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Analysis of histological and immunohistochemical patterns of benign and malignant adrenocortical tumors by computerized morphometry☆

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ABSTRACT

Diagnosis of benign and purely localized malignant adrenocortical lesions is still a complex issue. Moreover, histology-based diagnosis may suffer of a moment of subjectivity due to inter- and intra-individual variations. The aim of the present study was to assess, by computerized morphometry, the morphological features in benign and malignant adrenocortical neoplasms.

Eleven adrenocortical adenomas (ACA) were compared with 18 adrenocortical cancers (ACC). All specimens were stained with H&E, cellular proliferation marker Ki-67 and reticulin. We generated a morphometric model based on the analysis of volume fractions occupied by Ki-67 positive and negative cells (nuclei and cytoplasm), vascular and inflammatory compartment; we also analyzed the surface fraction occupied by reticulin. We compared the quantitative data of Ki-67 obtained by morphometry with the quantification resulting from pathologist's visual reading.

The volume fraction of Ki-67 positive cells in ACCs was higher than in ACAs. The volume fraction of nuclei in unit volume and the nuclear/cytoplasmic ratio in both Ki-67 negative cells and Ki-67 positive cells were prominent in ACCs. The surface fraction of reticulin was considerably lower in ACCs.

Our computerized morphometric model is simple, reproducible and can be used by the pathologist in the histological workup of adrenocortical tumors to achieve precise and reader-independent quantification of several morphological characteristics of adrenocortical tumors.

1. Introduction

Tumors of the adrenal cortex are mostly adenomas (ACA), often found incidentally (*incidentalomas*) during workup for unrelated indication [1,2]. ACA is usually a well-circumscribed nodular lesion with cells organized in alveoli, cords or nests similar to those of the normal adrenal cortex, with minimal pleomorphism and generally unremarkable mitotic activity [3].

On the other side, adrenocortical carcinoma (ACC) is a highly aggressive malignancy with an estimated worldwide prevalence of 4–12 cases per million adults and a five-year-survival ranging between 16 and 38% [4]. Microscopically, ACCs are composed of cell cords organized in large bands in a fine sinusoid network and have capsular and vascular infiltration. Necrosis and fibrosis are frequent. Nuclear pleomorphism is often prominent and the mitotic rate is quite variable [5].

Although several different scoring systems have been proposed to assess malignancy in adrenocortical tumors, Weiss score remains the most used in separating benign from malignant adrenocortical neoplasms [6]. This score counts 9 histopathologic criteria: eosinophilic ("dark") cytoplasm in more than 75% of tumor cells, a "patternless" diffuse architecture, necrosis, nuclear atypia, mitotic index above 5 per 50 high-power fields, atypical mitoses,

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Table 1
Characteristics of patients.

Lesion	*Dimensions (cm)*	*Age*	*M/F*
ACA	*4–11*	*28–59*	*4/7*
ACC	*5–19*	*25–68*	*5/13*

sinusoidal, venous, and capsular invasion [7]. An adrenocortical neoplasm is classified as malignant when it meets 3 or more criteria [8]. However, low Weiss score (2–3) generates ambiguous results, especially in small sized and purely localized lesions and in large tumors without invasive features or cellular atypia in which well-differentiated cells resemble those seen in ACA [9]. This "grey zone" needs further investigation to obtain a more precise characterization and to eventually reveal, if possible, an intermediate class of adrenocortical tumors. In this context, several studies already reported that ACCs with Weiss score 3 often do not recur; on the other side, adrenocortical tumors with Weiss score 2 with lung metastases have been described [10,11].

Several immunohistochemical markers have been proposed to improve the histological recognition of malignancy. Among molecular and phenotypic markers, the expression of Ki-67, a non-histonic protein involved in DNA replication, has been indicated as a useful marker of malignancy for adrenocortical tumors [12,13]. However, some overlaps between benign and malignant lesion have been described [14,15].

Furthermore, the disruption of the reticular network has been demonstrated to be present in the majority of ACCs [16] and an algorithm including reticulin disruption and additional parameters (mitotic index, necrosis and vascular invasion) has been proposed to simplify the diagnostic workup of adrenocortical tumors [17].

Nonetheless, histology-based diagnosis may suffer of a moment of subjectivity due to inter- and intra-individual variability. This is particularly evident when certain histological features (i.e. cell density, nuclear atypia and nuclear/cytoplasmic ratio, extent of architectural disruption) need to be quantified and subsequently integrated for interpretation in a semi-quantitative way. In this setting, relevant interobserver and intraobserver variability in histopathological evaluation of Ki-67 in several tumors has been documented [18–21].

