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Title:

AGO2 Protein: A Key Enzyme in the miRNA Pathway as a Novel Biomarker in Adrenocortical Carcinoma.

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Abstract:

Adrenocortical carcinoma (ACC) is a rare and aggressive malignancy characterised by diagnostic challenges, high recurrence rates, and poor prognosis. This study explored the role microRNA (miRNA) processing genes in ACC, and their potential role as diagnostic and prognostic biomarkers. We analysed the mRNA expression levels of miRNA machinery components (DROSHA, DGCR8, XPO5, RAN, DICER, TARBP2 and AGO2) utilising mRNA-Seq data from The Cancer Genome Atlas (TCGA) and The Genotype-Tissue Expression (GTEx) projects. Additionally, protein levels were quantified in tissue samples from the Kolling Institute of Medical Research's tumour bank. Our results demonstrated that among all miRNA processing components, AGO2 exhibited significant overexpression in ACC compared to the normal adrenal cortex (NAC) and benign adrenal adenoma (AA) ($p < 0.001$). Kaplan–Meier survival analysis indicated that higher AGO2 expression correlated with significantly worse overall survival in ACC patients (HR 7.07, $p < 0.001$). Among 32 cancer types in TCGA, the prognostic significance of AGO2 was most prominent in ACC. This study is the first to report AGO2's potential as a diagnostic and prognostic biomarker in ACC, emphasising its significance in ACC pathogenesis and potential application as a non-invasive liquid biopsy biomarker.

Introduction

Adrenocortical carcinoma (ACC) is a rare and highly aggressive malignancy of the adrenal gland. Five-year survival rates vary based on disease stage at diagnosis, ranging from 60-80% for localized tumours to 0-28% for metastatic disease (Fassnacht *et al.*, 2018). Currently, surgical resection remains the only curative therapeutic option. For unresectable disease, systemic therapy is recommended by clinical practice guidelines; however, the efficacy of these treatments is limited, with objective response rates of less than 25% and significant side effects(Fassnacht *et al.*, 2018; Turla *et al.*, 2022). Even after curative resection, disease recurrence occurs in more than 60% of patients and poses a significant therapeutic challenge (Amini *et al.*, 2016). To date, two comprehensive multi-omics studies have laid the foundation for understanding the molecular

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classification of adrenocortical carcinoma (ACC) and its prognostic implications (Assié *et al.*, 2014; Zheng *et al.*, 2016). Zheng and colleagues classified the molecular signature of ACC into three groups based on a Cluster of Cluster (CoC) analysis of DNA copy number, DNA methylation, mRNA expression and miRNA expression — COC1, COC2, and COC3 — each reflecting distinct prognostic outcomes, with COC1 showing the best prognosis and COC3 showing the worst. Moreover, despite advances in the genomic characterisation of ACC (Assié *et al.*, 2014; Zheng *et al.*, 2016), there are currently no biomarkers that facilitate diagnosis, pathological prognostication, or monitoring for recurrent disease after curative resection(Fassnacht *et al.*, 2016; Sinclair *et al.*, 2020; Hazimeh *et al.*, 2021).

MicroRNAs (miRNAs) are small non-coding RNAs that regulate more than 60% of protein coding genes by interacting with messenger RNA (mRNA) (Friedman *et al.*, 2009). The differential expression of miRNAs between ACC and adrenal adenoma has recently emerged as a potential diagnostic and prognostic indicator. Specific miRNAs, such as the upregulation of miR-503, miR-210, miR-483-5p, and miR-483-3p and the downregulation of miR-195, miR-497, and miR-335, have been identified as potential markers for ACC (Decmann *et al.*, 2020). However, the lack of significant differences in the expression of hsa-miR-483-3p and hsa-miR-483-5p between adrenal myelolipoma and ACC limits their clinical utility (Decmann *et al.*, 2018). Furthermore, conflicting patterns of miRNA expression in ACC and adrenocortical adenoma (AA) have been reported (Özata *et al.*, 2011; Koperski *et al.*, 2017). These discrepancies highlight the complexity of miRNA regulation in ACC and the need for standardized quantification protocols and rigorous validation. Currently, the utility of miRNAs as biomarkers is limited by their low expressed concentrations, lack of standardised analytical methodologies and lack of specificity to tumour types(Mytareli *et al.*, 2021).

The miRNA biogenesis pathway consists of tightly regulated, interdependent steps involving key components such as DGCR8, Drosha, Exportin-5 (XPO5), RAN, Dicer1, TARBP2, and AGO2, which are essential for miRNA maturation and function. This pathway has previously been extensively described (Moore and Blobel, 1993; Bernstein *et al.*, 2001; Hutvágner *et al.*, 2001; Yi *et al.*, 2003;

Han *et al.*, 2004; Lee *et al.*, 2004; Chendrimada *et al.*, 2005). In various cancers, such as clear cell renal carcinoma (Lee *et al.*, 2019), ovarian carcinoma (Vaksman *et al.*, 2012), leiomyosarcoma (Papachristou *et al.*, 2012), and breast cancer (Yan *et al.*, 2012), deregulation of miRNA-processing complexes has been observed, indicating their potential role in tumorigenesis. In this study, we evaluated the expression of microRNA (miRNA) biogenesis components in adrenocortical carcinoma (ACC). Among these components, Argonaute 2 (AGO2)—a key regulator directing miRNAs to their target genes and modulating gene expression at the post-transcriptional level (Hutvagner and Simard, 2008). —emerged as a candidate for further investigation. Through a comprehensive analysis of AGO2 and related miRNA genes, we aimed to explore their potential as novel diagnostic and prognostic biomarkers for ACC.

2. Materials and Methods:

2.1. RNA-Seq Data analysis for miRNA biogenesis genes in ACC:

We obtained RNA-Seq data from two public repositories: The Cancer Genome Atlas (TCGA) for cancer samples and The Genotype-Tissue Expression (GTEx) project for normal tissue samples. Our bioinformatic analysis focused on the mRNA expression of core components in the miRNA biogenesis pathway, specifically AGO2, DGCR8, XPO5, RAN, DROSHA, DICER, and TARBP2, in adrenocortical carcinoma (ACC). Normalized RNA sequencing (RNA-seq) data specific to miRNA biogenesis genes for normal adrenal cortical tissue were obtained from the Genotype-Tissue Expression (GTEx) project and from The Cancer Genome Atlas (TCGA) for adrenocortical carcinoma (ACC). The TNMplot bioinformatics web tool was used for data retrieval(Bartha and Győrffy, 2021).

2.2. Survival analysis

Survival analysis paired gene expression data and survival data from The Cancer Genome Atlas (TCGA), using the Encyclopedia of RNA Interactomes (ENCORI) database (Li *et al.*, 2014). Kaplan-Meier survival analysis was performed on the UCSC Xena platform(Goldman *et al.*, 2020). To explore

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the specificity of the prognostic value of AGO2 expression for ACC, survival data for 32 different cancers, including clinicopathological data, were obtained from the TCGA.

2.3. Tumour samples:

The study received ethics approval from the Northern Sydney Local Health District Human Research Ethics Committee (2020/ETH01931). Tissue samples, including adrenocortical carcinoma (ACC), benign adrenocortical adenoma (AA), and normal adrenal cortex (NAC) samples, were obtained from the Tumour Bank of the Kolling Institute of Medical Research. The Kolling Institute Tumour Bank Access Committee granted access to these samples (reference NETBMC #20-49). All participating patients provided informed consent for the use of their tissue samples and the collection of associated clinical data. At the time of adrenalectomy, tissue samples were immediately snap-frozen in liquid nitrogen and subsequently stored at -80°C. All ACC samples utilized in this study were histologically confirmed according to accepted diagnostic criteria (https://www.rcpa.edu.au/Library/Practising-Pathology/Structured-Pathology-Reporting-of-Cancer/Cancer-Protocols).

