AperTO - Archivio Istituzionale Open Access dell'Università di Torino

Sarcomatoid adrenocortical carcinoma: a comprehensive pathological, immunohistochemical, and targeted next-generation sequencing analysis

This is a pre print version of the following article:

Original Citation:

Availability:

This version is available <http://hdl.handle.net/2318/1622115> since 2018-11-16T12:12:38Z

Published version:

DOI:10.1016/j.humpath.2016.08.006

Terms of use:

Open Access

Anyone can freely access the full text of works made available as "Open Access". Works made available under a Creative Commons license can be used according to the terms and conditions of said license. Use of all other works requires consent of the right holder (author or publisher) if not exempted from copyright protection by the applicable law.

(Article begins on next page)

Manuscript Number: YHUPA-D-16-00233R1

Title: Sarcomatoid Adrenocortical Carcinoma: A Comprehensive Pathological, Immunohistochemical and Targeted Next-Generation Sequencing Analysis.

Article Type: Original Article

Keywords: adrenocortical carcinoma; sarcomatoid; mutations; epithelial-mesenchymal transition; nestin

Corresponding Author: Dr. Thomas Papathomas,

Corresponding Author's Institution: King's College Hospital

First Author: Thomas Papathomas

Order of Authors: Thomas Papathomas; Eleonora Duregon ; Esther Korpershoek ; David Restuccia ; Ronald van Marion; Rocco Cappellesso ; Nathalie Sturm; Giulio Rossi ; Antonella Coli ; Nicola Zucchini ; Hans Stoop ; Wolter Oosterhuis ; Laura Ventura ; Marco Volante ; Ambrogio Fassina ; Winand Dinjens ; Mauro Papotti ; Ronald de Krijger

Abstract: Adrenocortical carcinomas (ACCs) with sarcomatous areas represent an extremely rare type of highly aggressive malignancy of unknown molecular pathogenesis. The current study was planned to gain insight into its molecular genetics using a targeted next-generation sequencing approach and to explore the status of epithelial-mesenchymal transition (EMT)-associated markers (E-/P-/N-Cadherins, MMP-2/-9 and Caveolin-1), downstream transcriptional regulators of EMT-related signaling pathways (ZEB-1/-2, Slug), stem cell factors (Oct3/4, LIN28, SOX2, SO17, NANOG, CD133, nestin) and markers of adrenocortical origin/tumorigenesis (SF-1, β -catenin, p53) in phenotypically diverse tumor components of six cases. Thirteen pathogenic variants of ACC-associated TP53 and CTNNB1 genes were detected in epithelial and/or non-epithelial components in 4 out of 6 tumors. Three cases had identical mutations in distinct components, one of which containing TP53/CTNNB1 in 3 out of 5 components, while one harbored a single TP53 mutation only in the non-epithelial component. By immunohistochemistry, SF-1 and E-/P-/N-Cadherins were found positive only in the epithelial component of all cases, whereas the non-epithelial components were mainly enriched for nestin, ZEB-1 and MMP-2/-9. β -catenin demonstrated an aberrant nuclear localization in the sarcomatoid component of 5 cases, while p53 was strongly positive in non-epithelial constituent in 4 of 6 cases. In summary, we have shown that Wnt/ β -catenin signaling pathway dysregulation and mutational inactivation of TP53 are common genetic events in sarcomatoid ACCs, a subset of which being monoclonal in origin. These tumors are enriched for EMT-related markers and stem cell factors, potentially conferring a poor prognosis, which might be exploited as novel therapeutic targets.

To whom it may concern,

I clearly state that this **manuscript, or parts of it** (*entitled* Sarcomatoid Adrenocortical Carcinoma: A Comprehensive Pathological, Immunohistochemical and Targeted Next-Generation Sequencing Analysis), **have not been and will not be submitted elsewhere for publication. All authors have read and approved the manuscript.** Attached you will find the relevant document as well.

I am looking forward to hearing from you soon.

Kind Regards,

Thomas Papathomas, MD

Please wait...

If this message is not eventually replaced by the proper contents of the document, your PDF viewer may not be able to display this type of document.

You can upgrade to the latest version of Adobe Reader for Windows®, Mac, or Linux® by visiting http://www.adobe.com/go/reader_download.

For more assistance with Adobe Reader visit <http://www.adobe.com/go/acrreader>.

Windows is either a registered trademark or a trademark of Microsoft Corporation in the United States and/or other countries. Mac is a trademark of Apple Inc., registered in the United States and other countries. Linux is the registered trademark of Linus Torvalds in the U.S. and other countries.

Dear Sir,

Please find enclosed our manuscript entitled: Sarcomatoid Adrenocortical Carcinoma: A Comprehensive Pathological, Immunohistochemical and Targeted Next-Generation Sequencing Analysis, by Papathomas *et al.*

We think this would be a study with high relevance to the endocrine pathology community and indeed the entire pathology community, as it unravels the unknown molecular pathogenesis of Adrenocortical carcinomas (ACCs) with sarcomatous areas, an extremely rare type of highly aggressive malignancy, by a targeted next-generation sequencing (NGS) approach and an immunohistochemical investigation of epithelial-mesenchymal transition (EMT)-associated markers (E-/P-/N-Cadherins, MMP-2/-9 and Caveolin-1), downstream transcriptional regulators of EMT-related signaling pathways (ZEB-1/-2, Slug), stem cell factors (Oct3/4, LIN28, SOX2, SO17, NANOG, CD133, nestin) and markers of adrenocortical origin/ tumorigenesis (SF-1, β -catenin, p53) in phenotypically diverse tumor components of six cases.

Highlights are summarized, as follows:

(1) Wnt/ β -catenin signaling pathway dysregulation is common genetic event in sarcomatoid ACCs

(2) mutational *TP53* inactivation is common genetic event in sarcomatoid ACCs

(3) monoclonal evolution is displayed at least in a subset of sarcomatoid ACCs

(4) sarcomatoid ACCs are enriched for EMT-related markers and stem cell factors

We feel this paper would be of interest to the broad readership of your journal and would attract ample attention in the field of pathology and beyond.

We are looking forward to hearing from you soon.

Best Regards,

Thomas Papathomas, M.D.

Dear Editor,

First, I would like to thank you for your interest our manuscript number YHUPA-D-16-00233 *entitled* “Sarcomatoid Adrenocortical Carcinoma: A Comprehensive Pathological, Immunohistochemical and Targeted Next-Generation Sequencing Analysis”

In this context, I would like to state with regard to the Editor comments, as annotated in red:

Editors:

1. The references are generally limited to 35 total. Please choose between eliminating nonessential references or offering a rationale for exceeding the limit. Also, please update reference #35 when more information becomes available.

(1) The reference #35 has been updated as suggested.

(2) we kept the references despite the fact the limit is exceeded given our approach to provide a comprehensive review of all reported sarcomatoid ACCs and a required body of the literature referring not only to similar molecular techniques, but also to immunohistochemical investigations on EMT and stem cell factors. We feel that in this way our current research work is additionally highlighted and strengthened.

2. Please condense abstract to meet our 250-word limit.

This is now amended (word count 247).

3. Please note that the highlights should consist of only 3-5 bulleted key points, each no more than 85 characters. Please condense the highlights provided. More information can be found here: <http://www.elsevier.com/journal-authors/highlights>.

