Steroidogenic Acute Regulatory Protein is a Useful Marker for Leydig Cells and Sex-Cord Stromal Tumors

Lei Dong, MD, Huan Wang, MD, Zhitao Su, MD, Sanqiang Niu, MD, Rongrong Wang, MD, Liang Wu, MD, and Guorong Chen, MD

Abstract: Steroidogenic acute regulatory (StAR) protein is a rate-limiting protein, which is essential for transporting cholesterol into the mitochondria for steroidogenesis. StAR protein could be a marker for steroidogenic tissues. In this study, we investigated StAR protein levels in sex-cord stromal tumors (SCSTs) including 31 adult granulosa cell tumors, 3 juvenile granulosa cell tumors, 10 fibrothecomas, 2 luteinized thecomas, 4 Sertoli-Leydig cell tumors (SLCTs), 4 sclerosing stromal tumor and 3 Leydig cell tumors (LCTs), and 219 non-SCSTs. SCSTs were used for immunohistochemical staining of StAR protein, α-inhibin, calretinin, and CD99. All the 3 LCTs (100%) strongly stained for StAR protein; 30 of the 31 adult granulosa cell tumors (96%) showed focal staining of StAR protein; all the 10 fibrothecomas and 4 sclerosing stromal tumors (100%) were negative for StAR protein staining; StAR protein stained in Leydig cells but not in Sertoli cells in the 4 SLCTs. All the non-SCSTs were negative for StAR protein except for tumor cells in 4 adrenocortical adenomas and 2 adrenocortical carcinomas. Results of the study indicate that StAR protein is a useful marker for differential diagnosis of SCSTs. It is sensitive and specific for Leydig cells in tumors containing this component (LCT, SLCT), and can express focally in granulosa cell tumors. It is negative for Sertoli cells and nonluteinized theca cells.

Key Words: StAR, ovary, testis, sex-cord stromal tumors, immunohistochemistry

(Suppl Immunochemistry Mol Morphol 2011;00:000–000)

Steroidogenic acute regulatory (StAR) protein, first purified from mouse Leydig cells1 is a 30-Kd phosphoprotein, which localizes to the mitochondria and plays an important role in steroidogenesis. StAR protein transfers cholesterol from the outer to the inner mitochondrial membrane, and is a rate-limiting protein for steroidogenesis.2 Loss of StAR protein function after mutation can cause a lethal condition known as congenital lipoid adrenal hyperplasia, indicating that StAR protein is essential for steroidogenesis.3 StAR protein has been found to be present in many steroidogenic cells including adrenocortical, corpus luteal, and Leydig cells.4–8 Besides steroidogenic tissues, StAR protein is also found to be expressed in renal distal tubules. This relatively organ-specific expression spectrum indicates that StAR protein might be a good marker for the diagnosis of some tumors with steroidogenic origin.

Sex-cord stromal tumors (SCSTs) are a group of uncommon tumors, which are from the gonadal stroma. These tumors contain granulosa cells, theca cells, Sertoli cells, Leydig cells, and fibroblasts of stromal origin. SCSTs show a wide range of morphologic patterns depending on the variants of a single component as in granulosa cell tumors, or the various combinations of different components as in Sertoli-Leydig cell tumors (SLCTs). Therefore, they can cause numerous issues in differential diagnosis. Although in most circumstances, distinctive diagnosis can be made by gaining information from the clinical history, examining the gross specimen, and hematoxylin and eosin (H&E) staining of samples, immunohistochemistry might be very useful for differential diagnosis. Several useful markers of SCSTs including α-inhibin, calretinin, CD99, melan-A, and müllerian-inhibiting substance have been used for their differential diagnosis.9–15 However, new biomarkers are also required for accurate diagnosis. SCSTs usually contain steroidogenic cells; therefore, we postulate that StAR protein, the essential component for steroidogenesis, could be a marker for differential diagnosis of SCSTs.

In this study, we examined the expression patterns of StAR protein in SCSTs including adult granulosa cell tumors (AGCTs), juvenile granulosa cell tumors (JGCTs), thecomas, SLCTs, Leydig cell tumors (LCTs), and sclerosing stromal tumors of the ovaries and testes.

