Intratumor heterogeneity of prognostic DNA-based molecular markers in adrenocortical carcinoma

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Abstract

Background: The prognosis of adrenocortical carcinoma (ACC) is heterogeneous. Genomic studies have identified ACC subgroups characterized by specific molecular alterations, including features measured at DNA level (somatic mutations, chromosome alterations, DNA methylation), which are closely associated with outcome. The aim of this study was to evaluate intratumor heterogeneity of prognostic molecular markers at the DNA level.

Methods: Two different tissue samples (primary tumor, local recurrence or metastasis) were analyzed in 26 patients who underwent surgery for primary or recurrent ACC. DNA-related biomarkers with prognostic role were investigated in frozen and paraffin-embedded samples. Somatic mutations of p53/Rb and Wnt/β-catenin pathways were assessed using next-generation sequencing (N=26), chromosome alteration profiles were determined using SNP arrays (N=14) and methylation profiles were determined using 4-gene bisulfite pyrosequencing (N=12).

Results: Somatic mutations for ZNRF3, TP53, CTNN1B and CDKN2A were found in 7, 6, 6 and 4 patients respectively, with intratumor heterogeneity in 8/26 patients (31%).

Chromosome alteration profiles were “Noisy” (numerous and anachronic alterations) in 8/14 and “Chromosomal” (extended patterns of loss of heterozygosity) in 5/14 of the study samples. For these profiles, no intratumor heterogeneity was observed.

Methylation profiles were hypermethylated in 5/12 and non-hypermethylated in 7/12 of the study samples. Intratumor heterogeneity of methylation profiles was observed in 2/12 patients (17%).

Conclusions: Intratumor heterogeneity impacts DNA-related molecular markers. While somatic mutation can differ, prognostic DNA methylation and chromosome alteration profile seem rather stable, and might be more robust for the prognostic assessment.
Introduction

Adrenocortical carcinoma is a rare cancer with poor but heterogeneous prognosis. The 5-year overall survival of ACC patients remains below 40% in most series(1).

The main prognostic factors used in clinical practice at present are the tumor extension, best reflected by the ENSAT stage(2), and the tumor proliferation, estimated either by mitotic count(3) or Ki67 proliferation index(4, 5). However, the prognosis still varies widely among tumors with the same tumor stage and proliferation index(6).

Recently, pan-genomic studies have identified molecular subtypes closely associated with prognosis(7, 8). A first subtype associates a “C1A” transcriptome profile – characterized by upregulation of proliferative genes -, a CpG island hypermethylation, a “Noisy” chromosome alteration profile – i.e. numerous and anarchic alterations -, and an accumulation of mutations in p53/Rb and Wnt/β-catenin related genes. This subgroup is associated with very poor outcome. Conversely, another subtype of ACC associates a “C1B” transcriptome profile – enriched in immune-related genes -, no hypermethylation, a “chromosomal” genome profile – i.e. extended patterns of loss of heterozygosity (LOH) -, and a low mutation rate. This subgroup is associated with a better outcome. Hence, targeted molecular markers have been proposed to complete the prognostic assessment of ACC(9, 10, 11).

These markers are either measured at the tumor DNA level – including somatic mutations, chromosome alteration and DNA methylation profile-, or at the RNA level - including transcriptome and targeted gene expression profiles.

Intratumor heterogeneity of somatic mutations has been reported in many cancer types(12, 13, 14). In a small series of exome sequencing in 14 ACC patients, intratumor heterogeneity was reported in 43 to 63% of somatic mutations among different metastatic sites from the same patient(15). In addition to somatic mutations, intratumor heterogeneity of DNA methylation has been reported in several cancer types(16, 17). Whether molecular alterations identified as major prognostic features of ACC are exposed to intratumor heterogeneity remains to be determined.

The aim of our study was to assess the robustness of targeted molecular markers measurable at the DNA level - somatic mutations, chromosome alteration profile and targeted DNA methylation profile -
, by performing multiple measures for different tumor manifestations in same patients. Variability of
the prognostic molecular assay was determined for each measurement.
Materials and Methods

Tumor samples

Two cohorts of ACC patients were included in this study: 14 patients from Cochin Hospital, Paris, France, and 12 patients from Wuerzburg University Hospital, Germany. Tissue samples were obtained from two different tumor sites for each patient (Supplementary Table 1, Supplementary Figure 1), including primary tumor (P), local recurrence (R) or distant metastasis (M). Tumor specimens were collected between 2001 and 2015 and were either frozen in the Cochin cohort or formalin-fixed paraffin-embedded (FFPE) samples in the Wuerzburg cohort, as previously described (9, 11). The diagnosis of ACC was confirmed by an expert endocrine pathologist according to Weiss criteria (18). Written informed consent for the molecular analysis and the collection and use of the clinical data was obtained from all patients and the study was approved by the Comité de protection des personnes Île de France I (application #13311) and the Ethics Committee of the University of Würzburg (registration number 88/11).