This is now amended, as follows:

(1) Wnt/ β -catenin signaling pathway dysregulation is common genetic event in sarcomatoid ACCs

(2) mutational *TP53* inactivation is common genetic event in sarcomatoid ACCs

(3) monoclonal evolution is displayed at least in a subset of sarcomatoid ACCs

(4) sarcomatoid ACCs are enriched for EMT-related markers and stem cell factors

4. The addition of author degrees, conflict of interest disclosure and a running head (condensed version of full title limited to 60 characters) on the title page would be helpful.

This is now amended in Pages 1-3.

5. Please use superscript letters not symbols to label table footnotes.

This is now amended in all tables.

6. Consider adding magnification information to figure legends where appropriate.

Given that all figures have been extracted from scanned virtual slides, the magnification information has been automatically preserved at the left bottom of each figure.

Second, I would like to point out all of the amendments according to the valuable Reviewer 2 comments as annotated in red:

1. The authors are advised to mention the proportion of sarcomatoid component in ACC and demonstrate by H&E staining the border between these two components, sarcomatoid and epithelial.

(1) In all unpublished cases (n=3), the sarcomatoid component is estimated >10%; this is now amended in Table 1; nevertheless, the exact quantification is dependent on extensive sampling; when confronted with such large tumours is very challenging to sample the tumour in its entirety and hence accurately quantify the diverse components.

(2) This is now illustrated in Figure 2.

2. Figures. Even with the better quality, the images of H&E staining is too low in magnification and very hard to observe details. The authors are advised to demonstrate the H&E images of higher power as "Insets".

This is now amended for both Figures.

3. In the cases of mixed adeno-neuroendocrine carcinoma (MANEC) in neuroendocrine neoplasms (NEN), the same mutation of p53 gene was noted in both neuroendocrine carcinoma and adenocarcinoma, which suggested the monoclonal origin for both components. As a reference, the authors may include Neuroendocrinology 2014,100:310-316. PMID: 25342539

Given the limitations concerning the number of references and as we exceeded the limit, we feel uncomfortable to add this reference.

We would really appreciate if our latest revised version is taken into your precious consideration and looking forward to hearing from you soon.

On behalf of all authors,

Thomas Papathomas MD, PhD

Title Page

Sarcomatoid Adrenocortical Carcinoma: A Comprehensive Pathological, Immunohistochemical and Targeted Next-Generation Sequencing Analysis.

Thomas G. Papathomas MD, PhD ^{1,2 *}, Eleonora Duregon MD ^{3 *}, Esther Korpershoek PhD ⁴, David F. Restuccia PhD ⁵, Ronald van Marion ⁶, Rocco Cappellesso MD ⁷, Nathalie Sturm MD, PhD ⁸, Giulio Rossi MD, PhD ⁹, Antonella Coli MD ¹⁰, Nicola Zucchini MD ¹¹, Hans Stoop PhD ¹², Wolter Oosterhuis MD, PhD ¹³, Laura Ventura PhD ¹⁴, Marco Volante MD, PhD ¹⁵, Ambrogio Fassina MD, PhD ¹⁶, Winand N.M. Dinjens PhD ¹⁷, Mauro Papotti MD, PhD ^{18 *}, Ronald R. de Krijger MD, PhD ^{19, 20}

*** equal contribution**

1. Department of Pathology, Erasmus MC Cancer Institute, University Medical Center Rotterdam, Rotterdam 3015 GE, The Netherlands. Electronic address: t.papathomas@erasmusmc.nl

2. Department of Histopathology, King's College Hospital, Denmark Hill, London SE5 9RS, UK. Electronic address: thomaspapathomas@nhs.net

3. Department of Oncology, University of Turin, Orbassano 10043, Turin, Italy. Electronic address: eleonora.duregon@unito.it

4. Department of Pathology, Erasmus MC Cancer Institute, University Medical Center Rotterdam, Rotterdam 3015 GE, The Netherlands. Electronic address: e.korpershoek.1@erasmusmc.nl

5. Department of Pathology, Erasmus MC Cancer Institute, University Medical Center Rotterdam, Rotterdam 3015 GE, The Netherlands. Electronic address: restuccia.david@gmail.com

6. Department of Pathology, Erasmus MC Cancer Institute, University Medical Center Rotterdam, Rotterdam 3015 GE, The Netherlands. Electronic address: r.vanmarion@erasmusmc.nl

7. Department of Diagnostic Medical Sciences and Special Therapies, Surgical Pathology and Cytopathology Unit, University of Padova, Padova 35121, Italy. Electronic address: rocco.cappellesso@gmail.com

8. Department of Pathology, CHU Albert Michallon, Grenoble 38043, France. Electronic address: NSturm@chu-grenoble.fr

- 38
- 39 **9. Division of Pathology, University of Modena, Modena 41100, Italy. Electronic address:**
40 rossi.giulio@policlinico.mo.it
41
- 42 **10. Division of Pathology, Policlinico Universitario Gemelli, Rome 00168, Italy. Electronic address:**
43 antonella.coli@rm.unicatt.it
44
- 45 **11. Division of Pathology, San Gerardo Hospital, Monza 20900, Italy. Electronic address:**
46 n.zucchini@hsgerardo.org
47
- 48 **12. Department of Pathology, Erasmus MC Cancer Institute, University Medical Center Rotterdam,**
49 **Rotterdam 3015 GE, The Netherlands. Electronic address:** j.stoop@erasmusmc.nl
50
- 51 **13. Department of Pathology, Erasmus MC Cancer Institute, University Medical Center Rotterdam,**
52 **Rotterdam 3015 GE, The Netherlands. Electronic address:** j.w.oosterhuis@erasmusmc.nl
53
- 54 **14. Department of Statistical Sciences, University of Padova, Padova 35121, Italy. Electronic**
55 **address:** laura.ventura@unipd.it
56
- 57 **15. Department of Oncology, University of Turin, Orbassano 10043, Turin, Italy. Electronic**
58 **address:** marco.volante@unito.it
59
- 60 **16. Department of Diagnostic Medical Sciences and Special Therapies, Surgical Pathology and**
61 **Cytopathology Unit, University of Padova, Padova 35121, Italy. Electronic address:**
62 ambrogio.fassina@unipd.it
63
- 64 **17. Department of Pathology, Erasmus MC Cancer Institute, University Medical Center Rotterdam,**
65 **Rotterdam 3015 GE, The Netherlands. Electronic address:** w.dinjens@erasmusmc.nl
66
- 67 **18. Department of Oncology, University of Turin, Orbassano 10043, Turin, Italy. Electronic**
68 **address:** mauro.papotti@unito.it
69
- 70 **19. Department of Pathology, Erasmus MC Cancer Institute, University Medical Center Rotterdam,**
71 **Rotterdam 3015 GE, The Netherlands. Electronic address:** r.dekrijger@erasmusmc.nl
72
- 73 **20. Department of Pathology, Reinier de Graaf Hospital, Delft 2625 AD, The Netherlands.**
74 **Electronic address:** r.dekrijger@erasmusmc.nl

Contact details of the corresponding author:

Thomas G. Papathomas, MD
Department of Histopathology,
King's College Hospital, Denmark Hill,
London SE5 9RS, UK
Tel: +44-7437416650
thomaspapathomas@nhs.net

Acknowledgements: Funding: This study was supported by the Seventh Framework Programme (FP7/2007-2013) under grant agreement no. 259735 (ENS@T-Cancer) and partially supported by a grant from the Italian Association for Cancer Research (AIRC, Milan; grant no. IG/14820/2013 to M.P.). D.F.R. was supported by a ParaPheoAlliance Fellowship.

Conflict of Interest/ Disclosure: The authors declare no conflict of interest.