MATERIALS AND METHODS

Sample Preparation

Most samples used in this study were retrieved from the Department of Pathology of the first Affiliated
Hospital of Wenzhou Medical College from 2000 to 2007. Some of them were kindly donated by other hospitals (2 JGCTs were from the second Affiliated Hospital of Wenzhou Medical College and 1 SLCT from Yueqing People’s Hospital). All patients were evaluated by 2 pathologists based on the 2003 World Health Organization classification of tumors of female genital organs.16 Ovarian or testicular sex cord tumors included in this study are shown in Table 1. Fifteen testicular biopsies and 10 normal ovarian tissues from patients, who undertook radical hysterectomy, were also included. To determine StAR protein specificity in SCSTs, 219 non-SCST tumors were also included in this examination: 10 endometrioid adenocarcinomas, 4 ovarian malignant teratomas, 10 seminomas, 4 yolk sac tumors, 10 embryonal carcinomas, 5 ovarian serous adenocarcinomas, 11 ovarian mucinous adenocarcinomas, 4 ovarian clear cell carcinomas, 4 ovarian transitional cell tumors, 8 ovarian borderline serous cystadenomas, 5 adenocortical adenomas, 2 adenocortical carcinomas, 5 intestinal carcinoids, 3 choriocarcinomas, 4 lung carcinoids, 8 lung adenocarcinomas, 5 diffuse large B-cell lymphomas, 10 hepatocellular carcinomas, 9 thyroid carcinomas, 10 urothelial cell carcinomas, 5 esophageal squamous cell carcinomas, 10 gastric adenocarcinomas, 4 gallbladder adenocarcinomas, 4 mesotheliomas, 6 meningiomas, 5 ependymomas, 5 oligodendrogliomas, 5 astrocytomas, 3 pituitary adenomas, 5 prostate adenocarcinomas, 5 schwannomas, 5 skin sebaceous carcinomas, 5 skin basal cell carcinomas, 4 malignant melanomas, 5 rhabdomyosarcomas, 5 fibrosarcomas, 5 malignant fibrous histiocytomas, 5 synoviosarcomas, 4 liposarcomas, hemangiosarcomas, and 8 kidney clear cell carcinomas. All specimens were fixed in formalin and were routinely processed in paraffin wax. The study was approved by the Hospital Ethics Committee.

Western Blot Analysis
Western blot was performed on protein lysates of 4 representative normal tissues, including the endometrium, corpus luteum, parotid, and ovary. Forty micrograms protein extracts were electrophoresed on 10% polyacrylamide gels. Proteins were electroforetically transferred onto nitrocellulose membranes and blocked in 1 × tris-buffered saline tween-20 containing 5% non-fat milk at room temperature with agitation for 60 minutes. The membranes were incubated with a 1:500 dilution of a rabbit polyclonal StAR protein antibody and a 1:1000 dilution of a rabbit polyclonal β-tubulin antibody followed by overnight incubation at 4°C with agitation. The goat anti-rabbit horse radish peroxidase-conjugated secondary antibody was then applied at a dilution of 1:5000 at room temperature for 60 minutes. Immunoreactive bands were visualized by enhanced chemiluminescence and exposure to ECL films (Amersham, Braunschweig, Germany) for appropriate times.

Immunohistochemical Staining
Immunohistochemistry was performed on 4-μm-thick histologic sections of formalin-fixed, paraffin-embedded tissues. Antibodies used in this study included anti-StAR protein (Clone: FL-285, dilution: 1:300, Santa Cruz Biotechnology, Santa Cruz, CA), anti-α-inhibin (Clone: R1, prediluted, Zeta Corporation, Sierra Madre, CA), anti-CD99 (Clone: O13, prediluted, Invitrogen, Camarillo, CA), and anti-calretinin (Polyclonal Antibody Designation: DC8, prediluted, Invitrogen, Camarillo, CA). The tissue sections were deparaffinized and dehydrated by sequential passages through xylene, graded ethanol, and water, and then boiled in citrate buffer (pH 6.0) in a pressure cooker and kept for 2 minutes after full pressure was reached. The sections were blocked with 3% H2O2 for 10 minutes to quench endogenous peroxidase activity. After washing with phosphate-buffered saline (pH 7.2), the sections were incubated with the primary antibodies at 37°C for 1 hour. The horseradish peroxidase polymer conjugate from Super Pic Ture Polymer Detection Kit (Cat. No. 87-8963, Zymed Laboratories, San Francisco, CA) was applied for 30 minutes. The reaction product was detected with 3, 3-diaminobenzidine chromogen. For negative controls, the tissue sections were incubated with phosphate-buffered saline in the absence of primary antibody. Counter staining was carried out with hematoxylin.