DNA isolation

Tumor DNA was extracted and purified by proteinase K digestion and ethanol extraction, followed by a clean-up step on columns (Qiagen, Courtaboeuf, France) in Cochin cohort, and with the GeneRead DNA FFPE Kit (Qiagen, Hilden, Germany) in Wuerzburg cohort, according to the manufacturer’s protocols. The quality of DNA was analyzed by the Nanodrop ND-1000 spectrophotometer (Nyxor Biotech) in Cochin cohort and by the GeneRead DNA QuantiMIZE Assay Kit (Qiagen, Hilden, Germany) in Wuerzburg cohort.

Leukocyte DNA was isolated with the DNA isolation kit for mammalian blood (Roche, Mannheim, Germany) in Cochin cohort and with the NucleoSpin Blood L Kit (Macherey-Nagel, Bethlehem, PA) in Wuerzburg cohort, according to the manufacturer’s instructions.

Sequence mutations and copy number alterations
Somatic alterations in p53/Rb (TP53, RB1, CDK4, and CDKN2A) and Wnt/ß-catenin (CTNNB1, and ZNRF3) pathways were determined in both cohorts (N=26).

For each patient, two samples from two different tumor regions were studied. In the Cochin cohort, sequence variants were called using exome sequencing (HiSeq, Illumina, San Diego, USA) for 4/28 samples, and targeted next generation sequencing (NGS) with a custom panel (Ampliseq and PGM, Life Technologies, Saint-Aubin, France) for 24/28 samples, as previously reported(11).

In the Wuerzburg cohort, sequence variants were called using targeted NGS with the GeneRead DNAseq Human Comprehensive Cancer Panel V2 and the GeneRead DNAseq Panel PCR Kit V2 (Qiagen, Hilden, Germany and NextSeq500, Illumina) and Sanger sequencing for ZNRF3 for 20/24 samples, as previously reported(9), and with a QIAseq Targeted DNA Custom Panel (Qiagen and NextSeq500, Illumina) for 4/24 samples.

Sequence mutations were defined as somatic variants that fulfill the following criteria: coverage > 100x in targeted NGS or > 30x in exome sequencing, rare in population database (<0.02 in Exome Aggregation Consortium), allelic ratio > 0.2, exonic or splicing, non-synonymous, and not predicted as benign by at least one prediction algorithm including SIFT(19), Popyphen2(20) and MutationTaster2(21). All non-synonymous somatic variants were validated visually using the Integrative Genomics Viewer(22) or GensearchNGS (PhenoSystems®, Braine le Chateau, Belgium).

In case of subclonal variant – called in one tumor region but not in the other –, the coverage depth of the negative region was also checked. Comparison of single nucleotide polymorphisms genotypes was used to verify the proper matching of the two samples for each patient (data not shown).

In addition to sequence variations, copy number alterations were called by analyzing SNP array data in the Cochin cohort (N=14). Homozygous deletions and amplifications were called if log R ratio (LRR) was lower or higher than 3-fold the standard deviation, respectively(11).

Calculation of recurrence rate was performed in both cohorts (N=26) for genes usually subject to sequence mutations (TP53, CTNNB1, RB1) and in the Cochin cohort only (N=14) for genes mostly subject to copy-number alterations (ZNRF3, CDKN2A, CDK4).
**Chromosome alterations**

Chromosome alteration profiles were determined in the Cochin cohort only (N=14) using Infinium HumanOmniExpress and HumanCore SNP arrays (Illumina, San Diego, USA).

Abnormal chromosome segments were generated with Genome Alteration Print(23), then filtered and smoothed. Filters included a minimal size – at least 20 SNPs with germline heterozygosity -, a reduced noise within the segment – measured by the standard deviation of mirrored B-allele frequency (MBAF) (a value between 0.5 and 1, reflecting the allelic ratio), expected lower than the standard deviation of MBAF from normal chromosomes-, and a validated segment – the MBAF generated by GAP was expected close (no more or less than 0.2) to the computed MBAF of the segment -. Smoothing was performed by merging any consecutive segments with close allelic ratios – defined by MBAF values lower than the standard deviation of MBAF from normal chromosomes-.

Each sample was assigned to “Noisy” profile – i.e. numerous and anarchic chromosome alterations—, “Chromosomal” – i.e. extended LOH- or “Quiet” – a limited number of chromosome alterations, following a classification rule based on the alterations of nine chromosome arms (1p, 1q, 2p, 2q, 11p, 11q, 18p, 18q, 22q), as previously reported(8, 11). A sample was called “Chromosomal” if at least seven chromosome arms presented complete LOH; a sample was called “Quiet” if less than 4 chromosome arms were altered; a sample was called “Noisy” if 20 or more altered chromosome segments were identified(11).