To address this problem, morphometric analysis could minimize the subjectivity of the individual morphological observations.

In our study, we generated a computerized morphometric model to evaluate the morphological features of adrenocortical lesions stained with reticulin method and Ki-67. The quantitative data of Ki-67 expression obtained by computerized morphometry have also been compared with the quantification obtained by the pathologist. Furthermore, to assess the reproducibility of the morphometric evaluation, 10 randomly selected specimens were recounted by another blinded operator and compared with the initial counting.

2. Materials and methods

In this study, we retrospectively examined 11 ACAs (median size = 6.2 cm, range 5–16 cm) and 18 ACCs (median size = 11 cm, range 4–19 cm). Specimens were obtained from the archival files of 29 patients (9 M/20F, 18 ACCs and 5 ACAs from Niguarda Ca' Granda Hospital and 6 ACAs from Azienda Ospedaliera Universitaria San Luigi Gonzaga Orbassano) submitted to adrenal surgery (Table 1). According to Weiss score, it was found that all ACC but one possessed 3 or more of these criteria of malignancy, and 10 (55,5%) had six or more. The most frequent criteria seen in all the carcinomas were nuclear grade III or IV based on Fuhrman criteria (83,3%), diffuse architecture (77,7%) and clear or vacuolated cells comprising 25% or less of tumor (77,7%). One ACC case had Weiss score 2 (nuclear grade III and capsular invasion) with invasion of vena cava. ENSAT stage in ACC was I (61,1%), II (27,8%) and III (11,1%). Five patients (Weiss score 7, ENSAT III-IV) had metastases, 6 patients (Weiss score 4–7, ENSAT II-IV) had recurrence after treatment.

The ACA showed 0–2 criteria of malignancy (high nuclear grade was the most frequent feature).

All patients were regularly followed up after surgery.

ACAs and ACCs were defined grossly and microscopically following the criteria and the nomenclature system of pathological features proposed by Weiss et al. (3) All primary malignant adrenal tumors reviewed as part of this study demonstrated three or more of the histopathologic criteria needed for the diagnosis of ACC as defined by Weiss.

All specimens have been reviewed by two experienced pathologists blinded to clinical history or outcome.

In all cases investigated, from a representative formalin-fixed, paraffin-embedded block three consecutive 4 μm thick sections were obtained.

Each section series was stained with different methods:

- ○ Hematoxylin-Eosin (HE) to confirm the diagnosis of adrenal nodular lesions.
- ○ Monoclonal antibodies against Ki-67 (Rabbit anti-human Ki-67 monoclonal Ab Clone SP6, 1:400 – Thermo Fisher Scientific, CA – USA) to assess volume fractions of Ki-67 positive cells [22].
- ○ Silver impregnation for reticulin fibers using the Gordon and Sweets method to assess the tumor structural network [23].

Table 2
Morphometric model.

● Ki-67 (40 X magnification):
□ Volume fractions referred to the entire tissue occupied by:
♦ nuclei of Ki-67 negative cells ($V_v$nucneg)
♦ cytoplasm of Ki-67 negative cells ($V_v$cytneg)
♦ nuclei of Ki-67 positive cells ($V_v$nucpos)
♦ cytoplasm of Ki-67 positive cells ($V_v$cytpos)
♦ ratio nuclei/cytoplasm of Ki-67 negative cells (N/Cneg)
♦ ratio nuclei/cytoplasm of Ki-67 positive cells (N/Cpos)
♦ other structure (vessels and inflammatory infiltrate) ($V_v$other)
● **Reticulin (10 X magnification):**
□ **Surface fraction (surface in unit volume) of reticulin (Svret)**

The variables assessed by morphometry are listed in Table 2 and include cellular compartment, fibrous stroma, and vascular supply in both groups of lesions. The morphometric analysis was performed at two magnification levels using an interactive approach with a high-resolution computerized image analyzer (Kontron-Zeiss KS 400) that included a color video camera (JVC TK-C1381EG) attached to a light microscope equipped with a motorized stage with 10X and 40X objectives and auto-focusing software. The software system, tailored on the research needs of our team, consisted of different programs to control interactively the scanning stage and autofocus functions [24–26].

The analysis works as follows. Images acquired by video camera are displayed on the monitor. The analyzer automatically superimposes to each microscopic field different grids of points and lines, included in a test area 504 × 504 pixels, allowing an evaluation of the stereological variables [27,28].

The observer can interactively apply techniques of enhancement for a better definition of the different structures. It is also possible to exclude fields in which the tissue section may not be suitable for analysis due to technical artifact. An algorithm automatically controls the scanning stage operation in order to avoid duplicate measurements of the same structures.