Running Head: A Comprehensive Analysis of Sarcomatoid Adrenocortical Carcinoma

Abstract

Adrenocortical carcinomas (ACCs) with sarcomatous areas represent an extremely rare type of highly aggressive malignancy of unknown molecular pathogenesis. The current study was planned to gain insight into its molecular genetics using a targeted next-generation sequencing approach and to explore the status of epithelial-mesenchymal transition (EMT)-associated markers (E-/P-/N-Cadherins, MMP-2/-9 and Caveolin-1), downstream transcriptional regulators of EMT-related signaling pathways (ZEB-1/-2, Slug), stem cell factors (Oct3/4, LIN28, SOX2, SO17, NANOG, CD133, nestin) and markers of adrenocortical origin/ tumorigenesis (SF-1, β -catenin, p53) in phenotypically diverse tumor components of six cases. Thirteen pathogenic variants of ACC-associated *TP53* and *CTNNB1* genes were detected in epithelial and/or non-epithelial components in 4 out of 6 tumors. Three cases had identical mutations in distinct components, one of which containing *TP53/CTNNB1* in 3 out of 5 components, while one harbored a single *TP53* mutation only in the non-epithelial component. By immunohistochemistry, SF-1 and E-/P-/N-Cadherins were found positive only in the epithelial component of all cases, whereas the non-epithelial components were mainly enriched for nestin, ZEB-1 and MMP-2/-9. β -catenin demonstrated an aberrant nuclear localization in the sarcomatoid component of 5 cases, while p53 was strongly positive in non-epithelial constituent in 4 of 6 cases. In summary, we have shown that Wnt/ β -catenin signaling pathway dysregulation and mutational inactivation of *TP53* are common genetic events in sarcomatoid ACCs, a subset of which being monoclonal in origin. These tumors are enriched for EMT-related markers and stem cell factors, potentially conferring a poor prognosis, which might be exploited as novel therapeutic targets.

Keywords: adrenocortical carcinoma; sarcomatoid; mutations; epithelial-mesenchymal transition; nestin

Introduction

Adrenocortical carcinomas (ACCs) with sarcomatous areas represent an extremely rare type of malignancy being the least common ACC variant following oncocytic and myxoid ACCs in decreasing order of frequency [1]. As a matter of fact, only 16 cases have been previously published, all of which as single case reports [2-17]. ACCs with sarcomatous areas have been variably designated carcinosarcomas or sarcomatoid carcinomas due to the presence of a specialized mesenchymal component, including osteosarcomatous, chondrosarcomatous or rhabdomyosarcomatous differentiation, or devoid of specific differentiation, respectively [2-17]. Nonetheless, this variant is characterized by a highly aggressive biologic behaviour and a worse prognosis in comparison with its conventional counterparts [1]. The question whether this aggressive clinical course may be attributed not only to advanced stage at presentation, but also to inherent biologic properties, still remains unanswered.

Epithelial-mesenchymal transition (EMT) is a key developmental program that can be aberrantly activated during tumor progression, endowing cells with invasive and migratory properties, inducing stem cell properties, preventing apoptosis and senescence and contributing to chemo-resistance and immunosuppression [18]. During tumor progression, epithelial cells are driven towards a mesenchymal state, which subsequently enables them to evade hostile microenvironments generated by hypoxia, mechanical constraints and/or nutrient deprivation [19]. In this context, it has been shown that the induction of EMT endows breast epithelial cells with stem cell traits [20], while these EMT-derived cells share similarities with mesenchymal stem cells in terms of gene expression, multilineage differentiation, and ability to migrate towards tumor cells and wound sites [21]. Despite these lines of experimental evidence implying a link between EMT and cancer stem cells (CSCs), it still remains unsettled whether CSCs derive from EMT-induced differentiated cancer cells or from transformed tissue-specific stem cells [22]. Immunohistochemical expression of EMT markers has been documented in sarcomatoid carcinomas/carcinosarcomas [23-25], implying a potential role for EMT in such tumors.

The purpose of the current study was to gain insight into the molecular genetics of this rare ACC variant by using a targeted next-generation sequencing (NGS) approach. In addition, we investigated the expression of EMT-associated markers, downstream transcriptional regulators of EMT-related signaling pathways and stem cell factors in morphologically diverse tumor components of sarcomatoid ACCs.

Materials & Methods

Case series

Six sarcomatoid ACCs were collected from five Departments of Pathology, three of which had previously been reported from the clinico-pathological viewpoint [8-9, 11]. Detailed clinico-pathological data of all cases included in the current study are summarized in **Table 1**. All cases were reviewed by two investigators (TP/ED) to identify the sarcomatoid areas. The study received ethical approval from the local Institutional Review Boards.

Tissue preparation and immunohistochemistry (IHC)

Sections serial to those used for conventional hematoxylin-eosin were obtained from one or two representative formalin-fixed paraffin-embedded (FFPE) blocks. The following markers were evaluated: (i) general ones of adrenocortical origin and differential diagnosis: pan-cytokeratin AE1/AE3, vimentin, Steroidogenic Factor 1 (SF-1), melan A, α -inhibin, synaptophysin, chromogranin A, neuron specific enolase (NSE), CD56 & Ki-67; (ii) markers involved in ACC tumorigenesis: β -catenin & p53; (iii) EMT-related markers: E-Cadherin, P-Cadherin, N-Cadherin, Matrix-Metalloproteinases (MMP)-2, MMP-9 & Caveolin-1; (iv) downstream transcriptional regulators of EMT-related signaling pathways: ZEB1, ZEB2 & Slug; (v) stem cell factors: Oct3/4, LIN28, NANOG, SOX2, SOX17, CD133 & nestin. Antibodies employed, experimental conditions, staining interpretation and scoring systems are detailed in **Supplementary Table 1**.

Adrenal cortical tumor series

To investigate the specificity of those immunohistochemical markers expressed in sarcomatoid ACCs, i.e. EMT-related markers (N-Cadherin, P-Cadherin, E-Cadherin, MMP2, MMP9 and Caveolin-1), downstream transcriptional regulators of EMT-related signaling pathways (ZEB1) and stem cell factors (nestin, SOX2, SOX17 and LIN28), these were also assessed in 38 conventional ACCs and 17 adrenocortical adenomas (ACAs) (including 12 control tissue samples) arranged in a tissue microarray (TMA) format using an automated TMA constructor (ATA-27 Beecher Instruments, Sun Prairie, WI, USA; available at the Department of Pathology, Erasmus MC Cancer Institute) as previously described [26].

DNA Isolation

From six sarcomatoid ACCs, 16 morphologically distinct tumour components (8 epithelial/ 8 sarcomatoid) were identified, as follows: 1 epithelial/1 sarcomatoid (cases No 1-2/4/6); 2 epithelial/ 3 sarcomatoid (case No 5); and 2 epithelial/ 1 sarcomatoid (case No 3). DNA isolation from 13 tumor areas (cases No 1-2/4-6) was carried out using standard procedures following manual microdissection. DNA isolation from the remaining 3 tumor areas (case No 3) following laser capture microdissection (Zeiss PALM Microbeam IV;

available at the Department of Pathology, Erasmus MC Cancer Institute). All tumor samples were estimated to contain at least 80% neoplastic cells.