Immunohistochemical Assessment
Immunohistochemical staining for StAR protein, α-inhibin, CD99, and calretinin were carried out on all the cases of SCSTs in this study. Staining for only StAR protein was carried out for all other miscellaneous tumors. Positive immunohistochemical staining for StAR protein, calretinin and α-inhibin were granular cytoplasmic staining, and for CD99 both membrane and cytoplasmic staining were carried out. The extent of positive cells and the intensity of immunohistochemical staining were evaluated by a semi-quantitative method. Scores were calculated as follows: 0, representing no positive cells on the whole section; 1+ (1 point), < 10% positive cells; 2+ (2 points), 11% to 50% positive cells; 3+ (3 points), 51% to 100% positive cells. Five to 10 positive fields were examined for each section. Intensity was arbitrarily graded as 2 grades: weak (1 point) and strong (2 points).

### TABLE 1. List of SLCT and Their Tissue Types

<table>
<thead>
<tr>
<th>Tumor Type</th>
<th>No. Cases</th>
<th>Tissue Type</th>
</tr>
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<tbody>
<tr>
<td>AGCT</td>
<td>31</td>
<td>Ovary</td>
</tr>
<tr>
<td>JGCT</td>
<td>3</td>
<td>Ovary</td>
</tr>
<tr>
<td>Fibrothecoma</td>
<td>10</td>
<td>Ovary</td>
</tr>
<tr>
<td>Luteinized thecoma</td>
<td>2</td>
<td>Ovary</td>
</tr>
<tr>
<td>SLCT</td>
<td>4</td>
<td>Ovary</td>
</tr>
<tr>
<td>Sclerosing stromal tumor</td>
<td>4</td>
<td>Ovary</td>
</tr>
<tr>
<td>LCT</td>
<td>3</td>
<td>1 testis, 2 ovaries</td>
</tr>
</tbody>
</table>

AGCT indicates adult granulosa cell tumor; JGCT, juvenile granulosa cell tumor; LCT, Leydig cell tumor; SCT, Sertoli cell tumor; SLCT, Sertoli-Leydig cell tumor.
Statistical Analysis

The comparison of the staining scores of StAR protein and those of calretinin, CD99, and α-inhibin were statistically analyzed using Student *t* test (2-tailed). All *P* values < 0.05 were considered to be statistically significant.

RESULTS

Western Blot Analysis

The specificity of the anti-StAR protein antibody was determined by Western blot analysis. A single band with a molecular weight of about 30 Kd, which corresponded to the StAR protein, was detected in the human ovary, corpus luteum, but was absent in the endometrium and parotid, which do not contain steroidogenic cells. There was no cross-reactivity with other proteins as showed in the Western blot analysis (Fig. 1).

StAR Protein in Non-Neoplastic Testicular and Ovarian Tissues

Non-neoplastic testicular tissue sections from 15 testicular biopsies and 10 normal ovarian tissue sections from patients who undertook radical hysterectomy were evaluated for StAR protein expression. Ovarian follicles were classified according to the Clement description.17 The results are summarized in Table 2. In testicular tissues, Leydig cells were intensively stained for StAR protein in all cases (Fig. 2), whereas Sertoli cells and spermatocytes were all negative for StAR protein. In ovarian tissues, ovarian surface epithelial cells, fibroblast stromal cells, granulosa cells, and inner theca cells from primary follicles were negative for StAR protein staining, whereas the inner theca cells from the antral follicle, the granulosa cells, and the inner theca cells from the corpus luteum were positive for StAR protein (Fig. 2, Table 2). These results indicate that StAR protein is specifically expressed in steroidogenic cells.

