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Intratumoral heterogeneity of steroidogenesis in aldosterone-producing adenoma revealed by intensive double- and triple-immunostaining for CYP11B2/B1 and CYP17

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Abstract

Introduction—Cytochrome P450 11B2 (CYP11B2) plays a pivotal role in aldosterone synthesis, while cytochrome P450 11B1 (CYP11B1) and cytochrome P450 17A1 (CYP17) are involved in cortisol synthesis in normal human adrenal glands. However, their detailed distribution in aldosterone-producing adenoma (APA) remains incompletely settled.

Materials and methods—We examined the status of CYP11B1/CYP11B2 and CYP11B2/CYP17A1 expressions in 27 APA (double staining) cases and 21 APA (triple staining) cases by using immunofluorescence staining and semi-quantitative evaluation.

Results—Tumor cells co-expressing CYP11B1/B2 (hybrid cell type A), CYP11B2/17 (hybrid cell type B), CYP11B1/17 (hybrid cell type C), and CYP11B1/B2/17 (triple-positive cell) were identified. The area and cell number of these cells were relatively small, but the size of individual hybrid cells were different between three hybrid cell types (A/B/C) and triple-positive cells.

Conclusion—The presence of hybrid cells indicated the marked intratumoral heterogeneity of steroidogenesis in APAs, particularly in those producing glucocorticoids and mineralocorticoids.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.mce.2015.11.014>.

Keywords

Aldosterone-producing adenoma; CYP11B1; CYP11B2; CYP17; Double-immunostaining; Triple-immunostaining

1. Introduction

Aldosterone-producing adenoma (APA) is known to be the major cause of primary aldosteronism (PA), which causes secondary hypertension. It is therefore pivotal to study the detailed status of aldosterone-related steroidogenic enzymes in APA tissues. The over-production of aldosterone in APA is generally thought to result from elevated expression of the aldosterone synthase (CYP11B2) (Fallo et al., 2002a; Bassett et al., 2005). We recently reported the patterns of CYP11B2 expression by using a newly developed, highly specific monoclonal antibody against CYP11B2 in normal human adrenal glands and PA cases including APA (Gomez-Sanchez et al., 2014; Nakamura et al., 2014; Ono et al., 2014; Konosu-Fukaya et al., 2015). In these studies, the distribution and immunointensity of CYP11B2 were not always diffuse and abundant in all APA cases, but the status of CYP11B2 was significantly inversely correlated with the size of APA (Ono et al., 2014).

CYP11B2 is present in the zona glomerulosa (ZG) of normal human adrenal glands, where aldosterone is synthesized. 11-Hydroxylase (CYP11B1) and cytochrome P450 17A1 (CYP17) are expressed in the zona fasciculata (ZF), where cortisol is produced via deoxycorticosterone (DOC) and corticosterone. However, CYP11B1 expression was also detected in APA cases (Nakamura et al., 2014). In addition, CYP11B1 status was significantly correlated with APA size (Ono et al., 2014). A previous study also demonstrated that CYP11B1-transfected cells converted DOC into corticosterone, 18-OH-DOC, and small amounts of 18-OH-corticosterone *in vitro*, indicating that CYP11B1 may be involved in the production of mineralocorticoids including aldosterone (Zhou et al., 1995a). In addition, both CYP11B1 and CYP11B2 were reported to coexist in APA tumor cells based on immunofluorescence examination (Nakamura et al., 2014). Therefore, the distribution of these double-positive cells and the functional significance of CYP11B1 in the synthesis of mineralocorticoids or glucocorticoids in APA tissue must be further examined. In addition, the expression of CYP17 in APA was reported to be absent or markedly decreased (Sasano, 1994). However, CYP17A-positive tumor cells were reported to be heterogeneously distributed, and their relative immunoreactivity was weak in APA cases (Ono et al., 2014). Analysis of APA was further complicated by the histological nomenclature of the tumor cells, ZG-like cells, or small tumor cells with scant lipid in the cytoplasm and the ZF-like cells or large tumor cells containing relatively abundant intracellular lipids (Chen et al., 1999). This classification was based on histological findings alone, but the endocrinological significance should be further explored in order to avoid confusion.