**Targeted DNA methylation analysis**

Methylation profiles were determined in the Wuerzburg cohort only (N=12), using bisulfite pyrosequencing of 4 genes (PAX5, PAX6, PYCARD, and GSTP1), as previously reported(9, 24). The samples were classified as “Not hypermethylated” if the mean methylation of all assays of all genes was ≤ 25%. If the mean methylation was > 25% the samples were categorized as “Hypermethylated”.

**Statistical analysis**

Calculations were performed using R statistical software(25).
Comparisons between groups were assessed using Mann-Whitney Wilcoxon test for quantitative variables and Fisher’s test for qualitative variables. Paired tests were used for the comparison of the matched tumor samples.

Overall survival was defined as the time elapsed between surgery of the primary tumor and death or last follow-up visit.

All p-values were two-sided, and the level of significance was set at P < .05.
Results

Patient characteristics

Patient characteristics are presented in Table 1. Two different tumor samples were analyzed for each patient, including 24 primary tumor, 7 local recurrence and 21 distant metastasis. Compared to the Cochin cohort, patients from the Wuerzburg cohort included more males (10/12 vs 5/14, p=0.02) and more metachronous surgeries (11/12 vs 5/14, p=0.005). Age, ENSAT stage, Weiss score and Ki67 proliferation index were similar in the two cohorts. Median OS was 40.2 months in Cochin cohort and 30.2 months in Wuerzburg cohort. Two patients died from post-operative complications. All other patients received adjuvant mitotane after the first surgery. No patient received chemotherapy or radiation therapy before surgery of primary tumor or metastases.

Sequence mutations and copy number alterations profiles

Sequence mutations were determined using NGS and Sanger sequencing in 26 patients, and copy-number alterations were determined using SNP array in 14 patients. Combining sequence mutations and copy-number alterations, the most recurrently altered genes were ZNRF3 (7/28 samples), CDKN2A (6/28 samples), TP53 (10/52 samples), and CTNNB1 (10/52 samples, Figure 1, Table 2). No alteration of CDK4 and RB1 was observed. For 8/26 patients, no alteration was identified in the two tumor samples. Subclonal alterations were observed in 8/26 patients (31%) −4/14 in the Cochin cohort and 4/12 in the Wurzburg cohort−, with one or several gene alterations found in one ACC region but not in the other (Figure 1, Table 2, Supplementary Figure 2), including ZNRF3 (N=5), CDKN2A (N=2), TP53 (N=3), and CTNNB1 (N=2). For subclonal mutations - detected in only one ACC region -, the coverage depth of the negative region was at least 30x.

Chromosome alteration profiles
Chromosome alterations were determined using SNP array in 14 patients (Figure 2).

The percentage of tumor cells was estimated from SNP profiles and ranged from 40 to 100% (median 80%, Supplementary Table 2). The proportion of genome alteration ranged from 11.6% to 88.2%. The proportion of altered genome was not significantly different between primary tumors (median 38.8%) and matched local or metastatic recurrences (median 53.2%, p=0.30, Supplementary Table 2). Intratumor heterogeneity of chromosome alterations was observed to a variable extent in all matched tumor samples (Figure 2), and subclonal events could be identified within individual profiles (Supplementary Figure 3).

However, beyond these subclonal focal events, the global chromosome alteration profile remained stable. More precisely, chromosome alteration profiles were classified as “Noisy” for 16/28 samples and “Chromosomal” for 10/28 samples. Neither the “Noisy” nor the “Chromosomal” status changed between the different tumor locations. Especially, no “Noisy” subclone could be found in a “Chromosomal” tumor. Of note, for the 2 samples of one patient, the chromosome alteration profiles showed no extended LOH and an intermediate number of chromosome segments, and could not be reliably classified as “Chromosomal”, “Noisy” or “Quiet” profiles (Figure 2, Supplementary Table 2).

**Methylation profiles**

Methylation profiles were determined using bisulfite pyrosequencing of 4 genes (PAX5, PAX6, PYCARD, and GSTP1) in 12 patients.

Average methylation of 4 genes ranged from 3 to 72% and was not significantly different between primary tumor (median 31%) and matched local or metastatic recurrence (median 36%, p=0.72). Methylation profiles were classified as “Hypermethylated” in 10/24 samples and “Not hypermethylated” for 14/24 samples (Figure 3). Intratumor heterogeneity of methylation profile was observed in 2/12 patients (17%): in one case (patient F), the retroperitoneal metastasis was classified as “Hypermethylated” whereas the primary tumor was not; conversely, in the other case (patient H), the primary tumor was classified as “Hypermethylated” whereas the lung metastasis was not (Figure 3).
Association of intratumor heterogeneity with clinical and genomic features

The association between intratumor heterogeneity of gene alterations (sequence mutations and copy-number alterations in p53/Rb and Wnt/β-catenin pathways) and clinical and genomic features was tested in the 18 patients with at least one gene alteration in one of the two tumor samples.