Targeted Next-Generation Sequencing (NGS) analysis

Based on (i) mutations in ACCs, carcinosarcomas occurring at various anatomic locations, anaplastic thyroid carcinomas, and sarcomas; (ii) EMT-related pathways: Wnt pathway, MAPK pathway and PI3K/AKT pathway; and (iii) signalling pathways involved in the pathogenesis of adrenocortical tumors i.e. cAMP/PKA pathway, 18 genes were selected for mutational analysis: (1) *APC* (exons 12-14), (2) *AXIN1* (exons 1-6), (3) *AXIN2* (exon 7), (4) *CTNNB1* (exon 3), (5) *BRAF* (exons 11/15), (6) *KRAS* (exons 2-4), (7) *NRAS* (exons 2-4), (8) *HRAS* (exons 2-4), (9) *EGFR* (exons 18-21), (10) *PI3KCA* (exons 9/20), (11) *AKT1* (exon 2), (12) *AKT2* (exon 2), (13) *AKT3* (exon 2), (14) *PTEN* (exons 3-5/7), (15) *ALK* (exons 23-25), (16) *ERBB2* (exons 19-20), (17) *PRKAR1a* (exons 4-8) and (18) *TP53* (exons 2-11).

An Ion AmpliSeq Custom Panel was created and sequenced on the Ion Torrent Personal Genome Machine (PGM; Life Technologies) on 10 nanograms (per sample) of FFPE tumor DNA according to the manufacturer's protocols. In short, libraries were made using the Ion AmpliSeq Library Preparation Kit. Template was prepared using the Ion OneTouch Template Kit and sequencing was performed with the Ion Sequencing Kit v2.0 on an Ion 318 chip.

Data were analyzed with Torrent Suite Software, version 3.6 (Life Technologies). Annotation of variant calls was performed with Annovar (<http://www.openbioinformatics.org/annovar/>) [27] and facilitated using an in-house galaxy platform/server on which Annovar wrapper was installed [28-30]. The variants (i) with a read frequency higher than 30%, (ii) not known as common polymorphisms according to 1000G2012 April and ESP6500, (iii) non-synonymous with a minimum of 5 forward/ reverse variant read and 100 total depth read were retained as variants (mutations) and confirmed by alternate platforms i.e. Sanger direct sequencing. Sequences of all primers and probes are available upon request.

TERT promoter mutation analysis

In two cases displaying identical mutations in morphologically diverse components, hotspot *TERT* promoter mutations were analyzed by a SNaPshot assay using the ABI Prism SNaPshot Multiplex Kit (Applied Biosystems, Foster City, CA, USA) as previously described [26].

Statistical analysis

Non-parametric tests (Wilcoxon rank sum test) were used to study differences of immunohistochemical expression between the sarcomatoid series and control ACAs and conventional ACCs. Reciprocal correlations among the different expression of immunohistochemical markers among control ACCs and ACAs were made using a two-tailed Spearman's test.

Results

Clinical and pathological features of sarcomatoid ACCs

Three unpublished cases analyzed herein further added to the existing body of the literature; thus accounting for 19 cases (**Supplementary Table 2**). Sarcomatoid ACCs are rare tumors characterized by a wide age range (23-79 years; median age 55; mean age 53.89) and no gender or laterality predilection. These are usually non-functioning tumors of large size (average 14cm; range 6.5cm up to 24cm). Microscopically, they predominantly display a pure spindle cell component (n=11), which can be occasionally admixed with foci of rhabdomyosarcomatous or osteosarcomatous differentiation or undifferentiated PNET-like areas (n=3). Less frequently a pure specialized mesenchymal component i.e. rhabdomyosarcomatous (n=3), osteosarcomatous (n=1) and osteosarcomatous/chondrosarcomatous constituents (n=1), can be detected. These are highly aggressive neoplasms with a high propensity for metastatic and/or recurrent disease and an average post-operative survival of 6.9 months. Two cases developed a second primary malignancy and associated metastatic disease.

Immunohistochemical profile of sarcomatoid ACCs

SF-1 was the sole adrenocortical marker positive in the epithelial component of all cases, but always negative in sarcomatoid areas. Cytokeratin AE1/AE3 was negative in all cases except for a focal positivity in the epithelial component of case *No 1* and the sarcomatoid part of cases *No 3* and *6*. Conversely, vimentin was positive in both components of all cases, except for the epithelial component of case *No 2*. Neuron specific enolase had always a positive reactivity, while synaptophysin was positive in the epithelial component of four cases. Ki-67 proliferation index as estimated in the epithelial areas had a median value of 28% (range 1%-54%). β -catenin showed an aberrant nuclear localization in the sarcomatoid component of all but one case (*No 2*). β -catenin and p53 immunohistochemical data are summarized in **Table 2 (Figure 1)**.

Expression and/or specificity of EMT-related markers, downstream transcriptional regulators of EMT-related signaling pathways and stem cell factors in sarcomatoid ACCs

Cadherins were positive only in the epithelial component. In particular, E- and P-cadherins were always negative except for case *No 2*; N-cadherin was negative in all components of cases *No 1-2* and positive only in epithelial components of the other cases. Conversely, MMP-2 and/or MMP-9 were positive in sarcomatoid areas of all cases. ZEB-1 immunoreactivity/ ZEB-2 immunonegativity were noted in both components of all cases. Slug and Caveolin-1 were positive in the sarcomatoid component of five and four cases, respectively. Among stem cell markers, nestin was positive in sarcomatoid areas of all cases, while SOX-2, SOX-17 and LIN28 were positive in the sarcomatoid component of four, one and three cases, respectively. OCT3/4, NANOG and CD133 were always negative (**Table 3**).

Comparing the immunohistochemical expression of those markers demonstrated in sarcomatoid areas of sarcomatoid ACCs with a control series of conventional ACCs, MMP-2 and MMP-9 were found significantly more often expressed in sarcomatoid areas (Wilcoxon rank test, $p<0.0001$ and $p=0.0002$ respectively), while ZEB1 was always positive in sarcomatoid components and mostly negative in conventional ACCs (Wilcoxon rank test, $p=0.003$). ACAs had a significant positive expression of N-cadherin and P-cadherin as compared to ACCs ($p<0.0001$), while ZEB-1 and Caveolin-1 were significantly expressed in ACCs rather than in ACAs ($p<0.0001$). ACA stained mostly positive for N-cadherin and P-cadherin ($p<0.0001$ for both markers). ZEB1 was always absent in ACA ($p=0.005$), while Caveolin 1 was positive in the majority of ACC ($p<0.0001$).

Mutational analysis of sarcomatoid ACCs

Targeted NGS revealed 13 pathogenic, nonsynonymous, variants of ACC-associated genes in epithelial and/or non-epithelial components in 4 out of 6 tumors. Three cases had identical mutations in phenotypically diverse components, one of which containing *TP53/CTNNB1* in 3 out of 5 components, whereas one case harbored a single *TP53* mutation in the non-epithelial component (**Table 2**). The epithelial and sarcomatoid tumor components displayed concordant p53 expression patterns in 5 out of 6 cases. However, the aberrant p53 expression pattern, i.e. loss of expression, could not be entirely explained by p53 mutations in the exons examined. Aberrant nuclear β -catenin expression, as detected in all but one sarcomatoid components, was the result of *CTNNB1* mutations only in case No 5 (**Table 2**). No *TERT* promoter mutations were detected in four phenotypically diverse samples from two cases harboring *TP53* or *TP53/CTNNB1* mutations, respectively.

Discussion

Adrenocortical carcinomas (ACCs) with sarcomatous areas are rare tumors of unknown molecular pathogenesis with respect to associated genetic alterations and potential clonal relationships between the phenotypically diverse tumor components. Herein, we provide novel mutational findings and molecular evidence of monoclonal origin. In addition, we elucidate the immunohistochemical profile as well as mechanisms potentially conferring such a highly aggressive biological behavior to these rare tumor variants, which are enriched for EMT-related markers and stem cell factors.