Therefore, in this study, we examined the correlation between key enzymes involved in mineralocorticoid and corticosteroid biosynthesis, including CYP11B2, CYP11B1, and

CYP17A1, in APA tissues using immunofluorescence analysis in order to increase the understanding of steroid biosynthesis in APA.

2. Materials and methods

2.1. Human adrenal glands

Twenty-seven cases (double staining) and 21 cases (triple staining) of APA surgical pathology materials were retrieved from the surgical pathology files of Tohoku University Hospital (Sendai, Japan). The specimens were fixed in 10% formalin and embedded in paraffin. The research protocol was approved by the ethics committee at Tohoku University Graduate School of Medicine (Sendai, Japan) (No. 2011-544).

2.2. Antibodies

A mouse monoclonal antibody for CYP11B2 and rat monoclonal antibody for CYP11B1 were recently developed in the laboratory of Dr. Gomez Sanchez (University of Mississippi Medical Center, Jackson, MS, USA) (Gomez-Sanchez et al., 2014; Nakamura et al., 2014; Ono et al., 2014; Konosu-Fukaya et al., 2015). The details of the rabbit polyclonal antibody against CYP17 have been previously reported (Ono et al., 2014; Konosu-Fukaya et al., 2015).

2.3. Double immunofluorescence analysis

The tissue slides were de-paraffinized. Antigen retrieval was performed by heating the glue-coated slides in an autoclave for 5 min in a pH 9.0 buffer (Nichirei, Tokyo, Japan). Blocking was performed for 1 h by using buffer with 2.5% goat serum and 0.5% SDS at room temperature (Vector Laboratories, Burlingame, CA, USA). The slides were then incubated with a mixture of two of the following three primary antibodies: rat monoclonal anti-hCYP11B1 (1:50), mouse monoclonal anti-hCYP11B2 (1:150), and rabbit polyclonal anti-CYP17 (1:300) antibody overnight in 0.01 M phosphate-buffered saline, 0.5% bovine serum albumin, and 0.05% NaN₂. The specimens were washed and then incubated with two of the following corresponding secondary antibodies: goat anti-rat IgG conjugated to Alexa 488 (112-545-167, Jackson ImmunoResearch, Allentown, PA, USA) (1:500), goat anti-mouse IgG conjugated to Alexa 594 (115-585-166, Jackson ImmunoResearch) (1:500), goat anti-rabbit IgG conjugated to Alexa 488 (111-545-144, Jackson ImmunoResearch) (1:500), and goat anti-rat IgG conjugated to Alexa 594 (112-585-167, Jackson ImmunoResearch) (1:500) for 1 h. Coverslips were mounted using VECTASHIELD mounting media (Vector Laboratories) with DAPI (Vector Laboratories).

2.4. Triple immunofluorescence analysis

The slides were de-paraffinized and antigen retrieval was performed by heating the glue-coated slides in an autoclave for 5 min in the pH 9.0 buffer (Nichirei). Blocking was performed for 1 h by using buffer with 2.5% goat serum and 0.5% SDS at room temperature (Vector Laboratories). The slides were then incubated with the mixture of primary antibodies: rat monoclonal anti-hCYP11B1 (1:50), mouse monoclonal anti-hCYP11B2 (1:150), and rabbit polyclonal anti-CYP17 (1:200) antibodies overnight in 0.01 M phosphate-buffered saline, 0.5% bovine serum albumin, and 0.05% NaN₂. The slides were

washed and then incubated with a mixture of corresponding secondary antibodies: goat anti-rat IgG conjugated to Alexa 488 (A-11034, Life Technologies, Carlsbad, CA, USA) (1:500), goat anti-mouse IgG conjugated to Alexa 594 (A-11030, Life Technologies) (1:500), and goat anti-rabbit IgG conjugated to Alexa 680 (A-21109, Life Technologies) (1:200) for 1 h. Coverslips were mounted using VECTASHIELD mounting media (Vector Laboratories) with DAPI (Vector Laboratories).