Intratumor heterogeneity of gene alterations was not significantly associated with the origin of the samples used for comparison – primary tumor vs local or metastatic recurrence, two samples from primary tumor or two samples from local or metastatic recurrence - (p=0.48) or with the delay between surgeries of the matched tumor samples (p=0.37, Supplementary Table 3). However, no intratumor heterogeneity was observed in patients for whom the gene alterations profiles were analyzed in two samples from local or metastatic recurrence (N=4).

Intratumor heterogeneity of gene alterations was neither associated with clinical prognostic factors such as age (p=0.90), ENSAT stage (p=0.72) or Ki67 proliferation index (p=0.44, Supplementary Table 3), nor with tumor purity (80 vs 88% in samples without intratumor heterogeneity, p=0.49), nor with other genomic alterations, such as proportion of altered genome (62 vs 38% in samples without intratumor heterogeneity, p=0.16), average methylation of 4 genes (22 vs 11% in samples without intratumor heterogeneity, p=0.48), chromosome alterations or methylation profiles (p=1, data not shown).
Discussion

Molecular classification has recently emerged as a major prognostic factor in ACC(7, 8, 11), and targeted molecular markers, based on gene expression, chromosome alterations, methylation or gene alterations, have been proposed to complete the routine prognostic assessment(9, 10, 11).

In the present study, intratumor heterogeneity of targeted chromosome alterations, methylation profiles and gene alterations was evaluated by analyzing two different tumor samples for each patient. Intratumor heterogeneity of gene alterations and methylation profiles was identified in 8/26 (31%) and 2/12 (17%) patients, respectively. Intratumor heterogeneity was observed in the comparison of primary/local or metastatic recurrence and primary/primary samples, but not in that of two recurrence sites (N=4). Conversely, the prognostic classification based on chromosome alteration profiles was identical in the two tumor samples for all patients studied.

Strikingly, intratumor heterogeneity of gene alterations was observed in genes usually considered as “drivers” of adrenocortical tumorigenesis, such as TP53, CTNNB1 and ZNRF3. Indeed, alterations of these genes are almost mutually exclusive, found each in 20% of ACC(7, 8, 26). Germline mutations of TP53 are also known to predispose to ACC in part of Li Fraumeni syndrome(27). Moreover, the role of CTNNB1 mutations in adrenocortical tumorigenesis has been validated by functional experiments in cell lines and mouse models(28, 29). Therefore, intratumor heterogeneity of such gene alterations appears to go against the hypothesis of early clonal events that drive the tumorigenesis.

Several explanations can be raised to conciliate our findings with this hypothesis. First, p53/Rb or Wnt/ß-catenin alterations could be drivers of aggressive subclones, associated with metastatic spread and therefore predominant in poor prognosis tumors(30, 31). In this hypothesis, random sampling may have led to underestimate the frequency of such alterations in primary tumor compared to local recurrence and metastasis. In ACC, data regarding intratumor heterogeneity are scarce. Vatrano et al. compared the mutational profile from matched primary and recurrent/metastatic samples in 10 ACC using targeted NGS and found a high degree of intratumor heterogeneity, extended to prognostic-associated genes such as TP53, CTNNB1 and ZNRF3(32). Recently, Gara et al. reported a series of multiregion exome sequencing in 14 ACC patients(15) and observed a 37 to 57% overlap in genes that are mutated among different metastatic sites within the same patient. Focusing on genes associated
with prognostic value, no intratumor heterogeneity was observed for **CTNNB1** and **CDKN2A**, and no alteration of **TP53** and **ZNRF3** was found in this study. However, unlike in our study, no comparison with the primary tumor was performed. Of note, gene alterations were explored in two different recurrence sites for 4 patients in our study. Two of them exhibited gene alterations in p53/Rb or Wnt/β-catenin pathways, with no intratumor heterogeneity (Figure 1), which is in line with the hypothesis of driver mutations of the aggressive subclone. Our results are also consistent with literature in other cancer types, in which intratumor heterogeneity has been fully revisited by the advances of genomics in the last decade. Gerlinger et al. have performed exome sequencing in up to 14 different tumor regions of patients with metastatic renal-cell carcinoma(12) and showed that only one third of somatic mutations are shared by all tumor regions. Interestingly, intratumor heterogeneity was also observed for genes recurrently altered and considered as “drivers” of the disease, such **MTOR** and **SETD2**.