The shared presence of *TP53* and *CTNNB1* gene mutations in both the epithelial and sarcomatoid compartments supports a common clonal origin at least in a subset of sarcomatoid ACCs. This is consistent with various molecular genetic studies, i.e. targeted NGS, mutational analysis, cytogenetic analysis, comparative genomic hybridization (CGH) analysis, as well as microdissection-based allelotyping/ loss of heterozygosity (LOH) analysis, in primary cutaneous carcinosarcomas [31-32], hepatic carcinosarcoma [33-34], maxillary carcinosarcoma [35] carcinosarcomas of the female genital tract [36-37], breast carcinosarcomas [38-39] and/or metaplastic breast carcinomas [40-41], salivary gland carcinosarcomas [42-43], sarcomatoid urothelial carcinomas of the urinary bladder [44-46], pharyngeal carcinosarcomas [44], esophageal carcinosarcomas [47], and pulmonary carcinosarcomas [48].

Three tumors harbored *TP53* mutations both in epithelial and non-epithelial constituents indicating that these mutations are early driver events in their pathogenesis most likely predisposing tumor cells to acquire additional genetic aberrations that might activate other critical signaling pathways for this particular sarcomatous differentiation lineage. One *CTNNB1* mutation concurred with a *TP53* mutation in three morphologically diverse tumor components of a single tumor (case No 5). This finding further reinforces a previously proposed association between the status of the *TP53* and *CTNNB1* genes in adrenocortical tumorigenesis, based on either co-occurrences of these mutations or *CTNNB1* gains in advanced ACCs [49]. Likewise, aberrant nuclear β -catenin localization was observed in all *TP53*-mutated sarcomatous components as well as in all *APC*-mutated tumor components of case No 3.

By using topographic genotyping with a targeted NGS technique combined with immunohistochemical investigation of EMT-related and stem cell-like markers, we tried to elucidate aspects of the molecular histogenesis of the sarcomatoid ACCs (**Supplementary Figure 1**). Notably, prior studies in carcinosarcomas, using a targeted NGS approach [33], microsatellite markers [47-48] or through deep sequencing [50] highlighted the complexity of the evolutionary process supporting either a continuous genetic progression model or a branched clonal divergent model with or without subclonal progression.

By immunohistochemistry, we observed a downregulation of Cadherin expression along with an enrichment of various EMT-related markers in sarcomatoid components, implying a potential role for EMT in a subset of sarcomatoid ACCs (**Figure 2**). Additionally, the sarcomatoid compartments of all cases

displayed Wnt pathway activation, as evidenced by aberrant nuclear and/or cytoplasmic β -catenin localization. This could be attributed either to *CTNNB1/APC* mutations or potentially to aberrations in other tumor suppressor genes related to the Wnt/ β -catenin pathway and/or negative crosstalk between SF-1 and Wnt/ β -catenin signaling [51-53]. Mutational inactivation of *TP53*, accompanied by p53 overexpression, was also a common genetic event (4 out of 6 cases). Given the critical role of the Wnt pathway in EMT [54] and the interplay between a fail-safe program escape and EMT [55-56], these molecular aberrations might mediate an EMT process in a subset of sarcomatoid ACCs. These observations are in agreement with data from mutational and immunohistochemical investigations of metaplastic breast carcinomas [41, 57-58] and primary cutaneous carcinosarcomas [31].

Nestin expression was noted almost exclusively in the sarcomatoid components of all examined cases along with Wnt/ β -catenin pathway deregulation. Four tumors displayed SOX2 co-expression in subpopulations of the sarcomatoid components and adjacent epithelial areas (transitional zones in cases No 1 & 5) indicative of an active ongoing process. Of note is that (i) β -catenin has been shown as an important binding partner of SOX2 and a regulator of its transcriptional activity in a subset of breast cancer cells [59]; (ii) an enhancer region on the nestin gene is dependent on SOX2 binding [60]; and (iii) a biologically significant linkage between nestin and SOX2 expression has been previously reported in human melanomas [61]. Moreover, nestin and/or SOX2 expression is enriched in various tumor types playing a potential role in cancer progression as well as conferring a poor prognosis [61-65]. Although it is unclear whether these participate in EMT [61, 66] or reflect a cancer stem cell phenotype [66], a molecular-driven multifaceted targeted approach (Wnt/ β -catenin signalling pathway, EMT program-associated molecules and nestin) seems to be of great therapeutic interest [64, 67-68].

EMT-associated markers were more significantly expressed in the sarcomatoid areas than in conventional ACCs (lacking sarcomatoid areas) adding further evidence for a role in its pathogenesis. Nestin immunoexpression was detected in 5 out of 38 ACCs (13.5%) and in none of ACAs examined (0%) as compared to other data of 13 out of 16 ACCs (81%) and 2 out of 20 ACAs (10%) [69]. In accordance with EMT-related zinc-finger transcription factor Snail immunoexpression in adrenocortical tumours [70], we detected significant differences in N-/P-Cadherin and ZEB1/Caveolin-1 expression between ACCs and ACAs. Further studies in larger cohorts are warranted to delineate the exact role of EMT in adrenocortical tumorigenesis.

In summary, we have shown that Wnt/ β -catenin signaling pathway dysregulation and mutational inactivation of *TP53* are common genetic events in adrenal cancers having a sarcomatous component. Targeted NGS approach provided molecular evidence of monoclonal evolution at least in a subset of cases. The term “sarcomatoid ACC” seems more appropriate than carcinosarcoma or others to address such a rare and aggressive variant of ACC. These tumors were enriched in EMT-related markers and stem cell factors, potentially conferring a poor prognosis and being exploited as novel therapeutic targets.

References

- [1] de Krijger RR, Papathomas TG. Adrenocortical neoplasia: evolving concepts in tumorigenesis with an emphasis on adrenal cortical carcinoma variants. *Virchows Arch* 2012;460:9-18.
- [2] Okazumi S, Asano T, Ryu M, et al. [Surgical resection of adrenal carcinoma extending into the vena cava, right atrium and ventricle: case report and review of the literature]. *Nihon Geka Gakkai Zasshi* 1987;88:231-8.
- [3] Collina G, Maldarizzi F, Betts CM, Eusebi V. Primary sarcomatoid carcinoma of the adrenal gland. First case report. *Virchows Arch A Pathol Anat Histopathol* 1989;415:161-7.
- [4] Decorato JW, Gruber H, Petti M, Levowitz BS. Adrenal carcinosarcoma. *J Surg Oncol* 1990; 45:134-6.
- [5] Fischler DF, Nunez C, Levin HS, McMahon JT, Sheeler LR, Adelstein DJ. Adrenal carcinosarcoma presenting in a woman with clinical signs of virilization. A case report with immunohistochemical and ultrastructural findings. *Am J Surg Pathol* 1992;16:626-31.
- [6] Barksdale SK, Marincola FM, Jaffe G. Carcinosarcoma of the adrenal cortex presenting with mineralocorticoid excess. *Am J Surg Pathol* 1993;17:941-5.
- [7] Lee MS, Park IA, Chi JG, Ham EK, Lee KC, Lee CW. Adrenal carcinosarcoma--a case report. *J Korean Med Sci* 1997;12:374-7.
- [8] Sturm N, Moulai N, Laverrière MH, Chabre O, Descotes JL, Brambilla E. Primary adrenocortical sarcomatoid carcinoma: case report and review of literature. *Virchows Arch* 2008;452:215-9.
- [9] Coli A, Di Giorgio A, Castri F, Destito C, Marin AW, Bigotti G. Sarcomatoid carcinoma of the adrenal gland: A case report and review of literature. *Pathol Res Pract* 2010;206:59-65.
- [10] Sasaki K, Desimone M, Rao HR, Huang GJ, Seethala RR. Adrenocortical carcinosarcoma: a case report and review of the literature. *Diagn Pathol* 2010;5:51.
- [11] Bertolini F, Rossi G, Fiocchi F, et al. Primary adrenal gland carcinosarcoma associated with metastatic rectal cancer: a hitherto unreported collision tumor. *Tumori* 2011;97:27e-30e.