2.4. Protein expression analysis:

Snap-frozen tissue samples, including 15 NAC, 15 AA, and 15 ACC, were obtained from the Kolling Institute Tumour Bank. Tissue homogenates were prepared by washing the tissue with pre-cooled phosphate-buffered saline (PBS) buffer (0.01M, pH=7.4). The tissue samples were then homogenized in Lysing Matrix A tubes (MP Biomedicals, Australia). Homogenization was performed using a FastPrep-24™5G (MP Biomedicals) bead beating grinder and lysis system according to the manufacturer's guidelines. Protein expression levels of miRNA biogenesis genes were measured using Human Protein ELISA Kits according to the manufacturer’s instructions, and included AGO2, DGCR8, DROSHA, RAN, XPO5 (Abebio-Co. Ltd.) and TARBP2 and DICER1 (Fine Biotech Co., Ltd.). Protein concentrations were measured by comparing the optical density to standard controls using a microplate reader (TECAN Spark absorbance reader).

2.5. Analysis of clinicopathological parameters and AGO2 expression:

We examined the relationships between AGO2 expression and key clinicopathological parameters, including age, sex, overall survival status, Weiss score, adrenal hormone excess and tumour stage. mRNA expression data for AGO2 and clinicopathological information were obtained from The Cancer Genome Atlas (TCGA) for 79 adrenocortical carcinoma (ACC) patients (Cerami *et al.*, 2012). Independent protein expression data were obtained from a cohort of 15 patients via the Kolling tumour bank.

2.6. miRNA-AGO2 correlation analysis:

We identified the top miRNAs highly expressed in TCGA-ACC patient clusters that are associated with distinct prognostic outcomes. The expression levels of these selected miRNAs were then correlated with AGO2 mRNA expression within the same patient cohort. For the identification of highly expressed miRNAs, we utilized supplementary data from the TCGA-ACC (The Cancer Genome Atlas - Adrenocortical Carcinoma) project (Zheng *et al.*, 2016) and assessed the correlation of these identified miRNAs with AGO2 mRNA expression levels using the ENCORI platform (The Encyclopedia of RNA Interactomes) (Li *et al.*, 2014). Our objective through this approach was to examine the correlation between the selected miRNAs and AGO2 expression, contributing to our understanding of AGO2's role in ACC pathogenesis.

2.7. Statistical analysis

Statistical analysis was performed using GraphPad Prism, version 9 (GraphPad Software, CA, USA). For gene expression data analysis, two-way analysis of variance (ANOVA) was used to compare the expression levels between groups. The log-rank test was used to compare survival outcomes between groups; for both gene expression and gene survival analysis, a p-value of <0.05 was considered statistically significant. To explore the correlation between gene expression and tumour staging in ACC, one-way ANOVA was utilized with a p-value threshold of < 0.05. ELISA absorbance levels were interpreted based on the construction of a standard curve in Microsoft Excel (Version

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2306 Build 16.0.16529.20166) and Curve Expert Basic (V.1.4-USA), with protein levels compared using one-way ANOVA and a p-value threshold of < 0.05. The receiver operating characteristic (ROC) curve was used to determine the optimal cut-off point for AGO2 protein levels, balancing sensitivity and specificity in the diagnosis of ACC.

Additionally, DataTab (https://datatab.net/) was utilized to perform statistical analyses of AGO2 mRNA expression in the TCGA-ACC cohort and AGO2 protein concentration in the collected ACC cohort. Independent t-tests were applied to assess the significance of correlations between AGO2 expression levels and various clinicopathological parameters, including age at diagnosis, sex, tumour stage, Weiss score, and overall survival outcomes. A significance threshold was established at a p-value of <0.05.

3. Results

3.1. Differential expression of miRNA biogenesis genes in ACC and normal adrenal cortex:

According to the RNA-Seq data from the GTEx project and TCGA, AGO2, RAN, and TARBP2 were significantly upregulated in ACC samples compared to normal adrenal cortex samples ($p <= 0.001$). Conversely, DGCR8 expression was slightly higher in the normal adrenal cortex than in ACC ($p=0.014$). No statistically significant differences were observed in the expression levels of DROSHA ($p=0.24$), DICER1 ($p=0.19$), and XPO5 ($p=0.66$) (Figure 1).

3.2. Among all miRNA biogenesis genes, AGO2 is the strongest prognostic indicator in ACC:

To assess the prognostic value of miRNA biogenesis genes in adrenocortical carcinoma (ACC), we utilized RNA-seq data from The Cancer Genome Atlas (TCGA). For the survival analysis, cancer samples were divided into two groups based on the median expression of each gene, as per the guidelines provided by ENCORI. Among the genes involved in the miRNA biogenesis pathway, AGO2

emerged as the strongest prognostic indicator in ACC, exhibiting a hazard ratio (HR) of 7.07 and a log-rank test p-value of 2.8e-06 (Figure 2). The Kaplan-Meier analysis further validated the strong association of AGO2 with poor prognosis in ACC patients (Figure 3). Other genes, such as DGCR8, XPO5, and RAN, also demonstrated prognostic potential, but to a lesser extent, with HRs of 5.9 ($p<0.0001$), 4.25 ($p=0.0004$), and 5.06 ($p=0.0001$), respectively. TARBP2 showed a weaker prognostic association, with an HR of 2.82 ($p=0.014$). On the other hand, DROSHA and DICER did not exhibit significant prognostic correlations, with HRs of 0.93 ($p=0.85$) and 1.24 ($p=0.57$), respectively.

3.3. The prognostic significance of the AGO2 gene in ACC is distinct from that in other cancers:

The prognostic correlation of AGO2 gene expression was strongest in ACC (HR 7.07, p=2.8e-06) compared to the 31 other TCGA cancer types studied. Although AGO2 gene expression held prognostic relevance in cholangiocarcinoma (HR 0.38, p=0.044), renal cell carcinoma (HR 2.15, p=0.016), mesothelioma (HR 2.36, p=0.00053), sarcoma (HR 1.71, p=0.0092) and endometrial carcinoma (HR 1.83, p=0.0052), in none of these other cancer types did AGO2 demonstrate such a significant prognostic impact as in ACC (Supplementary Table 1).

3.4. High AGO2 protein expression in ACC compared to benign and normal adrenal cortex:

AGO2 protein concentration was significantly higher in ACC than in adrenal adenoma or normal adrenal cortex ($p<0.0001$). Furthermore, there was no significant difference in AGO2 protein expression between normal and benign tumour (Figure 4). In contrast, XPO5, RAN, and DICER1 protein expression levels were significantly lower in ACC tissue homogenate samples compared to the non-malignant tissue samples ($p < 0.001$). No statistically significant differences were observed in the protein expression levels of DROSHA, DGCR8, or TARBP2 between the malignant and non-malignant groups.

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3.4.1. ROC analysis and specific cut-off point determination:

To explore the appropriate diagnostic threshold for determining the level of the AGO2 protein in ACC compared to non-malignant tissue, we performed receiver operating characteristic (ROC) curve analysis. The area under the curve (AUC) was 0.95 (95% CI: 0.86 to 1.00), indicating high diagnostic accuracy. Using a cut-off point of >3.9 ng/ml for AGO2 protein expression, a sensitivity of 89% (95% CI: 57% to 99%) and a specificity of 80% (95% CI: 55% to 93%) were achieved (Figure 5).