Several pan-cancer studies support a variable chronological timing of **CTNNB1** mutations(33, 34). In the TCGA ACC study, 5/9 **CTNNB1** mutations were late – i.e. occurring after whole-genome doubling- or subclonal events(8). Conversely, **TP53** mutations are usually described as early clonal events (34, 35). In a whole-exome analysis of paired primary tumors and metastases in 136 colorectal, lung or breast cancer patients, almost all **TP53** mutations (79/85, 93%) were fully clonal(35). In the TCGA ACC study, all 7 **TP53** mutations occurred before whole-genome doubling(8). In the present study, 2/6 **TP53** mutations showed intratumor heterogeneity. A similar result was reported by Vatrano et al., showing 3/7 private **TP53** mutations in matched primary and recurrent/metastatic samples(32). These results could be random due to the small number of patients, could suggest a technical artefact – for instance, all heterogeneous **TP53** mutations were found in FFPE samples – or a unique biology in ACC.

Finally, the hypothesis of revertant alleles may explain some cases of intratumor heterogeneity with alterations in “driver” genes found in the primary tumor, but not in recurrence. Some deletions of **BRCA2** mutation with restoration of the open reading frame have indeed been described as a mechanism of resistance to therapy in ovarian cancers(36).
Regarding targeted methylation markers, the prognostic classification was unstable for 2/12 patients in our cohort. CpG islands methylator phenotype (CIMP) was described in 50% of ACC and associated with poor prognosis(24, 37). This hypermethylated phenotype was also observed in other tumor types, such as colon cancer, paraganglioma and glioblastoma. In the last two cases, CIMP is related to the accumulation of an oncometabolite due to mutations in genes coding for metabolic enzymes, whereas in ACC, the origin of CIMP is still unknown. In a study evaluating the methylation profiles of 4 different tumor regions in glioblastoma patients(17), the global methylation profile, corresponding to IDH mutational status and its association to CIMP, was stable across the different tumor regions of a same patient. However, the methylation subclass varied from one tumor sample to another in nearly half of patients studied(17). In ACC, different levels of methylation were described among CIMP tumors(8, 37). This suggests a progressive rather than binary process, and may explain the 2 cases of intratumor heterogeneity of methylation pattern in our cohort. In the present study, we chose to focus on the 4-gene methylation marker that was previously validated in frozen and FFPE samples (9, 24). Targeted assessment of G0S2 methylation represents another prognostic marker in ACC (10). Whether this marker would be subject to intratumor heterogeneity remains to be determined.

Limitations of this study include the heterogeneity of the population study, mixing different sample comparisons –mostly primary-recurrence, but also primary-primary, and recurrence-recurrence comparisons- and different assessments of targeted markers in Cochin and Wuerzburg cohorts, resulting in small number of patients in each analysis. Therefore, the statistical power for the confrontation of mutational status with clinical and genomic features was limited. Moreover, prognostic markers based on gene expression were not evaluated in this study, due to the absence or insufficient amount of frozen tissue sample for RNA extraction. DNA-based markers were also assessed with different techniques, on frozen or FFPE samples, based on the local availability of tissue material and techniques. For instance, ZNRF3 mutations were identified by Sanger sequencing in 20/52 samples, whereas NGS was used for all remaining samples and other genes. Since Sanger sequencing has a lower sensitivity than NGS for detecting variants at low allelic ratio, this could have led to overestimate intratumor heterogeneity in patients with ZNRF3 mutations. However, only one patient was classified with intratumor heterogeneity based on the sole results of Sanger sequencing.
Finally, our study could suffer from a selection bias, as all patients were treated in referral centers and most were reoperated for the local or metastatic recurrence. Thus, the study patients may not reflect the general ACC population. For these reasons, our results are not definitive and should be validated in larger series.

Overall, our study shows that targeted molecular markers based on the pan-genomic profile, i.e. methylation and chromosome alteration profiles, seem more stable within different ACC regions than the gene alterations profile, and therefore more suitable for prognostic assessment. In a clinical perspective, intratumor heterogeneity of prognostic molecular markers must also be related to that observed for histo-prognostic markers such as Ki67 proliferation index(29). Thus, combining several molecular markers with clinical and pathological prognostic features remains the safest way to produce a robust prognostic classification(9, 11). Finally, intratumor heterogeneity of gene alterations should be taken into account for future therapeutics of ACC. With the recent development of cell-cycle and Wnt pathway inhibitors(38, 39), alterations of p53/Rb and Wnt/ß-catenin pathways could be considered as druggable targets in ACC in a near future. The present study suggests that the mutational status should be evaluated in progressive metastatic disease rather than in primary tumor for theranostic purpose.