- [12] Thway K, Olmos D, Shah C, Flora R, Shipley J, Fisher C. Oncocytic adrenal cortical carcinosarcoma with pleomorphic rhabdomyosarcomatous metastases. *Am J Surg Pathol* 2012;36:470-7.
- [13] Yan JJ, Sun AJ, Ren Y, Hou C. Primary adrenocortical sarcomatoid carcinoma: Report of a case. *Can Urol Assoc J* 2012;6:E189-91.
- [14] Kao CS, Grignon DJ, Ulbright TM, Idrees MT. A case report of adrenocortical carcinosarcoma with oncocytic and primitive neuroectodermal-like features. *Hum Pathol* 2013;44:1947-55.
- [15] Mark D, Boyd C, Eatock F. Adrenal sarcomatoid carcinoma: a case report and review of the literature. *Ulster Med J* 2014;83:89-92.
- [16] Shaikh AS, Bakhshi GD, Khan AS, Jamadar NM, Nirmala AK, Raza AA. Primary adrenal sarcomatoid carcinoma. *Clin Pract* 2014;4:604.
- [17] Wei YB, Gao YL, Wu HT, et al. Rare incidence of primary adrenocortical carcinosarcoma: A case report and literature review. *Oncol Lett* 2015;9:153-8.
- [18] Thiery JP, Acloque H, Huang RY, Nieto MA. Epithelial-mesenchymal transitions in development and disease. *Cell* 2009;139:871-90.
- [19] Ansieau S, Courtois-Cox S, Morel AP, Puisieux A. Failsafe program escape and EMT: a deleterious partnership. *Semin Cancer Biol* 2011;21:392-6.
- [20] Mani SA, Guo W, Liao MJ, et al. The epithelial-mesenchymal transition generates cells with properties of stem cells. *Cell* 2008;133:704-15.
- [21] Lan L, Luo Y, Cui D, et al. Epithelial-mesenchymal transition triggers cancer stem cell generation in human thyroid cancer cells. *Int J Oncol* 2013;43:113-20.
- [22] Battula VL, Evans KW, Hollier BG, et al. Epithelial-mesenchymal transition-derived cells exhibit multilineage differentiation potential similar to mesenchymal stem cells. *Stem Cells* 2010;28:1435-45.
- [23] Sung CO, Choi H, Lee KW, Kim SH. Sarcomatoid carcinoma represents a complete phenotype with various pathways of epithelial mesenchymal transition. *J Clin Pathol* 2013;66:601-6.

- [24] Conant JL, Peng Z, Evans MF, Naud S, Cooper K. Sarcomatoid renal cell carcinoma is an example of epithelial--mesenchymal transition. *J Clin Pathol* 2011;64:1088-92.
- [25] Gwin K, Buell-Gutbrod R, Tretiakova M, Montag A. Epithelial-to-mesenchymal transition in metaplastic breast carcinomas with chondroid differentiation: expression of the E-cadherin repressor Snail. *Appl Immunohistochem Mol Morphol* 2010;18:526-31.
- [26] Papathomas TG, Oudijk L, Zwarthoff EC, et al. Telomerase reverse transcriptase promoter mutations in tumors originating from the adrenal gland and extra-adrenal paraganglia. *Endocr Relat Cancer* 2014;21:653-61.
- [27] Wang K, Li M, Hakonarson H. ANNOVAR: functional annotation of genetic variants from high-throughput sequencing data. *Nucleic Acids Res* 2010;38:e164.
- [28] Goecks J, Nekrutenko A, Taylor J; Galaxy Team. Galaxy: a comprehensive approach for supporting accessible, reproducible, and transparent computational research in the life sciences. *Genome Biol* 2010;11:R86.
- [29] Blankenberg D, Von Kuster G, Coraor N, et al. Galaxy: a web-based genome analysis tool for experimentalists. *Curr Protoc Mol Biol* 2010;19:10.1-21.
- [30] Giardine B, Riemer C, Hardison RC, et al. Galaxy: a platform for interactive large-scale genome analysis. *Genome Res* 2005;15:1451-5.
- [31] Paniz-Mondolfi A, Singh R, Jour G, et al. Cutaneous carcinosarcoma: further insights into its mutational landscape through massive parallel genome sequencing. *Virchows Arch* 2014;465:339-50.
- [32] Kiuru M, McDermott G, Coit DC, Berger MF, Busam KJ. Basal cell carcinosarcoma with PTCH1 mutations in both epithelial and sarcomatoid primary tumor components and in the sarcomatoid metastasis. *Am J Surg Pathol* 2014;38:138-42.
- [33] Luchini C, Capelli P, Fassan M, et al. Next-generation histopathologic diagnosis: a lesson from a hepatic carcinosarcoma. *J Clin Oncol* 2014;32:e63-6.
- [34] Schaefer IM, Schweyer S, Kuhlitz J. Chromosomal imbalances in primary hepatic carcinosarcoma. *Hum Pathol* 2012;43:1328-33.

- [35] Ando M, Saito Y, Morikawa T, et al. Maxillary carcinosarcoma: Identification of a novel MET mutation in both carcinomatous and sarcomatous components through next generation sequencing. *Head Neck* 2015;**37**:E179-85
- [36] Taylor NP, Zigelboim I, Huettner PC, et al. DNA mismatch repair and TP53 defects are early events in uterine carcinosarcoma tumorigenesis. *Mod Pathol* 2006;**19**:1333-8.
- [37] Growdon WB, Roussel BN, Scialabba VL, et al. Tissue-specific signatures of activating PIK3CA and RAS mutations in carcinosarcomas of gynecologic origin. *Gynecol Oncol* 2011;**121**:212-7.
- [38] Teixeira MR, Qvist H, Böhler PJ, Pandis N, Heim S. Cytogenetic analysis shows that carcinosarcomas of the breast are of monoclonal origin. *Genes Chromosomes Cancer* 1998;**22**:145-51.
- [39] Zhuang Z, Lininger RA, Man YG, Albuquerque A, Merino MJ, Tavassoli FA. Identical clonality of both components of mammary carcinosarcoma with differential loss of heterozygosity. *Mod Pathol* 1997;**10**:354-62.
- [40] Geyer FC, Weigelt B, Natrajan R, et al. Molecular analysis reveals a genetic basis for the phenotypic diversity of metaplastic breast carcinomas. *J Pathol* 2010;**220**:562-73.
- [41] Lien HC, Lin CW, Mao TL, Kuo SH, Hsiao CH, Huang CS. p53 overexpression and mutation in metaplastic carcinoma of the breast: genetic evidence for a monoclonal origin of both the carcinomatous and the heterogeneous sarcomatous components. *J Pathol* 2004;**204**:131-9.
- [42] Vékony H, Leemans CR, Ylstra B, Meijer GA, van der Waal I, Bloemena E. Salivary gland carcinosarcoma: oligonucleotide array CGH reveals similar genomic profiles in epithelial and mesenchymal components. *Oral Oncol* 2009;**45**:259-65.
- [43] Fowler MH, Fowler J, Ducatman B, Barnes L, Hunt JL. Malignant mixed tumors of the salivary gland: a study of loss of heterozygosity in tumor suppressor genes. *Mod Pathol* 2006;**19**:350-5.
- [44] Torenbeek R, Hermsen MA, Meijer GA, Baak JP, Meijer CJ. Analysis by comparative genomic hybridization of epithelial and spindle cell components in sarcomatoid carcinoma and carcinosarcoma: histogenetic aspects. *J Pathol* 1999;**189**:338-43.