4. Associations between clinicopathological characteristics and AGO2 expression:

The prognostic potential of AGO2 in ACC was further explored by correlating clinicopathological characteristics with AGO2 mRNA expression in the TCGA-ACC cohort (Cerami *et al.*, 2012) and with the concentration of the AGO2 protein in a cohort from the Kolling Tumour Bank. The associations between clinicopathological characteristics and AGO2 mRNA expression and protein concentration are shown in supplementary Table 2. Our findings indicate that neither AGO2 gene expression ($p=0.672$) nor protein concentration ($p=0.833$) significantly correlates with age at diagnosis, suggesting that their prognostic relevance is not influenced by patient age. Similarly, sex did not significantly affect AGO2 levels in either analysis (gene expression, $p=0.254$; protein concentration, $p=0.484$). Notably, overall survival status was significantly associated with AGO2 levels; patients who were deceased exhibited higher levels of AGO2, both at the gene ($p<0.001$) and protein levels ($p=0.009$). The Weiss score, which reflects tumour aggressiveness, further confirmed this finding, with higher scores correlating with elevated AGO2 expression (gene $p=0.003$, protein $p=0.008$). Additionally, AGO2 expression levels varied significantly with pathological stage, with advanced-stage tumours (III-IV) showing increased levels compared to early-stage tumours (I-II) (gene $p=0.011$, protein $p=0.004$).

When correlating AGO2 mRNA expression within the TCGA-ACC dataset, which categorizes adrenocortical carcinoma (ACC) into three distinct molecular subtypes (CoC1, CoC2, and CoC3), we

observed notable prognostic disparities. Specifically, in the COC1 group, with a disease progression rate of 7%, the mean AGO2 mRNA expression was -0.16 ± 0.91 (log2). In the COC2 group, with a disease progression rate of 56%, the mean expression was -0.18 ± 1.14 (log2). Most notably, the COC3 group, characterized by the most adverse outcomes and a high disease progression rate of 96%, exhibited significantly higher levels of AGO2 expression (mean 0.31 ± 1.01 log2) compared to the COC1 group (p-value=0.036). This finding underscores the potential role of AGO2 as a prognostic indicator in ACC.

AGO2 expression in relation to overall survival and hormone production in ACC: AGO2 gene expression (log2-transformed) was analysed in relation to overall survival (OS) and excess adrenal hormone status in TCGA-ACC patients (Figure 6). The data were categorised into groups based on hormone secretion: No excess hormone production, Cortisol, Androgen, and Androgen|Cortisol. Elevated AGO2 expression was observed in deceased patients across all hormone statuses. Notably, patients with no excess hormone production also demonstrated higher AGO2 expression in the deceased cohort, suggesting that AGO2 expression is associated with poor prognosis independent of hormone production. Furthermore, deceased patients generally exhibited higher AGO2 expression compared to those alive within each hormone category, indicating the potential utility of AGO2 as a prognostic biomarker in ACC.

5. Differential AGO2-miRNA expression correlated with prognostic disparities in ACC clusters:

We conducted a correlation analysis to explore the relationship between AGO2 mRNA expression and miRNA expression profiles within TCGA-ACC patient clusters, which are distinguished by their prognostic outcomes. Notably, within the COC3 cluster—identified as having the worst prognosis and the highest rate of disease progression—a significant positive correlation was observed between AGO2 expression and the four most highly expressed miRNAs: hsa-miR-196a-5p (r = 0.351, p-value = 1.54e-03), hsa-miR-182-5p (r = 0.357, p-value = 1.25e-03), hsa-miR-139-3p (r = 0.324, p-value =

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3.56e-03), and hsa-miR-183-5p (r = 0.397, p-value = 2.90e-04). (Supplementary figure 1a). This positive correlation suggests a possible role of these miRNAs in conjunction with AGO2 in driving the aggressive nature of ACC within this patient group.

Conversely, the COC1 group, characterized by a more favourable prognosis, demonstrated an inverse correlation between AGO2 and miRNAs from the cluster Xq27.3 ((Yoshida *et al.*, 2021). Specifically, the miRNAs hsa-miR-513c-5p (r = -0.401, p-value = 2.51e-04), hsa-miR-506-3p (r = -0.393, p-value = 3.47e-04), hsa-miR-514a-3p (r = -0.389, p-value = 3.89e-04) and hsa-miR-513a-5p (r = -0.442, p-value = 4.52e-05) all exhibited a negative correlation with AGO2 expression. This inverse relationship may indicate the potential of these miRNAs, in conjunction with lower AGO2 expression, to mediate a less aggressive disease phenotype in COC1 patients (Supplementary figure 1b).

6. Discussion

In this study, we demonstrated the potential role of AGO2 as a diagnostic and prognostic marker in ACC. AGO2 is a key regulator of miRNA function and maturation (Connerty, Ahadi and Hutvagner, 2015), with variable expression across cancer types (Ye, Jin and Qian, 2015). Our analysis revealed a positive correlation between AGO2 expression and adverse clinical outcomes in ACC, including poorer survival, higher Weiss scores, and advanced tumour stages, emphasizing its potential as a biomarker. When evaluating AGO2 expression across TCGA-ACC clusters (COC1, COC2, and COC3) (Zheng *et al.*, 2016), the COC3 group, which has the worst prognosis, exhibited significantly higher AGO2 levels compared to COC1 and COC2, indicating a potential role of AGO2 in the pathogenesis of aggressive ACC. Although other proteins like TARBP2, RAN, and XPO5 showed expression differences, they lacked the concordance or prognostic significance of AGO2.

Previous studies that have examined the prognostic impact of miRNA biogenesis proteins have reported conflicting results. For example, Carmuta (Caramuta *et al.*, 2013) reported the upregulation of TARBP2 mRNA levels in ACC patients, whereas de Sousa (Sousa *et al.*, 2015) reported no difference in TARBP2 gene or protein (TRBP) expression between adrenocortical adenomas and ACC. In our study, although TARBP2 and RAN gene expression was significantly increased in ACC, a

corresponding increase in protein expression was not detected. Conversely, the gene expression of DROSHA, XPO5, and DICER did not differ between ACC and normal adrenal cortex, however, the protein expression levels were significantly lower in ACC. These discrepancies not only highlight the complexity of post-transcriptional and post-translational regulatory mechanisms on protein expression levels in ACC but also highlight the inherent challenges in comparing different methodological quantitative approaches.

Our investigation revealed a notable positive correlation between the expression of AGO2 and that of the four most highly expressed miRNAs in the COC3 cluster (hsa-miR-196a-5p, hsa-miR-182-5p, hsa-miR-139-3p, and hsa-miR-183-5p), which are associated with poor prognosis. In contrast, the COC1 cluster, which is associated with a more favourable prognosis, exhibited an inverse correlation with AGO2 expression (Zheng *et al.*, 2016). Considering the extensive progression rate of COC3, AGO2 merits further investigation to explore its role in ACC pathogenesis and its potential role as a diagnostic and prognostic biomarker.

In progressing toward clinical translation, several considerations must be addressed. Establishing the cut-off point for AGO2 protein expression is important. Furthermore, comparison of AGO2 protein levels in tissue samples and blood samples may facilitate further investigation into its potential application as a liquid biopsy. Similarly, further investigation into the quantitative significance of AGO2 protein levels in early-stage tumours may be useful in guiding adjuvant treatment and follow-up protocols.

7. Limitations

While we validated the elevated mRNA expression of AGO2 in the TCGA and GTEx cohorts, relevant cut-off values for AGO2 protein expression in ACC requires additional clinical trials and validation in larger cohorts.