In conclusion, intratumor heterogeneity was observed in 8/26 and 2/12 of ACC patients for targeted gene alterations profile and targeted methylation profile, respectively, but not for targeted chromosome alteration profile. Combination of multiple targeted molecular markers, along with clinical features, should be preferred to gene alterations profile alone for the prognostic assessment of ACC.
Declaration of interest, Funding and Acknowledgements

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Tables

**Table 1. Patients characteristics**
Results are expressed in median (range) for quantitative variables or numbers for qualitative variables.

**Table 2. Somatic mutations in matched ACC samples (Cochin and Wuerzburg cohorts, 26 patients)**
AA, amino acid; SNV, single nucleotide variation; P, sample from primary tumor; R, sample from local recurrence; M, sample from metastasis.

Patients from the Cochin cohort are numbered from 1 to 14 and patients from the Wuerzburg cohort are designated by letters A to L.
Figure Legends

**Figure 1. Detection of somatic mutations in two distinct ACC regions (Cochin and Wuerzburg cohorts, 26 patients)**

Patients from the Cochin cohort are numbered from 1 to 14 and patients from the Wuerzburg cohort are designated by letters A to L. Sequence mutations and gene copy number alterations are depicted with color in matched ACC samples for p53/Rb and Wnt/β-catenin genes. In each box, the lower left part represents the alterations of the first tumor sample and the upper right part represents the alterations of the second tumor sample.

P, sample from primary tumor; R, sample from local recurrence; M, sample from metastasis.

* mutations identified by Sanger sequencing

**Figure 2. Superimposed chromosome alterations profiles in two distinct ACC regions (Cochin cohort, 14 patients)**

Superimposed chromosome alteration profiles are represented in black and red plots for each patient. Different samples within the same patient are predominantly homogenous, especially regarding the 9 chromosome arms (in yellow) that were previously identified for classifying “Chromosomal” or “Noisy” prognostic profiles(11).

**Figure 3. Methylation profiles in two distinct ACC regions (Wuerzburg cohort, 12 patients)**

The cut-off of 25% in average methylation of 4 genes used to discriminate “Hypermethylated” from “Non-hypermethylated”(9, 24) samples is indicated in dashed red line.

P, sample from primary tumor; R, sample from local recurrence; M, sample from metastasis.
|                                | Total         | Cochin cohort | Würzburg cohort | p-value |
|--------------------------------|---------------|---------------|-----------------|---------|
| **Sex**                        |               |               |                 | 0.02    |
| Female                         | 11            | 9             | 2               |         |
| Male                           | 15            | 5             | 10              |         |
| **Age (years)**                |               |               |                 | 0.49    |
|                                | 44 (18 to 76) | 49 (24 to 76) | 44 (18 to 67)  |         |
| **ENSAT stage at diagnosis**   |               |               |                 | 0.15    |
| I                              | 4             | 4             | 0               |         |
| II                             | 10            | 4             | 6               |         |
| III                            | 6             | 2             | 4               |         |
| IV                             | 6             | 4             | 2               |         |
| **Weiss score**                |               |               |                 | 0.73    |
|                                | 6 (2 to 9)    | 6 (4 to 9)    | 6 (2 to 9)     |         |
| **Ki67 index (%)**             |               |               |                 | 0.27    |
|                                | 10 (0 to 70)  | 9 (0 to 70)   | 12 (2 to 40)   |         |
| **Origin of samples**          |               |               |                 | 0.34    |
| Primary / Recurrence or Metastasis| 20          | 9             | 11              |         |
| Primary / Primary              | 2             | 2             | 0               |         |
| Recurrence or Metastasis / Recurrence | 4         | 3             | 1               |         |
| **Delay between surgeries of the 2 samples** | |               |                 | 0.005   |
| Synchronous                    | 10            | 9             | 1               |         |
| Metachronous                   | 16            | 5             | 11              |         |
| Patient | Sample | Gene   | Alteration type | AA change | Allelic ratio |
|---------|--------|--------|----------------|-----------|---------------|
| 1       | P      | TP53   | nonsynonymous SNV | p.C176F  | 0.42          |
| M       | TP53   | nonsynonymous SNV | p.C176F  | 0.64      |
| 2       | P      | TP53   | nonsynonymous SNV | p.R342P  | 0.55          |
| R       | TP53   | nonsynonymous SNV | p.R342P  | 0.53      |
| 3       | P      | CTNNB1 | stopgain        | p.Y30X    | 0.39          |
| M       | CTNNB1 | stopgain | p.Y30X    | 0.48      |
| 4       | P      | CTNNB1 | nonsynonymous SNV | p.S45F   | 0.27          |
| M       | CTNNB1 | nonsynonymous SNV | p.S45P   | 0.44      |
| 8       | R      | ZNRF3  | stopgain        | p.Q167X   | 0.54          |
| M       | ZNRF3  | stopgain | p.Q167X    | 0.86      |
| 10      | P2     | ZNRF3  | frameshift deletion | p.A224fs | 0.77          |
| 11      | P      | TP53   | nonsynonymous SNV | p.R337C  | 0.81          |
| M       | TP53   | nonsynonymous SNV | p.R337C  | 0.93      |
| C       | P      | CTNNB1 | non-frameshift deletion | p.S45del | 0.26          |
| M       | CTNNB1 | non-frameshift deletion | p.S45del | 0.64      |
| E       | P      | ZNRF3  | frameshift deletion | p.E674Pfs*95 | 0.50          |
| M       | TP53   | nonsynonymous SNV | p.R248W  | 0.36      |
| F       | M      | TP53   | nonsynonymous SNV | p.R175H  | 0.38          |
| M       | ZNRF3  | splice site SNV | p.?      | 0.66      |
| G       | M      | TP53   | nonsynonymous SNV | p.R337C  | 0.89          |
| I       | P      | ZNRF3  | splice site SNV | p.?      | 0.50          |
| M       | ZNRF3  | nonsynonymous SNV | p.P179L  | 0.50      |
| J       | P      | CTNNB1 | non-frameshift deletion | p.E267del | 0.30          |
| P       | TP53   | nonsynonymous SNV | p.Q245S  | 0.81      |
| R       | CTNNB1 | non-frameshift deletion | p.E267del | 0.20      |
| R       | TP53   | nonsynonymous SNV | p.Q245S  | 0.34      |
| L       | P      | CTNNB1 | nonsynonymous SNV | p.S45P   | 0.44          |
| R       | CTNNB1 | nonsynonymous SNV | p.S45P   | 0.85      |
### Supplementary Table 1. Description of tissue samples