- [45] Sung MT, Wang M, MacLennan GT, et al. Histogenesis of sarcomatoid urothelial carcinoma of the urinary bladder: evidence for a common clonal origin with divergent differentiation. *J Pathol* 2007;211:420-30.
- [46] Armstrong AB, Wang M, Eble JN, et al. TP53 mutational analysis supports monoclonal origin of biphasic sarcomatoid urothelial carcinoma (carcinosarcoma) of the urinary bladder. *Mod Pathol* 2009;22:113-8.
- [47] Matsumoto T, Fujii H, Arakawa A, et al. Loss of heterozygosity analysis shows monoclonal evolution with frequent genetic progression and divergence in esophageal carcinosarcoma. *Hum Pathol* 2004;35:322-7
- [48] Dacic S, Finkelstein SD, Sasatomi E, Swalsky PA, Yousem SA. Molecular pathogenesis of pulmonary carcinosarcoma as determined by microdissection-based allelotyping. *Am J Surg Pathol* 2002;26:510-6.
- [49] De Martino MC, Al Ghuzlan A, Aubert S, et al. Molecular screening for a personalized treatment approach in advanced adrenocortical cancer. *J Clin Endocrinol Metab*. 2013;98:4080-8.
- [50] Murugaesu N, Chew SK, Swanton C. Adapting clinical paradigms to the challenges of cancer clonal evolution. *Am J Pathol* 2013;182:1962-71.
- [51] Ehrlund A, Jonsson P, Vedin LL, Williams C, Gustafsson JÅ, Treuter E. Knockdown of SF-1 and RNF31 affects components of steroidogenesis, TGFβ, and Wnt/β-catenin signaling in adrenocortical carcinoma cells. *PLoS One* 2012;7:e32080.
- [52] Bernard P, Ryan J, Sim H, et al. Wnt signaling in ovarian development inhibits Sf1 activation of Sox9 via the Tesco enhancer. *Endocrinology* 2012;153:901-12.
- [53] Assié G, Letouzé E, Fassnacht M, et al. Integrated genomic characterization of adrenocortical carcinoma. *Nat Genet* 2014;46:607-12.
- [54] Gheldof A, Berx G. Cadherins and epithelial-to-mesenchymal transition. *Prog Mol Biol Transl Sci* 2013;116:317-36.

- [55] Ansieau S, Courtois-Cox S, Morel AP, Puisieux A. Failsafe program escape and EMT: a deleterious partnership. *Semin Cancer Biol* 2011;21:392-6.
- [56] Kim T, Veronese A, Pichiorri F, et al. p53 regulates epithelial-mesenchymal transition through microRNAs targeting ZEB1 and ZEB2. *J Exp Med* 2011;208:875-83.
- [57] Hayes MJ, Thomas D, Emmons A, Giordano TJ, Kleer CG. Genetic changes of Wnt pathway genes are common events in metaplastic carcinomas of the breast. *Clin Cancer Res* 2008;14:4038-44.
- [58] Zhang Y, Toy KA, Kleer CG. Metaplastic breast carcinomas are enriched in markers of tumor-initiating cells and epithelial to mesenchymal transition. *Mod Pathol* 2012;25:178-84.
- [59] Ye X, Wu F, Wu C, et al. β -Catenin, a Sox2 binding partner, regulates the DNA binding and transcriptional activity of Sox2 in breast cancer cells. *Cell Signal* 2014;26:492-501.
- [60] Tanaka S, Kamachi Y, Tanouchi A, Hamada H, Jing N, Kondoh H. Interplay of SOX and POU factors in regulation of the Nestin gene in neural primordial cells. *Mol Cell Biol* 2004;24:8834-46
- [61] Laga AC, Zhan Q, Weishaupt C, Ma J, Frank MH, Murphy GF. SOX2 and nestin expression in human melanoma: an immunohistochemical and experimental study. *Exp Dermatol* 2011;20:339-45.
- [62] Liu K, Lin B, Zhao M, et al. The multiple roles for Sox2 in stem cell maintenance and tumorigenesis. *Cell Signal* 2013;25:1264-71.
- [63] Gauchotte G, Philippe C, Lacomme S, et al. BRAF, p53 and SOX2 in anaplastic thyroid carcinoma: evidence for multistep carcinogenesis. *Pathology* 2011;43:447-52.
- [64] Ishiwata T, Matsuda Y, Naito Z. Nestin in gastrointestinal and other cancers: effects on cells and tumor angiogenesis. *World J Gastroenterol* 2011;17:409-18.

[65] Yang XH, Wu QL, Yu XB, et al. Nestin expression in different tumours and its relevance to malignant grade. J Clin Pathol 2008;61:467-73.

[66] Lloyd RV, Hardin H, Montemayor-Garcia C, et al. Stem cells and cancer stem-like cells in endocrine tissues. Endocr Pathol 2013;24:1-10.

[67] Gaujoux S, Hantel C, Launay P, et al. Silencing mutated β -catenin inhibits cell proliferation and stimulates apoptosis in the adrenocortical cancer cell line H295R. PLoS One 2013;8:e55743.

[68] Mimeault M, Batra SK. Altered gene products involved in the malignant reprogramming of cancer stem/progenitor cells and multitargeted therapies. Mol Aspects Med 2014;39:3-32.

[69] Lachenmayer A, Lichtenauer UD, Cox T, et al. Nestin as a marker in the classification of adrenocortical tumors. Horm Metab Res 2009;41:397-401.

[70] Waldmann J, Feldmann G, Slater EP, et al. Expression of the zinc-finger transcription factor Snail in adrenocortical carcinoma is associated with decreased survival. Br J Cancer 2008;99:1900-7.

Figure Legends

Figure 1. H&E staining, β -catenin and p53 immunoexpression patterns in epithelial (e1 in **A-C**; e2 in **D-F**) and sarcomatoid components (s1 in **G-I**; s2 in **J-L**) of case No 5. All components harbored an identical *CTNNB1* p.S45F mutation displaying nuclear β -catenin immunoreactivity, whereas only those containing *TP53* p.R249W mutations demonstrated strong p53 immunopositivity. Please note that sarcomatoid component (s3) within the inferior vena cava, harboring *CTNNB1* p.S45F and *TP53* p.R249W mutations and exhibiting similar β -catenin/p53 immunoexpression patterns to (s2), is not included in the panel.

Figure 2. Immunohistochemical profile of case No 4: Note MMP2 (**A**)/Caveolin-1 (**C**)/ Nestin (**D**) immunoreactivity only in the sarcomatoid component abutting uninvolved pancreatic parenchyma (*) in contrast to N-Cadherin (**B**) as expressed in the epithelial compartment. ZEB-1 (**E**) is expressed both in the epithelial and adjacent sarcomatoid areas (transitional zone).