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8. Conclusion

This study is the first to identify Argonaute 2 (AGO2), a key regulator of miRNA function, as a potential diagnostic and prognostic biomarker in adrenocortical carcinoma (ACC). AGO2 is upregulated in ACC compared to adrenal adenoma and the normal adrenal cortex. This upregulation is evident at both the gene and protein levels, distinguishing AGO2 from other miRNA biogenesis proteins evaluated in this study. Compared to 31 other cancers in the TCGA dataset, the degree and significance of the prognostic impact of AGO2 expression are unique to ACC. The strong association between AGO2 expression and clinicopathological outcomes underlines its potential role in ACC progression. This study lays the groundwork for future research, especially in exploring the feasibility of AGO2 as a liquid biopsy biomarker—a promising direction that could revolutionize non-invasive cancer diagnostics and prognostication in ACC.

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Author Contributions: A.H. designed the study, performed the experiments, interpreted the results, and drafted the manuscript. G.H. and S.S. supervised the study design. A.H., G.H., S.S., and A.P. contributed to the scientific discussion, manuscript editing, and finalization of the manuscript. All the authors have read and agreed to the published version of the manuscript.

Ethics approval and consent to participate: The study received ethics approval from the Northern Sydney Local Health District Human Research Ethics Committee (2020/ETH01931). Tissue samples, including adrenocortical carcinoma (ACC), benign adrenocortical adenoma (AA), and normal adrenal cortex (NAC) samples, were obtained from the Tumour Bank of the Kolling Institute of Medical Research. The Kolling Institute Tumour Bank Access Committee

granted access to these samples (reference NETBMC #20-49). All participating patients provided informed consent for the use of their tissue samples and the collection of associated clinical data.

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Figure legends

Figure 1. mRNA expression analysis of genes related to miRNA biogenesis in adrenocortical carcinoma (ACC) and normal adrenal cortex (NAC) tissue samples. The expression levels of miRNA biogenesis genes (AGO2,DROSHA, DGCR8, XPO5, RAN, TARBP2, and DICER1) were compared in ACC and normal adrenal cortex tissue samples using RNA-seq data from the TCGA and GTEX datasets . Among these genes, AGO2 showed significantly higher expression in ACC samples than in normal samples ($p<0.001$), whereas minimal or no expression of AGO2 was detected in normal samples.

Figure 2. Association between miRNA biogenesis gene expression and survival rates in adrenocortical carcinoma (ACC) patients. Gene survival analysis of TCGA RNA-seq data was performed to explore overall survival rates in 79 ACC patients with adrenocortical carcinoma according to high (green) or low (brown) gene expression levels. The analysis revealed a poor prognosis associated with high expression levels of AGO2, DGCR8, XPO5 and RAN, with log-rank $p <0.001$. TARBP2 showed a weaker prognostic association (log-rank $p=0.014$). DROSHA and DICER did not exhibit significant prognostic correlations, with log-rank $p=0.85$ and $p=0.57$, respectively. Among the genes involved in the miRNA biogenesis pathway, AGO2 emerged as the strongest prognostic indicator in ACC, exhibiting a hazard ratio (HR) of 7.07 and a log-rank test p-value of 2.8e-06 .

Figure 3. Kaplan–Meier gene expression analysis of AGO2-ACC-TCGA. Kaplan–Meier curves comparing survival between ACC patients with low (< 15.52, blue) and high (≥ 15.52, red) AGO2 expression in the TCGA cohort. The difference in survival was statistically significant ($p = 0.0003335$,

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log-rank test statistic = 12.87), indicating a prognostic impact of AGO2 expression on patient outcome.

Figure 4. Protein expression analysis of miRNA biogenesis components in adrenocortical carcinoma (ACC), normal, and benign tissue samples. The protein expression levels of AGO2, DROSHA, DGCR8, XPO5, RAN, TARBP2, and DICER1 in normal, benign, and adrenocortical cancer tissue homogenate samples were measured using ELISA. The results revealed that XPO5, RAN, TARBP2, and DICER1 protein expression was downregulated in cancer samples compared to both normal and benign samples, suggesting a potential role for these proteins in cancer development through post-translational modification. In contrast, AGO2 protein expression was significantly higher in cancer samples than in both normal and benign samples. These findings highlight AGO2 as a strong candidate potential diagnostic biomarker for adrenocortical carcinoma among all the miRNA biogenesis factors analysed.

Figure 5. Receiver operating characteristic (ROC) curve for AGO2 protein expression in adrenocortical carcinoma (ACC) patients. The ROC curve illustrates the diagnostic ability of AGO2 protein expression to differentiate between ACC and non-malignant samples. The area under the curve (AUC) was 0.9481 (95% CI: 0.8641 to 1.000), indicating high diagnostic accuracy. A cut-off value of >3.9 for AGO2 protein expression yielded a sensitivity of 88.89% (95% CI: 56.50% to 99.43%) and a specificity of 80.00% (95% CI: 54.81% to 92.95%). The diagonal dashed line represents the line of no discrimination (AUC = 0.5).

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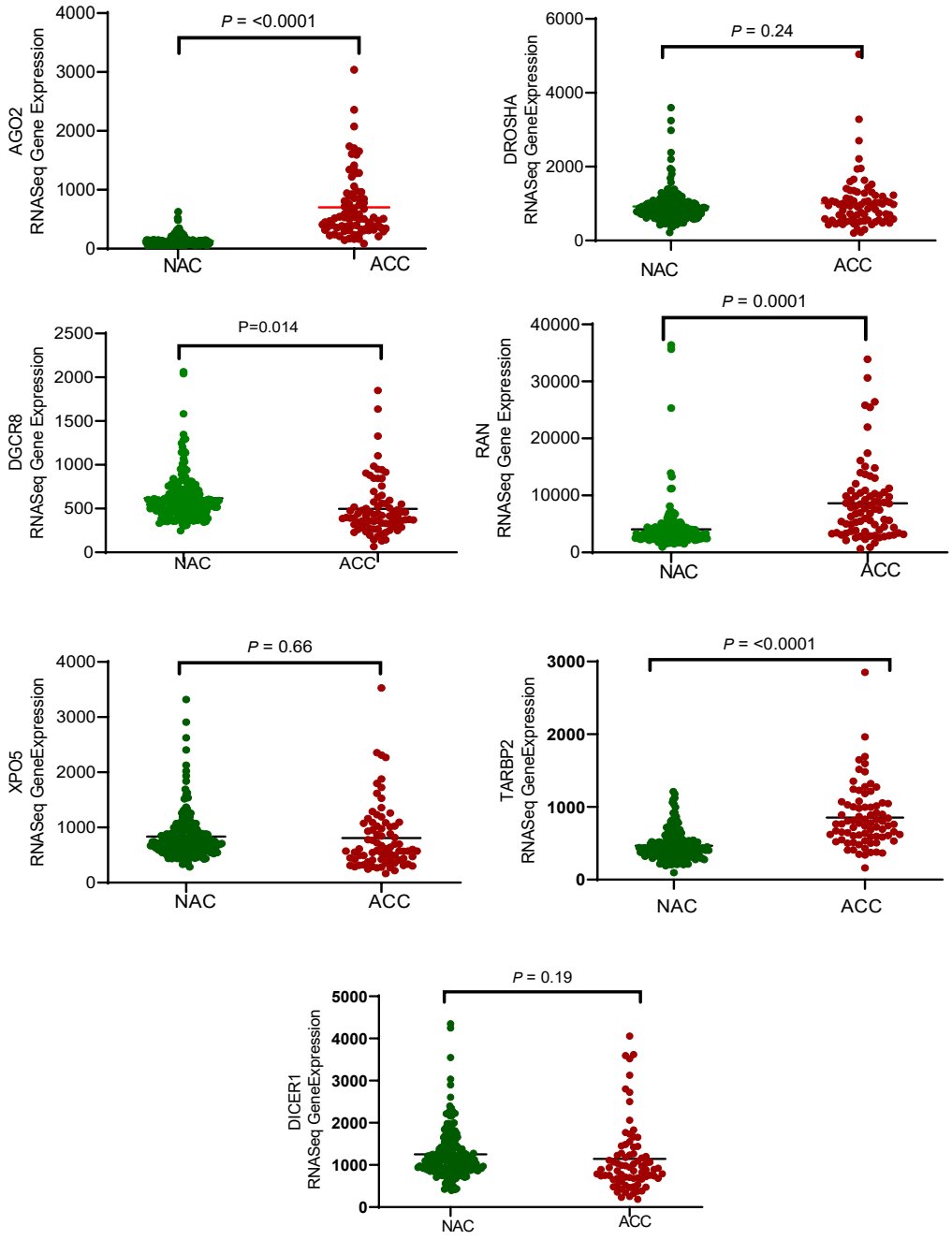