| Patient | Sample | Tissue of origin               | Patient | Sample | Tissue of origin               |
|---------|--------|--------------------------------|---------|--------|--------------------------------|
| 1       | P      | Primary tumor                  | A       | P      | Primary tumor                  |
|         | M      | Synchronous metastasis         |         | M      | Synchronous metastasis         |
| 2       | P      | Primary tumor                  | B       | R      | Local recurrence               |
|         | R      | Local recurrence               |         | M      | Metachronous metastasis        |
| 3       | P      | Primary tumor                  | C       | P      | Primary tumor                  |
|         | M      | Metachronous metastasis        |         | M      | Metachronous metastasis        |
| 4       | P      | Primary tumor                  | D       | P      | Primary tumor                  |
|         | M      | Metachronous metastasis        |         | M      | Metachronous metastasis        |
| 5       | P      | Primary tumor                  | E       | P      | Primary tumor                  |
|         | M      | Synchronous metastasis         |         | M      | Metachronous metastasis        |
| 6       | P      | Primary tumor                  | F       | P      | Primary tumor                  |
|         | M      | Metachronous metastasis        |         | M      | Metachronous metastasis        |
| 7       | R      | Local recurrence               | G       | P      | Primary tumor                  |
|         | M      | Synchronous metastasis         |         | M      | Metachronous metastasis        |
| 8       | R      | Local recurrence               | H       | P      | Primary tumor                  |
|         | M      | Synchronous metastasis         |         | M      | Metachronous metastasis        |
| 9       | M1     | Metachronous metastasis        | I       | P      | Primary tumor                  |
|         | M2     | Metachronous metastasis        |         | M      | Metachronous metastasis        |
| 10      | P1     | Primary tumor                  | J       | P      | Primary tumor                  |
|         | P2     | Primary tumor                  |         | R      | Local recurrence               |
| 11      | P      | Primary tumor                  | K       | P      | Primary tumor                  |
|         | M      | Synchronous metastasis         |         | M      | Metachronous metastasis        |
| 12      | P      | Primary tumor                  | L       | P      | Primary tumor                  |
|         | M      | Synchronous metastasis         |         | R      | Local recurrence               |
| 13      | P      | Primary tumor                  |         |       |                                |
|         | M      | Metachronous metastasis        |         |       |                                |
| 14      | P1     | Primary tumor                  |         |       |                                |
|         | P2     | Primary tumor                  |         |       |                                |
Supplementary Table 2. Chromosome alterations in matched ACC samples (Cochin cohort, 14 patients)