Table 1. Clinicopathological data of sarcomatoid ACCs investigated in the present series

Case #	Sex/Age	Location	Symptoms	Size / Weight	Gross Appearance	ACC component	Sarcomatoid component	Clinical Follow-up	Reference
1	M/31	L	Abdominal pain/ no endocrine dysfunction	12 cm / 620 g	Cystic degenerative cut surface, necrosis, hemorrhage	WS 8	Spindle cell	Locoregional recurrence with infiltration of the splenic hilum (2 mo) 3 months DOD	Sturm [8]
2	F/75	L	Abdominal pain/ no endocrine dysfunction	15 cm	Variegated cut surface with whitish-gray firm solid areas, extensive necrosis and cysts	WS 7	Spindle cell	Liver metastases (3 mo) 12 months DOD	Coli [9]
3	F/23	L	Occasional finding, synchronous rectal cancer in pregnancy/ no endocrine dysfunction	14 cm	Irregular gray surface, central cavity filled with mucinous material	WS 7 ^a	Osteosarcoma	widespread metastatic disease ^b 14 months DOD	Bertolini [11]
4	M/55	L	Abdominal pain/ no endocrine dysfunction	16 cm	Whitish gray cut surface, necrosis, hemorrhage	WS 9 ^c	Spindle cell ^d	widespread abdominal metastatic disease 4 months DOD	This study
5	F/70	R	Abdominal pain, diarrhea/ no endocrine dysfunction	15 cm	Cystic cut surface; cysts filled with clotted blood and viable appearing gray-white to partly yellow-white parenchyma Firm calcific areas Extensive necrosis and hemorrhage	WS 8	Osteosarcoma Spindle cell ^{d, e}	Liver metastasis (2 mo) Lung and bone metastases (5 mo) 8 months ^f DOD	This study
6	M/52	R	Abdominal pain, fatigue, malaise, weight loss/ no endocrine dysfunction	24 cm/ 3020 g	Solid and partially cystic cut surface. Multiple necrotic areas	WS 5	Spindle cell ^d	Liver metastasis (two months prior to surgical resection) Pleural metastasis (4 mo) 4.5 months ^f DOD	This study

Abbreviations: ACC, adrenocortical carcinoma; F, female; M, male; NE; not evaluated; L, left; R, right; WS, Weiss Score

^a a conventional ACC with adipocytic-like areas displaying **MDM2** gene polysomy as detected by FISH (experimental details available upon request) co-existing with a metastatic rectal adenocarcinoma

^b given the synchronous occurrence of a rectal adenocarcinoma, it becomes difficult to define the exact origin of the metastatic foci; due to the histopathologically proven metastasis of the rectal cancer to the adrenal gland, the patient was treated as having metastatic rectal cancer and thus mitotane was not an option

^c this case was originally reported as pheochromocytoma, due to a pheochromocytoma-like component comprising medium-sized cells, displaying scant nuclear atypia and a weakly eosinophilic cytoplasm, arranged in a nested or trabecular pattern. The diagnosis was amended (sarcomatoid ACC) following consultation.

^d the sarcomatoid component is estimated approximately >10% of the total tumour extent

^e two phenotypically diverse spindle cell components were identified (**Figure 1**)

^f postoperative time of death; Cases 5/6 were both treated with surgical resection and mitotane

Table 2. Summary of pathogenic variants as detected by targeted NGS approach & β-catenin/P53 IHC as evaluated in 16 morphologically distinct tumour components from 6 sarcomatoid ACCs

Case #	Pathogenic Mutations	β-catenin IHC	P53 IHC
1 (e)	---	m	nor
1 (s)	TP53 c.973G>T p.G325X (exon 9)	n & c	+
2 (e)	---	m	-
2 (s)	---	c	-
3 (e1)	--- ^a	n	-
3 (e2)	--- ^a	n	-
3 (s)	--- ^a	n	-
4 (e)	TP53 c.995T>G p.I332S (exon 10)	m	+
4 (s)	TP53 c.995T>G p.I332S (exon 10)	n	+
5 (e1)	CTNNB1 c.134C>T p.S45F (exon 3)	n	+
5 (e2)	CTNNB1 c.134C>T p.S45F (exon 3)	n & m	-
5 (s1)	CTNNB1 c.134C>T p.S45F (exon 3)	n	-
5 (s2)	CTNNB1 c.134C>T p.S45F (exon 3)	n & c	+
5 (s3)	CTNNB1 c.745A>T p.R249W (exon 7)	n & c	+
6 (e)	TP53 c.743G>A p.R248Q (exon 7)	m	+
6 (s)	TP53 c.743G>A p.R248Q (exon 7)	n	+

Abbreviations: +, overexpression; -, loss of expression; c, cytoplasmic; e, epithelial component; IHC, immunohistochemistry; m, membranous; nor; normal; n, nuclear; s, sarcomatoid component
^a known germline *APC* mutation [11]

Table 3. Expression of EMT-related markers, downstream transcriptional regulators of EMT-related signaling pathways and stem cell factors as evaluated in selected phenotypically distinct tumour components from 6 sarcomatoid ACCs.

Antibodies	Case # 1 [8]		Case # 2 [9]		Case # 3 [11]		Case # 4		Case # 5		Case # 6	
	e	s	e	s	e2	s	e	s	e2	s2	e	s
E-Cadherin	-	-	+	-	-	-	-	-	-	-	-	-
P-Cadherin	- ^a	-	+	-	-	-	-	-	-	-	-	-
N-Cadherin	-	-	-	-	+	-	+	-	+	-	+ ^b	-
MMP-2	-	-	-	+	-	+	-	+	-	+	-	+
MMP-9	-	+	-	+	-	-	-	+	-	+	-	+
ZEB-1	- ^c	+	+/-	+/-	+/-	+/-	+	+	+	+	+	+
ZEB-2	-	-	-	-	-	-	-	-	-	-	-	-
SLUG	+/-	+	-	-	+	+	+/-	+	-	+/-	-	+
Caveolin-1	-	+	-	-	-	-	-	+	-	+	-	+
OCT3/4	-	-	-	-	-	-	-	-	-	-	-	-
NANOG	-	-	-	-	-	-	-	-	-	-	-	-
CD133	-	-	-	-	-	-	-	-	-	-	-	-
LIN28	+	+	-	-	- ^a	- ^a	-	-	-	+	- ^a	+
SOX2	- ^c	+	-	-	-	+ ^d	-	-	- ^c	+	-	+
SOX17	-	-	-	-	-	-	-	-	-	-	-	+
Nestin	- ^c	+	-	+	+	+	-	+	-	+	-	+

Abbreviations: EMT, epithelial-mesenchymal transition; e, epithelial component; s, sarcomatoid component

^a only cytoplasmic localization

^b epithelial areas displayed also N-Cadherin immunonegativity

^c other epithelial areas adjacent to sarcomatous areas (transitional areas) displayed immunoreactivity

^d osteoblasts displayed immunoreactivity, while osteocytes immunonegativity

Figure(s)
[Click here to download high resolution image](#)

Figure(s)
[Click here to download high resolution image](#)

Supplementary Material

[Click here to download Supplementary Material: Supplementary Figure 1.tif](#)

Supplementary Material

[Click here to download Supplementary Material: Revised Supplementary Table 1.docx](#)

Supplementary Material

[Click here to download Supplementary Material: Revised Supplementary Table 2.docx](#)