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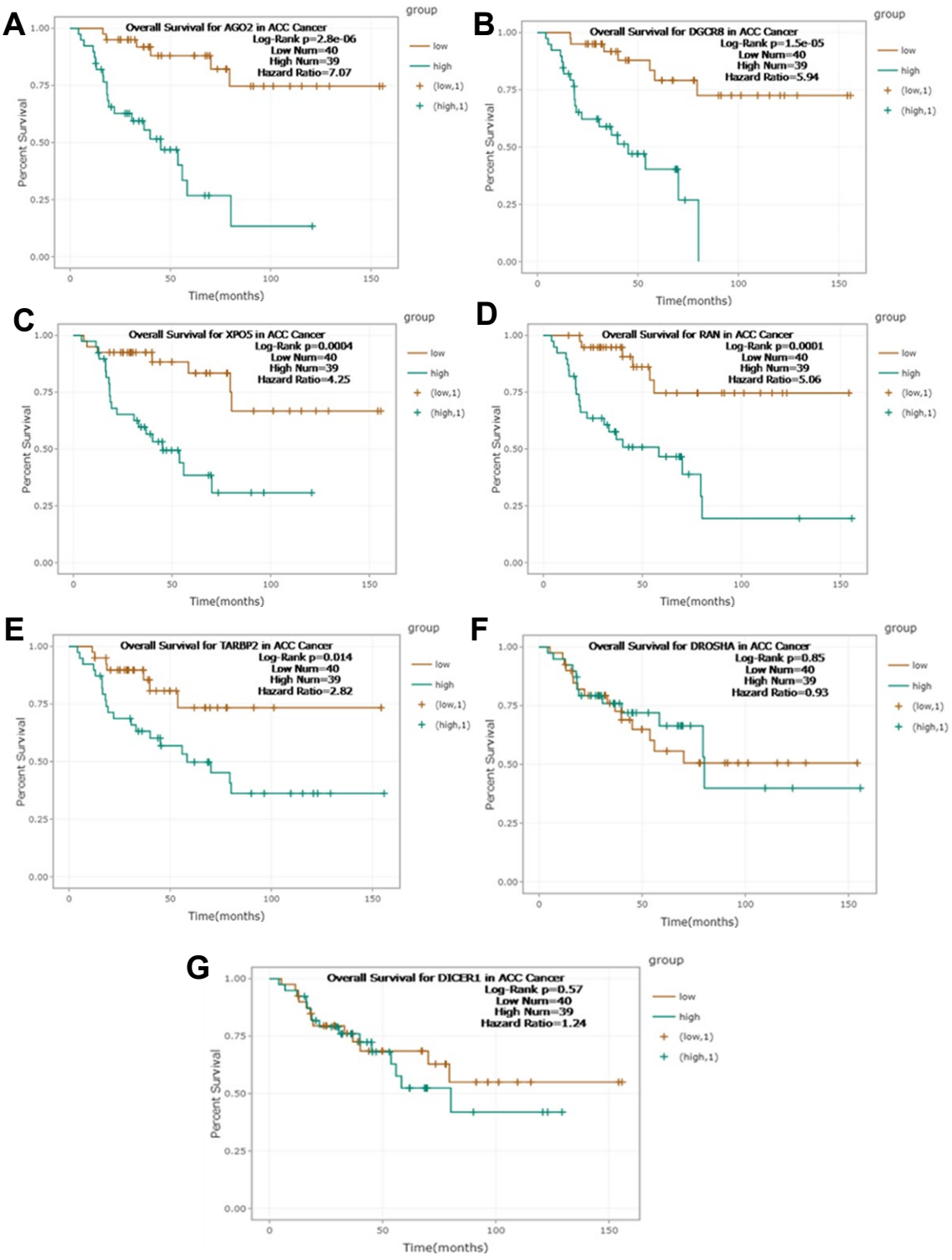

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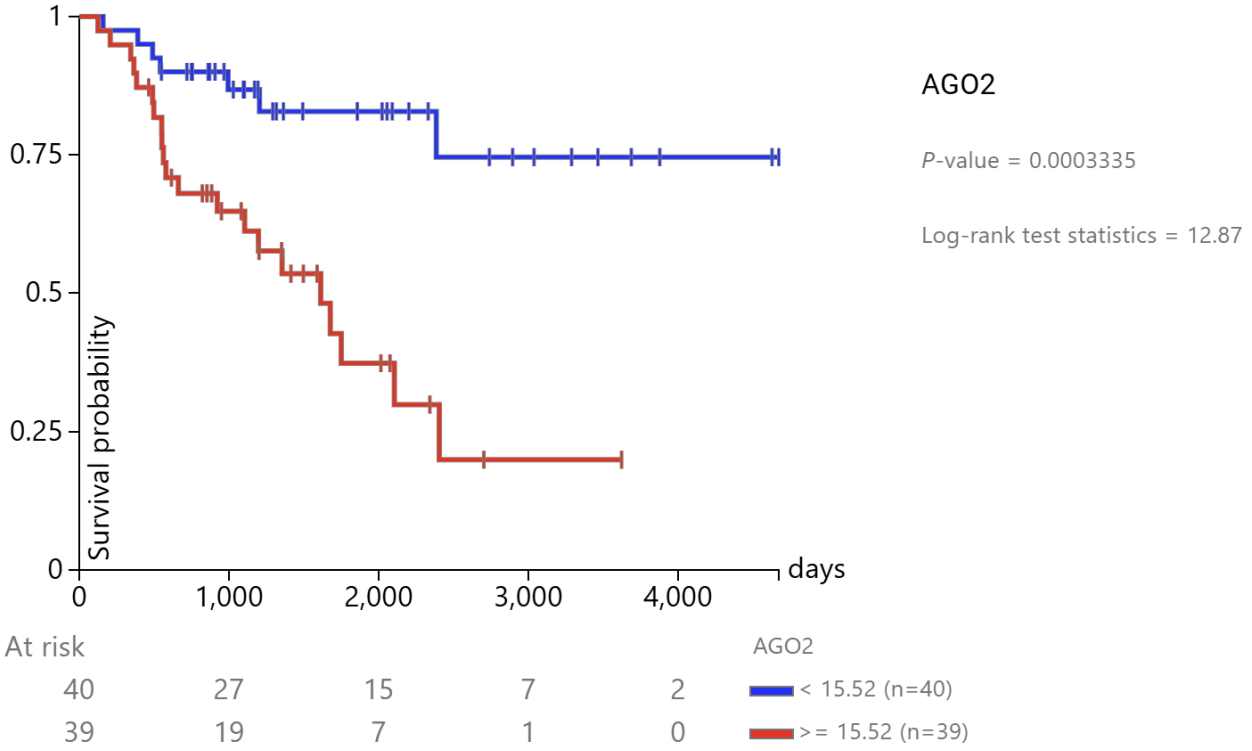