2.5. Immunofluorescence evaluation and analysis

Images of double and triple immunofluorescence staining were obtained using a confocal laser-scanning microscope system (A1R, Nikon, Tokyo, Japan), which was equipped a 32-channel multianode spectral detector with five laser lines. After intensive optimization of the image acquisition conditions for double or triple staining, we applied the following method: first, true signals of immunofluorescence excited by 488-nm and 561-nm laser lines were obtained by the un-mixing of overlapping spectral data in each pixel detected by the multianode spectral detector using two laser lines under the spectral un-mixing algorithm (Levenson, 2004); second, fluorescence of DAPI and immunofluorescence excited by the 633-nm laser line were obtained by the regular sequential acquisition mode; and third, these images were merged on Adobe Photoshop CS6 Extended (Adobe Systems, San Jose, CA, USA). The detailed protocol for detecting double or triple staining is available upon request.

Images were analyzed with ImageJ software (ver. 1.47) (NIH, Bethesda, MD, USA). Immunofluorescence signals were staged according to their immunointensity by >0–255. To classify the cells into positive/negative cells, threshold values for CYP11B1 and CYP11B2 were set as default values pre-determined in ImageJ (ver. 1.4.7). In addition, the threshold for CYP17 was 95/255 based on the intensity of non-specific immunoreactivity (Nakamura et al., 2014).

Following the determination of positive/negative cells, we evaluated the ratios of area and cell number of hybrid cell types A/B/C and triple-positive cells as well as single-positive cells and triple-negative cells (%). For example, the ratios of area and cell number, and the individual cell size of hybrid cell types A are as follows:

1. The ratio of area (hybrid cell types A) = the gross square formed by double-positive cells (CYB11B1/CYP11B2)/the entire square of APA tumor lesion (%) (0–100%)
2. The ratio of cell number (hybrid cell types A) = the total cell number of double-positive cells (CYB11B1/CYP11B2)/the total cell number of APA tumor lesion (%) (0–100%)
3. The individual cell size (hybrid cell types A) = the gross square formed by double-positive cells (CYB11B1/CYP11B2)/the total cell number of double-positive cells (CYB11B1/CYP11B2) (μm^2)

2.6. Antigen absorption test

We performed antigen absorption test for anti-CYP17 antibody. 1 μg of anti-CYP17 antibody and 10 μg of each peptide: LHHNEKEWHQPDQFC; KMNSDNGNAGPDQDSC; KKGKDFSGRPGMAC, which were used for the production of anti-CYP17 antibody, were

dissolved in 200 μ L of 0.5% bovine serum albumin/phosphate buffered saline (molar ratio of antibody : antigen was 1:160–200) and incubated at 4 °C overnight. This solution was used instead of the primary antibody.

2.7. Statistical analysis

Significant differences were evaluated to determine the analysis of variance using Tukey's test with JMP Pro software (ver. 11.0.0) (SAS, Inc., Cary, NC, USA). The correlation was evaluated using the Spearman rank method. $P < 0.05$ was considered statistically significant.

3. Results

A representative figure of APA with CYP11B1 and CYP11B2 immunofluorescence is shown in Fig. 1A. Cells exhibiting CYP11B1 (+)/CYP11B2 (+) (hybrid cell type A) were focally detectable (mean = 2.1%). The ratio of area was significantly smaller in the hybrid cell type A than in other three groups ($P < 0.05$) (Fig. 1B). The ratio of cell number was also significantly smaller in the hybrid cell type A than in other groups except for in the group showing CYP11B1 (–)/CYP11B2 (+) ($P < 0.05$) (Fig. 1C).

A representative figure of APA with CYP11B2 and CYP17 immunofluorescence is shown in Fig. 2A. The hybrid cell type B (CYP11B2 (+)/CYP17 (+)) was clearly focally detected (mean = 0.6%). Both the ratios of area and cell number were significantly smaller in the hybrid cell type B than in other groups of cells except for CYP11B2 (–)/CYP17 (+) ($P < 0.05$) (Fig. 2B and C).

A representative illustration of APA with CYP11B1 and CYP17 immunofluorescence is shown in Fig. 3A. The hybrid cell type C (CYP11B1 (+)/CYP17 (+)) was clearly focally detected (mean = 0.6%). The ratio of area was significantly smaller in the hybrid cell type C than in other groups except for those with CYP11B1 (–)/CYP17 (+) ($P < 0.05$) (Fig. 3B). The ratio of cell number was significantly smaller in the hybrid cell type C than the group with CYP11B1 (+)/CYP17 (–) and the one with CYP11B1 (–)/CYP17 (–), while was significantly larger than the group with CYP11B1 (–)/CYP17 (+) ($P < 0.05$) (Fig. 3C).