LOH, loss of heterozygosity

| Patient | Sample | Tumor cells (%) | Altered genome (%) | Number of selected chromosome arms with complete LOH | Chromosome alteration profile |
|---------|--------|-----------------|--------------------|-----------------------------------------------|-------------------------------|
| 1       | P      | 71              | 26.6               | 0                                             | Noisy                         |
|         | M      | 56              | 16.1               | 1                                             | Noisy                         |
| 2       | P      | 67              | 38.8               | 2                                             | Noisy                         |
|         | R      | 74              | 38.1               | 2                                             | Noisy                         |
| 3       | P      | 95              | 20.7               | 1                                             | Noisy                         |
|         | M      | 92              | 24.1               | 1                                             | Noisy                         |
| 4       | P      | 92              | 64.4               | 1                                             | Noisy                         |
|         | M      | 79              | 66.6               | 0                                             | Noisy                         |
| 5       | P      | 95              | 25.7               | 3                                             | Noisy                         |
|         | M      | 84              | 31                 | 3                                             | Noisy                         |
| 6       | P      | 72              | 40                 | 2                                             | Noisy                         |
|         | M      | 88              | 61                 | 4                                             | Noisy                         |
| 7       | R      | 40              | 62.5               | 9                                             | Chromosomal                   |
|         | M      | 46              | 83.8               | 9                                             | Chromosomal                   |
| 8       | R      | 60              | 77.2               | 9                                             | Chromosomal                   |
|         | M      | 92              | 79.9               | 9                                             | Chromosomal                   |
| 9       | M1     | NA              | 57.3               | 0                                             | Noisy                         |
|         | M2     | 91              | 57.4               | 0                                             | Noisy                         |
| 10      | P1     | 67              | 63                 | 9                                             | Chromosomal                   |
|         | P2     | 80              | 80.8               | 9                                             | Chromosomal                   |
| 11      | P      | 88              | 68.6               | 9                                             | Chromosomal                   |
|         | M      | 100             | 53.2               | 9                                             | Chromosomal                   |
| 12      | P      | 82              | 21.8               | 2                                             | Noisy                         |
|         | M      | 80              | 56.9               | 2                                             | Noisy                         |
| 13      | P      | 51              | 74.3               | 9                                             | Chromosomal                   |
|         | M      | 80              | 88.2               | 9                                             | Chromosomal                   |
| 14      | P1     | 73              | 11.6               | 1                                             | Undetermined                  |
|         | P2     | 88              | 11.7               | 1                                             | Undetermined                  |
Supplementary Table 3. Association of intratumor heterogeneity of somatic mutations with clinical and genomic features

|                                | No heterogeneity of somatic mutations | Heterogeneity of somatic mutations | p-value |
|--------------------------------|--------------------------------------|-----------------------------------|---------|
| **Sex**                        |                                      |                                   |         |
| Female                         | 4                                    | 5                                 | 0.64    |
| Male                           | 6                                    | 3                                 |         |
| **Age (years)**                | 50 (24 to 73)                        | 50 (30 to 76)                     | 0.9     |
| **ENSAT stage at diagnosis**   |                                      |                                   |         |
| I                              | 2                                    | 0                                 | 0.72    |
| II                             | 3                                    | 4                                 |         |
| III                            | 2                                    | 2                                 |         |
| IV                             | 3                                    | 2                                 |         |
| **Weiss score**                | 6 (2 to 9)                           | 6 (5 to 9)                        | 0.96    |
| **Ki67 index (%)**             | 5 (0 to 70)                          | 10 (3 to 30)                      | 0.44    |
| **Origin of samples**          |                                      |                                   |         |
| Primary / Recurrence or Metastasis | 8                                  | 7                                 | 0.48    |
| Primary / Primary              | 0                                    | 1                                 |         |
| Recurrence or Metastasis / Metastasis | 2                                  | 0                                 |         |
| **Delay between surgeries of the 2 samples** | 5                                  | 2                                 | 0.37    |
| Synchronous                    |                                      |                                   |         |
| Metachronous                   | 5                                    | 6                                 |         |
Supplementary Figure Legends

**Supplementary Figure 1. Hematoxylin/eosin staining of the two distinct regions sampled for the molecular analysis of a bulky primary tumor in patient 14.**

A. eosinophilic cells component (P1), 100X magnification  
B. eosinophilic cells component (P1), 200X magnification  
C. clear cells component (P2), 100X magnification  
D. clear cells component (P2), 200X magnification

**Supplementary Figure 2. Identification of sub-clonal events in mutational profiles**

Intratumor heterogeneity of *CTNNB1* mutation (A) and *CDKN2A* homozygous deletion B) in patient 4: IGV screenshot (A) shows *CTNNB1* « hotspot » mutation (p.S45F) only in the primary tumor whereas chromosome alterations in 9p21 region (B) shows a homozygous deletion (points below the green line) of *CDKN2A* (red points) only in the metastasis.

**Supplementary Figure 3. Identification of sub-clonal events in chromosome alterations profiles**

Chromosome alterations profile (A) and GAP plot (B) in one part of the primary tumor (clear-cell component, P2) of patient 14: pink, dark blue and light blue represent clonal amplification (copy number equal to 3), clonal deletion (copy number equal to 1) and sub-clonal deletion (copy number between 1 and 2), respectively.