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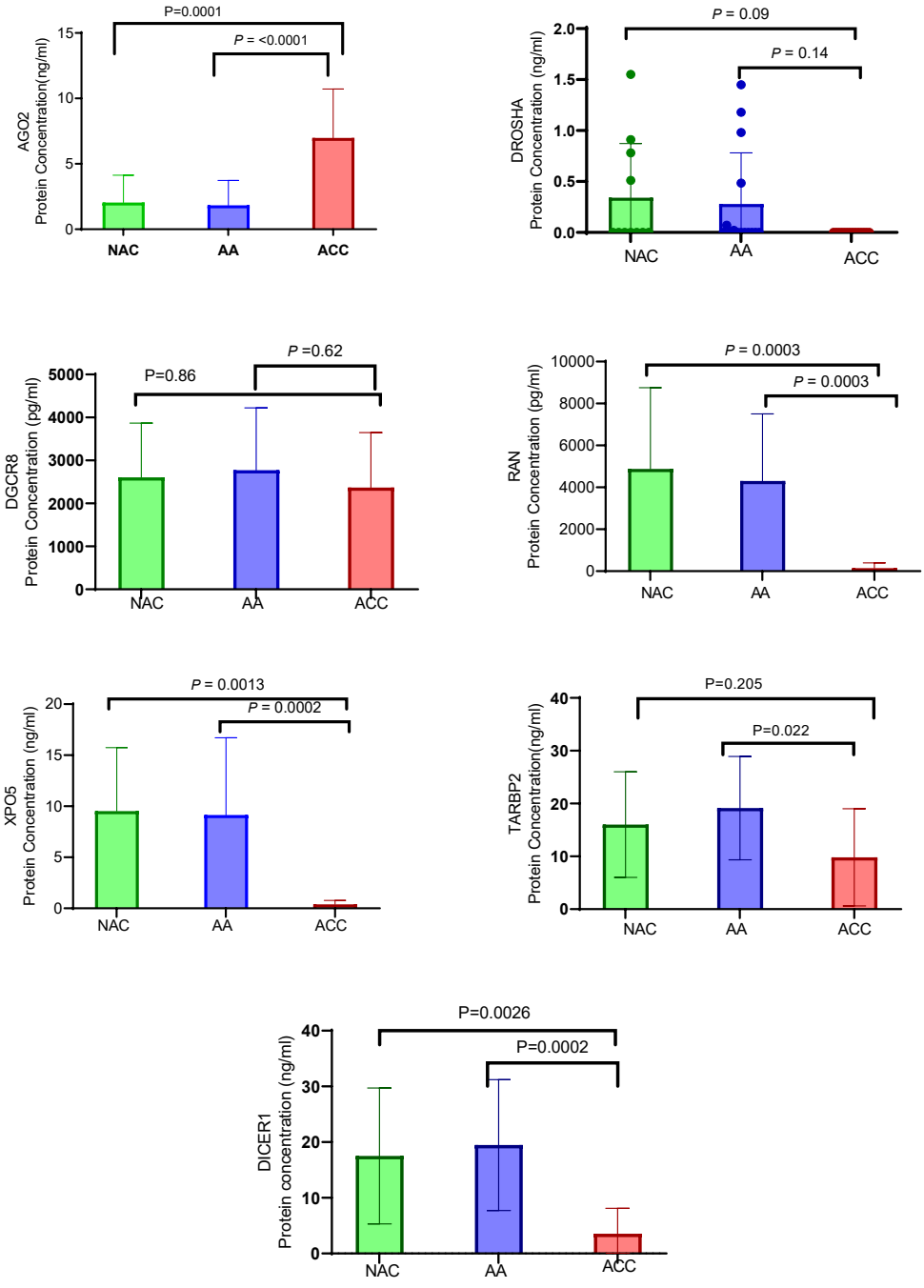

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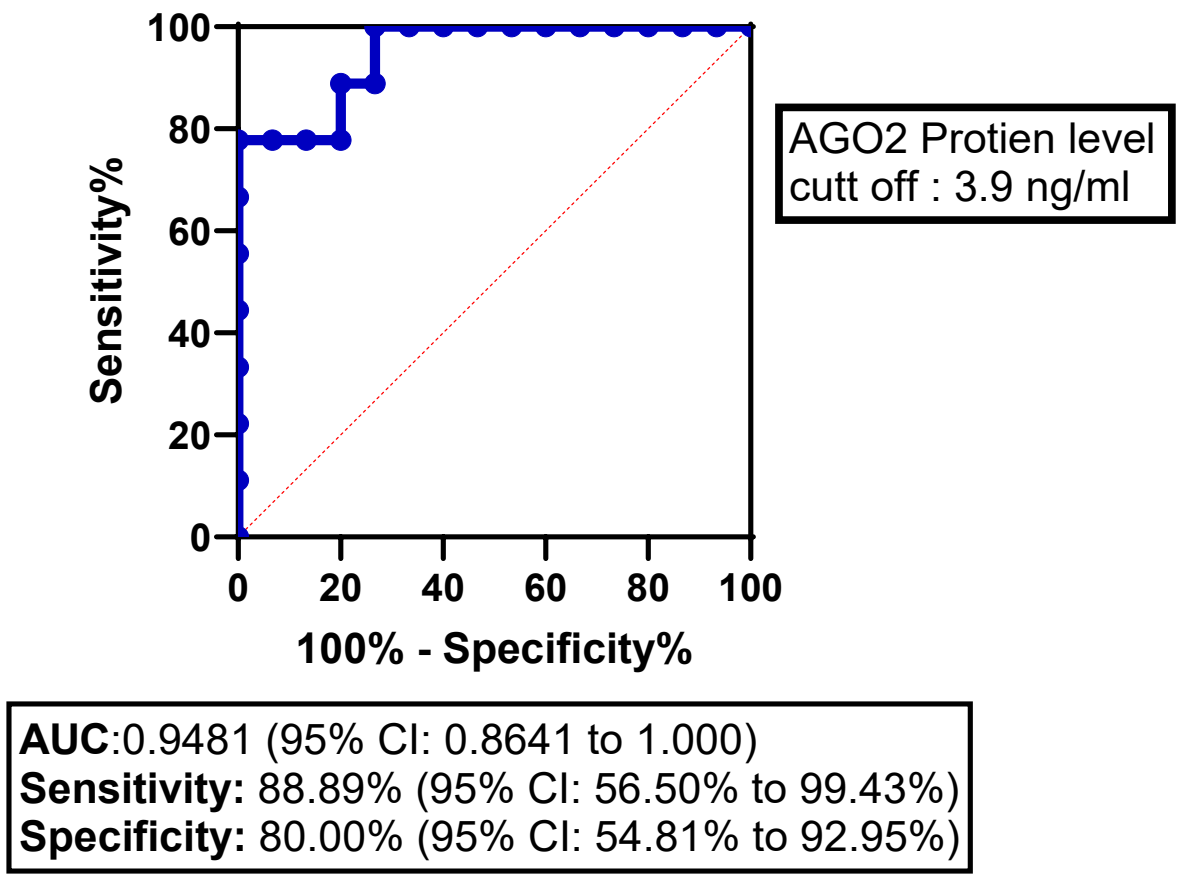

