and their susceptibility for novel target therapies. Platelet-derived growth factor receptors (PDGFRs) are catalytic receptors with intracellular tyrosine kinase activity [45] and are known to play an important role in

Table 3 Summary of mutational analysis of CSS using NGS

Case	Component	Variant	Frequency %	Coverage
Case 1	Epithelial/carcinoma	*TP53*C135Y TGC → TAC	30.67	600x
		KDR Q472H* CAA → CAT	56	1,449x
	Mesenchymal/sarcoma	*TP53*C135Y TGC → TAC	27.0	916x
		KDR Q472H* CAA → CAT	51.9	1,776x
	Whole tumor	*TP53* C135Y TGC → TAC	20.92	736x
		KDR Q472H* CAA → CAT	50.16	2,175x
Case 2	Epithelial/carcinoma	*TP53*exon 6, 11-bp deletion	NA	NA
	Mesenchymal/sarcoma	*TP53*exon 6, 11-bp deletion	NA	NA
	Whole tumor	*TP53*exon 6, 11-bp deletion	NA	NA
Case 3	Epithelial/carcinoma	*TP53*exon 6, 11-bp deletion	NA	NA
	Mesenchymal/sarcoma	*TP53*exon 6, 11-bp deletion	NA	NA
	Whole tumor	*TP53*exon 6, 11-bp deletion	NA	NA
Case 4	Whole tumor	*TP53*P278L CCT → CTT	36.95	203x
		*PIK3CA*E545K GAA → AAA	14.13	2,640x
		*PDGFRA*E556K GAA → AAA	6.24	1,215x
		*MET*N375S* AAC → AGC	65.32	3,019x
Case 5	Whole tumor	*PDGFRA*E556K GAA → AAA	8.07	1,698x
		*APC*P1361L CCC → CTC	12.18	1,215x
		MET N375S AAC →AGG	56.84	6,721x
Case 6	Epithelial/carcinoma	*SMARCB1* c.489_490delinsTA p.F164I	17.65	1,728x
		*PIK3CA*D350N GAC → AAC	9.49	643x
		KDR R961W CGG → TGG	24.61	2,903x
		CDKN2A c.171_172delinsTT p.R58*	72.55	1,224x
		TP53 c.585_586delinsTT p.R196*	37.03	1,407x
	Mesenchymal/sarcoma	*PIK3CA* D350N GAC → AAC	14.21	1,119x
		KDR R961W CGG → TGG	21.99	3,147x
		CDKN2A c.171_172delinsTT p.R58*	76.26	1,592x
		TP53 c.585_586delinsTT p.R196*	35.16	1,587x
	Whole tumor	*SMARCB1* c.489_490delinsTA p.F164I	7.47	2,154x
		PIK3CA D350N GAC → AAC	12.78	900x
		KDR R961W CGG → TGG	22.37	9,093x
		CDKN2A c.171_172delinsTT p.R58*	71.75	1,596x
		TP53 c.585_586delinsTT p.R196	38.78	1,566x

cell proliferation and differentiation of mesenchymal elements [46]. Moreover, PDGF production by all morphotypes across the BCC spectrum as well as its presence in associated stromal receptors suggests that the PDGF/PDGF receptor interplay may play a role in BCC progression [42]. To date, upregulation of the sonic hedgehog (SHH) signaling pathway has been the hallmark of BCC pathogenesis [47, 48], but its close relation with other pathways is also gaining relevance in the complex tumorigenic mechanisms behind BCC formation [42]. In a study by Xie et al., the authors showed the role of Gli1, a downstream player controlled by the smoothened (SMO) in activating PDGFR-α which, in turn, activates the Ras-ERK pathway leading to cell proliferation [42, 49]. This sequence of events correlates with the high levels of expression of PDGFR-α in BCCs, seen both in animals and humans [42, 49]. The identified mutations in PDGFR-α gene in two of our cases (4 and 5) provide further evidence on hedgehog signaling-mediated tumor development as well as to the expression of a BCC-permissive stroma [42, 50]. Most importantly, the data suggest that targeted inhibitors against PDGFR-α may have a role in inhibiting the progression of the BCC epithelial component in CCS. On the other hand, it is known that mutations in the *PIK3CA* and *AKT1* genes can cause activation of the PI3K/AKT pathway which has been linked to malignant transformation and behavior of affected cells in both SCC and BCC [51, 52], as well as in Merkel cell carcinoma [53]. In the skin, *PIK3CA* mutations have also been reported in a variety of benign entities such as epidermal nevi,

seborrheic keratoses [54], benign lichenoid keratosis [55], and other lesions such as verrucous keratosis [53, 56]. The presence of specific *PIK3CA* mutations in two of our cases (4 and 6) is of utmost importance since the PI3K/AKT signaling pathway represents a target for specific inhibitors [57]. Interestingly, in case 4 of our series, two targetable mutations in two different pathways (PDGFR-α and PIK3CA) were identified simultaneously. This finding can be explained by tumor heterogeneity [58, 59]. This phenomenon is well known in breast and gastric cancers as well as glioblastomas essentially when dealing with small biopsy samples [58–61]. Within the same tumor fragment, different subclones of cells harbor heterogeneous complex mutations affecting genes from different pathways or variable DNA copy number [62]. This tumor heterogeneity represents a specific signature that can be thought of as an evolutionary process fostering tumor adaptation and compromising the efficacy of targeted therapies [58–62]. Yet, this conclusion should be drawn very cautiously and should be further consolidated given our limited number of cases (only one case) and the important clinical impact.

Epithelial to mesenchymal transition (EMT) is a process by which malignant transformation in many carcinomas is associated with the loss of epithelial differentiation and gain of a mesenchymal phenotype [63, 64]. It has been described in many tumors including oral squamous cell carcinomas [65, 66]. EMT involves different trends and patterns of expression of many markers such E-cadherin, β-catenin, and vimentin, all of which are related to alterations affecting the WNT1 signaling pathway [66–68]. The loss of E-cadherin expression together with the upregulation of vimentin expression is known to be a hallmark of EMT changes in epithelial cells [66, 67]. Interestingly, all our cases showed a decreased membranous immunoreactivity with β-catenin in the malignant cells with an increased cytoplasmic β-catenin expression when compared to the normal epithelium. On the other hand, there was a significant decreased expression of E-cadherin in between the malignant components in comparison to the benign epithelium. Vimentin was strongly expressed in the mesenchymal component but not in the epithelial components. Our findings are in line with previously published literature examining the pattern of expression of different markers involved in EMT [66]. As such, one can conclude that CCSs are an additional group of carcinomas whose tumorigenesis may involve an EMT process.

In summary, CCS is a rare and heterogeneous group of tumors with specific underlying molecular signature correlating with the epithelial morphotype (BCC or SCC). These molecular events represent promising targets for personalized therapies. Although novel and promising, our findings need to be validated through further large studies.

Conflict of interest The authors declare no conflict of interest.

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