3.1. Triple immunofluorescence

A representative illustration of triple-positive cells (CYP11B1 (+)/CYP11B2 (+)/CYP17 (+)) is shown in Fig. 4A. Very small numbers of triple-positive cells were detected. The ratios of area and cell number of triple-positive cells were 0.14% (mean) and 0.03% (mean), respectively. The individual cell size of triple-positive cells was 1222 μm^2 (mean). These comparative data among the 8 groups based on immunoreactivity of individual antibodies are summarized in Fig. 4B–D. In particular, both the ratios of area and cell number were significantly smaller in triple-positive cells than in hybrid cell types A and C ($P < 0.05$) (Fig. 4B and C). However, the individual cell size was significantly larger in triple-positive cells than in any other cell types ($P < 0.05$) (Fig. 4D). The individual cell size and ratio of area of hybrid cell types A/B/C were significantly smaller than in triple-negative cells ($P < 0.05$) (Fig. 4D).

The ratios of area and cell number of hybrid cell types A/B/C and triple-positive cells were not significantly correlated with somatic mutations of ion channels (KCNJ5 mutation and ATPase mutation) or any clinicopathological features (tumor size, sex, age, plasma aldosterone concentration, blood pressure, and organ damage) (data not shown).

4. Discussion

In the present study, we examined the distribution of CYP11B1/CYP11B2/CYP17 in APA cases by using immunofluorescence and quantification. In addition, we confirmed the presence of three hybrid cell types (A/B/C) in APA. This is the first study to directly demonstrate the presence of hybrid or triple-positive cells that could produce and secrete both cortisol and aldosterone in the same tumor cells in APA. These cell types are not present in the normal adrenal cortex and may represent the result of neoplastic transformation.

Double immunofluorescence analysis revealed that the double-negative tumor cells of CYP11B1, CYP11B2, or CYP17 were the predominant type of tumor cells in APA. Therefore, most APA tumor cells are considered hormonally non-functioning and only a small number of tumor cells can synthesize excessive steroids, which is consistent with the histological findings of most APA studies examining the composition of large clear tumor cells (Hirano et al., 2001). These clear cells in APA contain large amounts of cholesterol and lipid droplets in their cytoplasm and display morphological ballooning; the sizes of clear cells are considered relatively large because of the accumulation of unutilized cholesterol (Hirano et al., 2001).

In general, CYP11B1 is required for cortisol synthesis, while CYP11B2 is required for aldosterone synthesis. CYP11B2 is not expressed in the ZF and CYP11B1 is not expressed in the ZG in normal adrenal tissue. However, *in vitro* models revealed that CYP11B1 may be involved in mineralocorticoid synthesis by sequentially converting deoxycorticosterone to corticosterone and corticosterone to 18-OH corticosterone in APA tumor tissue (Zhou et al., 1995b). Therefore, CYP11B1 in APA tumors may be associated with the neoplastic synthesis of corticosterone and 18-OH corticosterone (Fig. 5). In addition, in murine adrenal glands, CYP11B1 and CYP11B2 double-positive cells were identified in subcapsular lesions, which were recognized as immature, differentiating adrenocortical cells (King et al., 2009; Freedman et al., 2013). Therefore, the hybrid cell type A may represent undifferentiated adrenocortical parenchymal cells and may be associated with tumorigenesis; however, this hypothesis requires further analysis. The results of the present study also revealed that the ratio of area and individual cell size in the hybrid cell type A was significantly smaller compared to those in triple-negative cells. Tumor cells associated with biologically active steroidogenesis were reported to contain abundant mitochondria, were oxyphilic, and were small because free cholesterol was produced from the cholesterol ester and accumulated in mitochondria by the StAR protein, resulting in steroidogenesis (Hattangady et al., 2012). These cells may correspond to the previously reported nomenclature “ZG-like cells” (Chen et al., 1999), but the presence of CYP11B1 in these hybrid cells also indicated that this term should be avoided to prevent confusion. The hybrid cell type A may represent endocrinologically active tumor cells. Excessive production of

mineralocorticosteroids is not only dependent on the number and area of hybrid cells, but the hybrid cell type A may play important roles in mineralocorticoid synthesis in APA. Further functional or molecular analysis of the hybrid cell type A is necessary.