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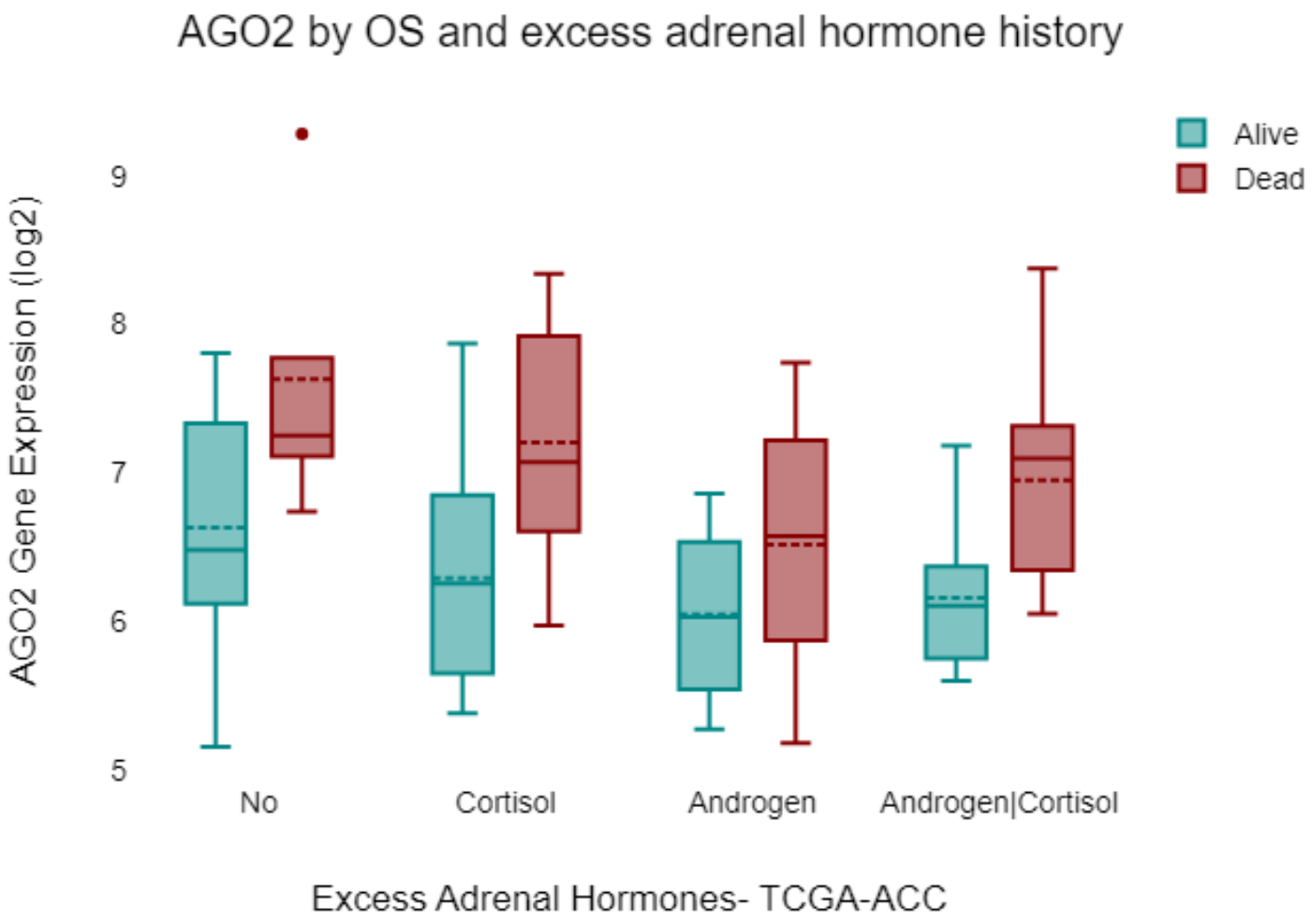

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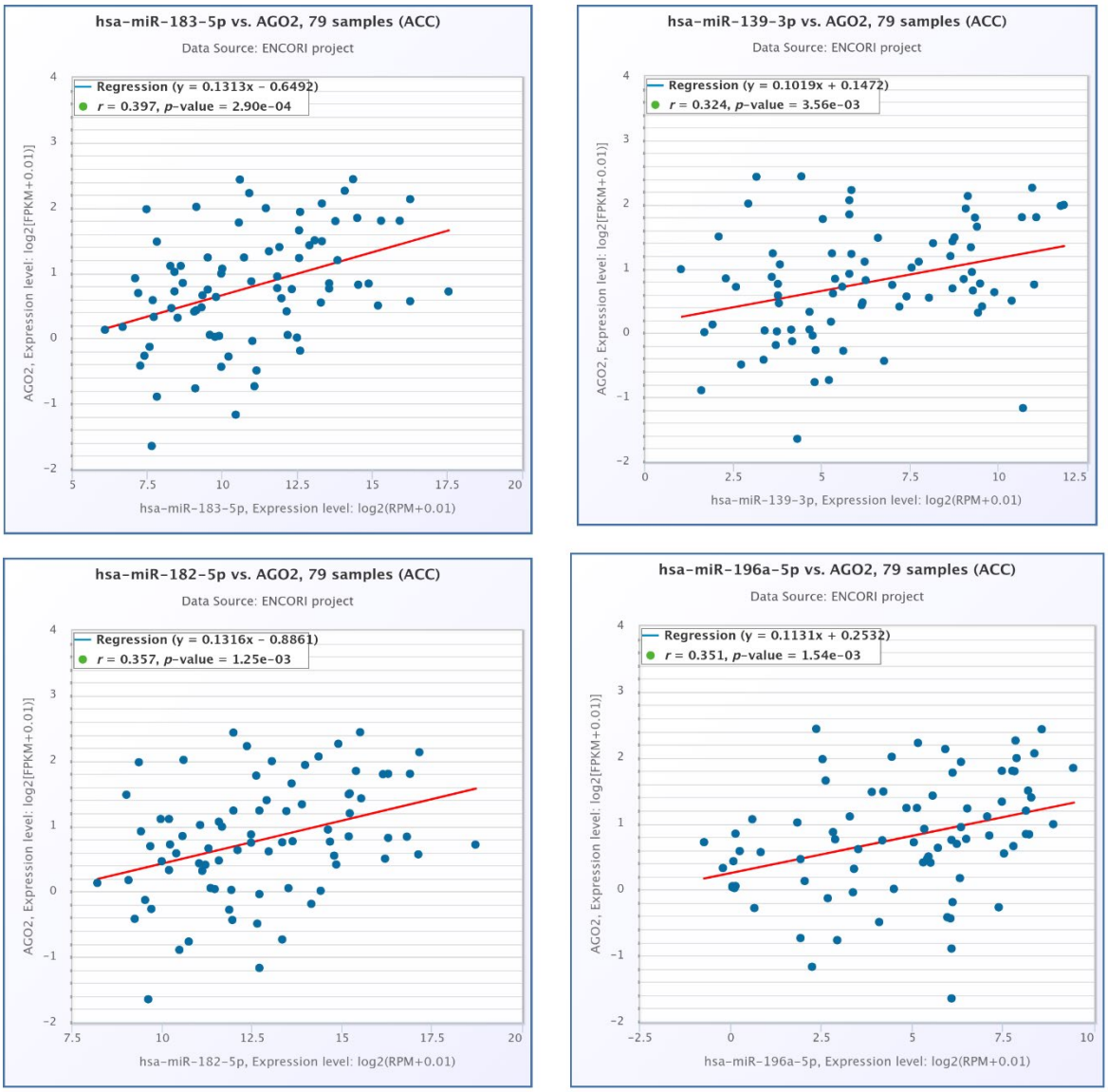

Supplementary Figure 1A:Co-expression of AGO2 with prognostically significant miRNAs in ACC The top four miRNAs highly expressed in the COC3 group-TCGA-ACC, which is associated with poor prognosis (hsa-miR-183-5p, hsa-miR-139-3p, hsa-miR-182-5p, and hsa-miR-196a-5p), demonstrated a positive correlation with AGO2 expression.

