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Original contribution

MicroRNA expression patterns in adrenocortical carcinoma variants and clinical pathologic correlations☆,☆☆

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Summary Several microRNAs (miRNAs) were shown to be deregulated in adrenocortical carcinoma (ACC) as compared with adenoma, but a detailed assessment of their expression in its histologic variants and correlation with clinicopathologic characteristics has not been performed, so far. Our aim was to assess the expression of 5 selected miRNAs (*IGF2* gene–related miR-483-3p and 5p and hypoxia-induced miR-210, miR-195, and miR-1974) in a series of 51 ACCs (35 classical, 6 myxoid, and 10 oncocytic) as compared with clinical and pathologic features and immunohistochemical expression of prognostic markers, including steroidogenic factor 1, p53, β-catenin, and glucose transporter 1. Oncocytic carcinomas had a reduced expression of miR-483-3p ($P = .0325$), miR-483-5p ($P = .0175$), and miR-210 ($P = .0366$), as compared with other histotypes. Overexpression of miR-210 was associated with the presence of necrosis ($P = .0035$), high Ki-67 index ($P = .0013$), and high glucose transporter 1 expression ($P = .0043$), whereas an inverse correlation with mitotic rate was observed in cases with high miR-493-3p ($P = .0191$) and miR-1974 ($P = .0017$) expression. High miR-1974 was also associated with low Ki-67 ($P = .0312$) and European Network for the Study of Adrenal Tumors stage ($P = .0082$) and negative p53 ($P = .0013$). At univariate analysis myxoid/classic histotype ($P = .026$), high miR-210 ($P = .0465$), high steroidogenic factor 1 protein ($P = .0017$), high Ki-67 ($P = .0066$), and high mitotic index ($P = .0006$) were significantly associated the shorter overall survival, the latter being the sole independent prognostic factor at multivariate analysis ($P = .017$). In conclusion, (*a*) miR-483-3p, miR-483-5p, and miR-210 are differentially expressed in ACC variants, and (*b*) high miR-210 is associated with clinicopathologic parameters of aggressiveness and a poor prognosis.
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1. Introduction

Adrenocortical carcinoma (ACC) is a rare malignancy with a highly aggressive biological behavior (20%-40% 5-year survival) [1,2]. The diagnosis of ACC is currently based on the Weiss system [3,4], a scoring procedure that can easily classify most cases but is particularly challenged in tumors with incomplete morphologic signs of malignancy, even if used by experts and specifically trained pathologists [5] and in ACC morphologic variants, that is, the myxoid and the oncocytic [2]. An interobserver validation study recently showed that the reticulin algorithm, previously proposed as a diagnostic approach alternative to the Weiss system, is highly accurate and reproducible to define malignancy also in ACC special histotypes [6]. However, although the morphologic distinctiveness of myxoid and oncocytic variants has already been thoroughly characterized and reviewed [7–10], their immunohistochemical and molecular phenotype has been addressed so far by very few studies only [8,10–13].

microRNAs (miRNAs) are short noncoding RNAs, 18 to 25 nucleotides in length, which regulate gene expression either by posttranscriptional regulation of gene expression leading to target messenger RNA degradation or by the repression of its translation with consequent decrease in the particular protein levels or even by up-regulation of their targets [14].

To date, several studies analyzed the miRNA expression profile in adrenocortical neoplasms, mainly aimed at finding those useful to differentiate adenomas from carcinomas. However, the expression of none of these miRNAs has been tested within the group of ACCs in terms of variability among variants or detailed association with clinical or pathologic features. Therefore, we designed a study to explore the expression of 5 miRNA in a series of 51 ACCs (including 35 classical, 6 myxoid, and 10 oncocytic) in comparison with clinical and pathologic features and immunohistochemical expression of markers related to biological or clinical aggressiveness, including steroidogenic factor 1 (SF-1), p53, β-catenin, and glucose transporter 1 (GLUT-1). Three miRNAs were selected because they are most consistently reported to be deregulated in both tissue and serum of ACC patients: miR-483-3p and 5p, which are transcribed from an intronic sequence of *IGF2* gene and are commonly overexpressed in ACC and miR-195, which is conversely down-modulated in ACC [15–20]; moreover, due to the peculiar metabolic and ultrastructural features of oncocytic cells, which are the hallmark of the oncocytic variant, 2 additional miRNAs—significantly deregulated in adrenocortical cancer—were selected based on their involvement in the hypoxic pathway (miR-210) [21] or a peculiar mitochondrial localization (miR-1974) [22].

We here show that (*a*) miR-483-3p, miR-483-5p, and miR-210 are differentially expressed among histologic variants of ACC, and (*b*) high miR-210 levels are associated with clinical and pathologic parameters of aggressiveness (necrosis and Ki-67 proliferation index) and a shorter overall survival.

2. Materials and methods

2.1. Tissue collection

Fifty-one adrenocortical tumors having a Weiss score at least 3 [3,4] (including 6 myxoid and 10 oncocytic) were retrieved from the pathology files of the University of Turin. The 10 oncocytic tumors were also reclassified according to the Lin-Weiss-Bisceglia scheme [9], and all proved malignant even with this more accurate score. A control series of 47 unpublished conventional adrenocortical adenomas (all with a Weiss score <1) was also tested. All these cases had paraffin-embedded tissue blocks suitable for miRNA extraction. Most ACC patients were treated at our institution, which serves as a referral center for this disease in Italy. The histopathologic features of all cases belonging to this data set have already been characterized as detailed in previous publications by our group [6,8,10,13,23,24]. For all of them, clinical data about treatment and outcome were updated (Table 1). The study received ethical approval from the local review board of our institution.

2.2. miRNA expression analysis by means of real-time polymerase chain reaction

miRNA were extracted from paraffin-embedded tissues using miRNeasy Kit (Qiagen srl Italy, Milan, Italy) according to manufacturer's instructions. Complementary DNA was synthesized with specific stemloop primers using 100 ng total RNA in 15-μL total reaction. The reaction consisted of the following components: 100 mmol/L deoxyribonucleotide triphosphates, 10× reverse transcriptase Buffer, RNase Inhibitor, 5× TaqMan (Life Technologies, Carlsbad, CA) microRNA reverse transcriptase primer, and Multiscribe reverse transcriptase enzyme and was carried out in a thermal cycler machine (Eppendorf, Hamburg, Germany) at the following conditions: 16°C for 30 minutes, 42°C for 30 minutes, 85°C for 5 minutes, and 4°C for 10 minutes.

The following Taqman microRNA assays were used by means of real-time polymerase chain reaction: hsa-miR-483-5p (code 002338), hsa-miR-483-3p (code 002339), hsa-miR-210 (code 000512), hsa-miR-195 (code 000494), and hsa-miR-1974 (code 121209_mat) (Life Technologies, Carlsbad, CA). Results were normalized using the $2^{-\Delta\Delta>CT}$ method [25] using expression of U6-small nuclear RNA and the value for each miRNA in the case with the lowest expression as the reference. Reactions were carried out according to the following parameters in an ABI Prism 7900HT Sequence Detection System (Life Technologies): 95°C for 10 minutes, 95°C for 15 seconds, and 60°C for 60 seconds for 40 cycles.

2.3. Immunohistochemistry

Of 51 ACCs herein analyzed for miRNA expression, 46 were included in Tissue MIcroArrays assembled as previously described [13] using 3 cores with a diameter of 2 mm from

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