The hybrid cell type C was also identified in APA. Both CYP11B1 and CYP17 are known to play roles in cortisol synthesis. The expression of CYP11B1 mRNA was reported to be moderate and that of CYP17 was moderate to high in some APA cases (Enberg et al., 2001). Therefore, the hybrid cell type C may be functionally associated with cortisol synthesis in APA (Fig. 5). However, the number of the hybrid cell type C was not significantly correlated with serum cortisol level (data not shown). Therefore, the role of the hybrid cell type C is not considered pivotal compared to that of the hybrid cell type A in APA. In addition, Fallo et al. recently reported that the CYP11B1 mRNA level was very low in APA and not correlated with basal aldosterone level, dexamethasone responsiveness, and proportion of clear cells, which is consistent with results of the present study (Fallo et al., 2002b).

CYP11B2 and CYP17 play quite different roles in the steroidogenic pathway. The former is involved in aldosterone synthesis, while the latter is involved in cortisol synthesis. However, in the present study, the hybrid cell type B was identified in APA tumors. In APA cases harboring the hybrid cell type B, plasma cortisol concentration was not suppressed by dexamethasone (1 mg) (data not shown); in the present study, APA cases with autonomous cortisol production were excluded and further investigations are required to clarify whether the hybrid cell type B is involved in steroidogenesis in APA (Fig. 6).

We demonstrated that a very small number of triple-positive cells were present in APA tumor lesions but that the sizes of these cells were relatively larger. This finding indicates that tumor cells expressing CYP11B1/CYP11B2/CYP17 may not be involved in efficient steroidogenesis because the direction of the steroidogenic pathway should be competitive between CYP11B2 (ZG-like) and CYP11B1/CYP17 (ZF-like) expressing cells (Fig. 6).

Recently, the genetic aspects, including somatic mutations of KCNJ5, ATP1A1 and ATP2B3 have been proposed to be deeply involved in the pathogenesis and pathophysiology of APA cases (Choi et al., 2011; Azizan et al., 2012, 2013; Beuschlein et al., 2013). As described above, the ratios of area and cell number of hybrid cell types A/B/C and triple-positive cells were not significantly correlated with the status of somatic mutations of ion channels (KCNJ5 mutation and ATPase mutation) in our present study. Therefore, it is considered that these mutations may be primarily involved in the onset of the hybrid and triple-positive cells. However, it awaits further studies to clarify the association of these occurrence and genetic aspects including a very recent finding on somatic CTNNB1 mutations with high expression of gonadal receptors in APA cases (Teo et al., 2015).

This study is considered highly dependent on the immunohistochemical technique and therefore we attempted to confirm the specificity of antibodies. We utilized three primary antibodies: anti-CYP11B1 rat monoclonal antibody (Gomez-Sanchez et al., 2014; Nakamura et al., 2014); anti-CYP11B2 mouse monoclonal antibody (Gomez-Sanchez et al., 2014; Nakamura et al., 2014), and anti-CYP17 rabbit polyclonal antibody (Gomez-Sanchez et al., 2014; Nakamura et al., 2014). We performed antigen absorption test for the CYP17

polyclonal antibody (Supplementary Fig. 1). The combination of the anti-CYP17 antibody and anti-rabbit goat antibody conjugated to Alexa 680 successfully yielded the specific immunoreactivity (Supplementary Fig. 1A) but this specific reaction was abolished when the anti-CYP17 antibody, which was pre-absorbed with the excess of the target peptides (see Materials and methods), was used for immunostaining (Supplementary Fig. 1B) similar to the reaction after immunostaining only with the secondary antibody (Supplementary Fig. 1C). We could not carry out antigen absorption test for anti-CYP11B1 and CYP11B2 antibodies, because both were monoclonal and the epitope of each antibody was not known. But results of our previous study have already demonstrated the specificity of both CYP11B1 and CYP11B2 antibodies (Gomez-Sanchez et al., 2014). We also tried to confirm the specificity of secondary antibodies. The immunoreaction with anti-CYP11B1 rat and CYP11B2 mouse antibodies and the antigen pre-absorbed-anti-CYP17 rabbit antibody yielded the specific signals for CYP11B1 and CYP11B2 but not that for CYP17, which did indicate that the secondary antibody against rabbit IgG did not recognize the primary antibodies raised in rat and mouse (Supplementary Fig. 2). Immunostaining with anti-CYP11B1 rat and anti-CYP17 rabbit antibodies and three secondary antibodies yielded no specific signal for CYP11B2 (Supplementary Fig. 3). Immunostaining with anti-CYP11B2 rat and anti-CYP17 rabbit antibodies and three secondary antibodies also yielded no signal of CYP11B1 (Supplementary Fig. 4). These data did demonstrate that the secondary antibodies used in this study were highly specific to immunoglobulin of each animal species.