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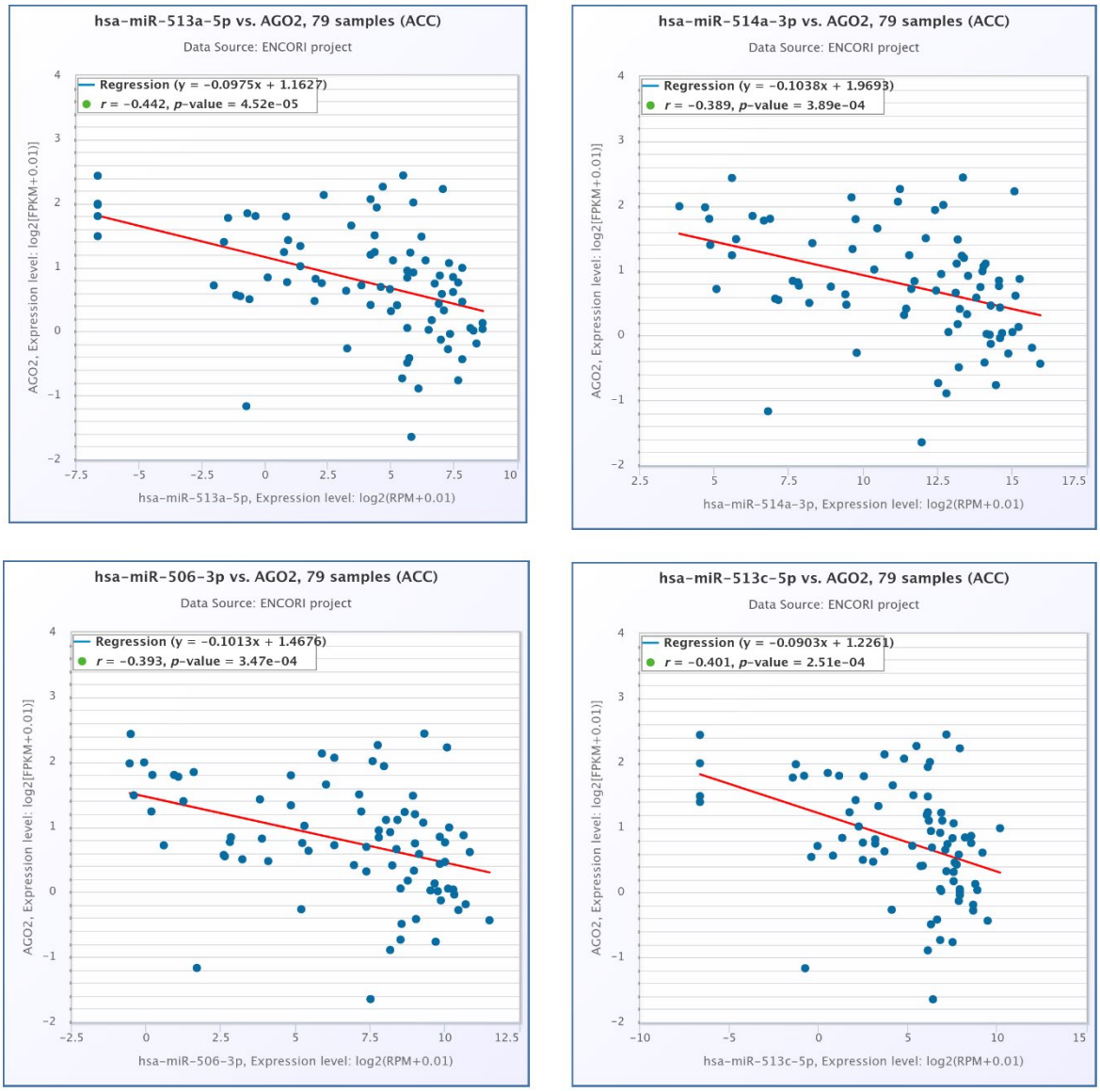

Supplementary Figure 1b AGO2 showed negative correlation with the top four upregulated miRNAs linked to the TCGA-ACC COC1 cluster with better prognosis (hsa-miR-513a-5p, hsa-miR-514a-3p, hsa-miR-506-3p, hsa-miR-513c-5p).

Supplementary Table 1: Table 1. **Pan-Cancer AGO2 expression and survival analysis in TCGA cohorts**. This table summarizes the hazard ratios (HR) for AGO2 expression across 32 TCGA cancer types, highlighting its prognostic significance, particularly in ACC with an HR of 7.07 (p value 2.80E-06)

Cancer	Cancer Number	p-value (significant thershold <0.05)	HR
ACC	79	2.80E-06	7.07
MESO	85	0.00053	2.36
UCEC	537	0.0052	1.83
SARC	261	0.0092	1.71
KIRP	288	0.016	2.15
CHOL	36	0.044	0.38
LGG	523	0.065	1.39
BRCA	1082	0.087	1.32
THYM	118	0.1	0.29
LIHC	369	0.12	1.32
KICH	64	0.21	2.35
UVM	80	0.23	1.69
CESC	306	0.27	1.3
OV	374	0.36	1.13
READ	159	0.36	0.69
LUAD	503	0.43	1.13
UCS	56	0.43	1.32
ESCA	162	0.49	0.84
STAD	365	0.5	0.89
HNSC	495	0.56	0.92
PCPG	183	0.57	1.52
BLCA	406	0.61	1.08
LUSC	469	0.67	1.06
SKCM	440	0.73	0.95
TGCT	139	0.75	0.72
PRAD	495	0.76	1.22
LAML	75	0.79	0.93
PAAD	178	0.8	1.06
KIRC	517	0.81	1.04
DLBC	47	0.82	1.18
THCA	509	0.84	1.11
COAD	447	0.87	1.03

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Supplementary Table 2: Comparative Analysis of AGO2 mRNA and Protein Levels Against Clinicopathological Parameters and Their Prognostic Significance in TCGA-ACC and an Independent ACC Cohort.

Parameters	TCGA-ACC cohort (n=79)	AGO2 mRNA expression in TCGA (log2)	p-value	Collected-ACC cohort (n=15) (Kollings Tumour bank)	AGO2 Protein concentration in collected cohort (ng/ml)	p-value
Diagnosis Age	46.7 ± 15.77		0.672	46.27 ± 15.47		0.833
<49	39	6.64 ± 0.77		9	6.66 ± 2.71	
>49	40	6.72 ± 0.9		6	7.03 ± 4.14	
Sex			0.254			0.484
Female	48	6.59 ± 0.8		8	6.24 ± 2.97	
Male	31	6.82 ± 0.88		7	7.46 ± 3.6	
Overall survival status			<0.001			0.009
Alive	51	6.44 ± 0.7		9	5.16 ± 2.42	
Deceased	28	7.11 ± 0.89		6	9.28 ± 2.72	
Weiss score			0.003			0.008
Weiss score (2-5)	30	6.32 ± 0.8		4	3.43 ± 1.86	
Weiss score (6-9)	31	6.9 ± 0.83		11	8.04 ± 2.69	
Pathologic stage			0.011			0.004
Stage I-II	46	6.49 ± 0.78		5	3.76 ± 1.77	
Stage III-IV	31	6.93 ± 0.87		10	8.33 ± 2.64	
Cluster of clusters (CoCs)	n=76		0.036 (COC1 vs COC3)			
COC1 (Disease progression rate: (7%)	33	-0.16 ± 0.91				
COC2 (Disease progression rate: (56%)	19	-0.18 ± 1.14				
COC3 (Disease progression rate: (96%)	24	0.31 ± 1.01				

The data are presented as the means ± SDs or n (numbers).
Abbreviations: TCGA, The Cancer Genome Atlas.