In addition, we optimized image acquisition conditions because the adrenal gland exhibited extremely high background autofluorescence under our experimental conditions. There were two types of autofluorescence, including 'green' fluorescence that was relatively strongly excited by the 488-nm laser line and 'red' fluorescence that was excited by the 561-nm laser. These made it extremely difficult to distinguish true signals because of the overlapping of true signals and two types of autofluorescence. Therefore, we used a confocal laser microscope system equipping a 32-channel multianode spectral detector. This enabled the spectral analysis excluding autofluorescence in each pixel, so that we successfully un-mixed the true signals from overlapping spectral data. This method is considered effective for analyzing double or triple immunofluorescence with the tissues harboring higher background autofluorescence such as dermis (Zeng et al., 1995) and injured tissues (Stirling et al., 2014) in addition to the adrenal gland.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Immunofluorescence evaluation and analysis for the hybrid cell type A. A. H&E and immunofluorescence figures (CYP11B1, CYP11B2, and CYP11B1 plus CYP11B2). The hybrid cell type A (double-positive for CYP11B1 and CYP11B2) is represented by white arrows. B. The ratio of area among groups of only CYP11B1-positive cells, only CYP11B2 positive cells, the hybrid cell type A, and double-negative cells (*, $P < 0.05$). C. The ratio of cell number among groups of only CYP11B1-positive cells, only CYP11B2-positive cells, the hybrid cell type A, and double-negative cells (*, $P < 0.05$).

Fig. 2.

Immunofluorescence evaluation and analysis for the hybrid cell type B. A. H&E and immunofluorescence figures (CYP11B2, CYP17, and CYP11B2 plus CYP17). The hybrid cell type B (double-positive for CYP11B2 and CYP17) is shown by white arrows. B. The ratio of area among groups of only CYP11B2-positive cells, only CYP17-positive cells, the hybrid cell type B, and double-negative cells (*, $P < 0.05$). C. The ratio of cell number among groups of only CYP11B2-positive cells, only CYP17-positive cells, the hybrid cell type B, and double-negative cells (*, $P < 0.05$).

Fig. 3.

Immunofluorescence evaluation and analysis for the hybrid cell type C. A. H&E and immunofluorescence figures (CYP11B1, CYP17, and CYP11B1 plus CYP17). The hybrid cell type C (double-positive for CYP11B1 and CYP17) is shown by white arrows. B. The ratio of area among groups of only CYP11B1-positive cells, only CYP17-positive cells, the hybrid cell type C, and double-negative cells (*, $P < 0.05$). C. The ratio of cell number among groups of only CYP11B1-positive cells, only CYP17-positive cells, the hybrid cell type C, and double-negative cells (*, $P < 0.05$).

Fig. 4. Immunofluorescence evaluation and analysis for triple-positive cells. **A.** Immunofluorescence figures (CYP11B1, CYP11B2, and CYP17). Triple-positive cells are shown by white arrows. **B.** The ratio of area among eight groups based on the expression pattern of CYP11B1, CYP11B2, and CYP17 (*, $P < 0.05$). **C.** The ratio of cell number among eight groups based on the expression pattern of CYP11B1, CYP11B2, and CYP17 (*, $P < 0.05$). **D.** The individual cell size among eight groups based on the expression pattern of CYP11B1, CYP11B2, and CYP17 (*, $P < 0.05$).

Fig. 5.
Presumption of adrenal steroidogenic pathway in hybrid cell type A and C.

Fig. 6.
Presumption of adrenal steroidogenic pathway in hybrid cell type B and triple-positive cells.