StAR Protein in Ovarian and Testicular LCTs

Of 2 ovarian and 1 testicular LCTs, Leydig cells strongly stained for StAR protein (Fig. 3, Table 3) with scores of extent 3 and intensity 2.

StAR Protein in Ovarian AGCTs and JGCTs

Among 31 AGCTs, 30 (96%) were positive for StAR protein. StAR protein staining showed focal or dotlike positive pattern (Fig. 4, Table 3). For extent scores, there were 28 cases scored as 1 and 2 cases as 2; for intensity scores, 11 cases were scored as 1 and 19 cases as 2. The staining in JGCTs (2 cases) was stronger than that in AGCTs. Both the cases were scored as extent 2 and intensity 2. StAR protein was also positive in some individual luteinized stromal cells.

StAR Protein in Ovarian Fibrothecomas and Sclerosing Stromal Tumors

All 10 fibrothecomas and 4 sclerosing stromal tumors were negative for StAR protein staining, except for some scattered individual luteinized theca cells stained as dotlike pattern. In 2 luteinized thecomas, the luteinized tumor cells showed strong staining for StAR protein.

StAR Protein in Ovarian SLCTs

In 4 SLCTs (1 well differentiated, 3 intermediate differentiated), mild-to-strong staining of StAR protein was observed in Leydig cells but not in Sertoli cells (Fig. 5, Table 3).

StAR Protein in Non-SCSTs

To explore the specificity of StAR protein staining, 219 non-SCST tumors from various organs and sites were also stained with StAR protein. All of them were negative for StAR protein, except for the tumor cells of adrenocortical adenomas (4 of 5 cases), which showed strong staining; a yolk sac tumor (1/4) and 5 borderline serous cystadenomas (5/8) showed positively scattered individual luteinized stromal cells.

Comparison of the Stain of StAR Protein With Those of Inhibin, Calretinin, and CD99 in SCSTs

To compare StAR protein with other SCST markers, we carried out inhibin, calretinin, and CD99
immunostaining for SCSTs. In 3 LCTs, StAR protein, inhibit, and calretinin were strongly stained, and CD99 was negative. In 4 SLCTs, StAR protein was uniquely positive in Leydig cells, and negative in Sertoli cells; calretinin and CD99 were stained in both types of cells and negative in Leydig cells in 1 case; inhibit was positive in both types of cells and negative in Sertoli cells in 1 case. For StAR protein, in 31 AGCTs, although only a few cells were stained in 96% of the AGCT cases, the staining intensity scores were as high as those in inhibit and calretinin (P > 0.05), and higher than those in CD99 (P < 0.05). For the extent staining scores in AGCTs, StAR protein was lower than those in other 3 markers (P < 0.05). None of them were positive for all AGCTs; the positive rate of StAR protein was 96% (30/31), inhibit was 87% (27/31), calretinin was 93%, and CD99 was 66%.

**DISCUSSION**

StAR protein is an essential phosphoprotein in the regulation of steroid biosynthesis. Translational inhibition of StAR production results in a dramatic decrease in steroid biosynthesis.\(^4,18\) In human tissues, StAR protein has been reported in the adrenal glands, testis, ovary, endometrium, and kidney.\(^3,5–7,19\) Consistent with the immunostaining studies by Pollack et al\(^7\) and Kiriakidou et al,\(^6\) our results showed that StAR protein was present in the granulosa and theca cells in mature follicles and corpora lutea, but not in primary and antral follicles. In this study StAR protein was only detected in Leydig cells, and not in Sertoli cells. In the study by Pollack et al,\(^7\) some specimens of Sertoli cells were also stained positive for StAR protein. This discrepancy might be due to the sources of primary antibody against StAR protein, as it has been detected in cultured rat Sertoli cells.\(^20\) Given the present conception that the function of StAR protein is associated with the transportation of cholesterol for steroidogenesis, its role in rodent Sertoli cells is not clear. The expression of StAR protein in human Sertoli cells is worth further investigation.

This study showed the sensitivity of StAR protein to Leydig cells. StAR protein was 100% positive for Leydig cells in normal testes and in ovarian and testicular tumors with a component of Leydig cells (3 LCTs and 4 SLCTs). This staining pattern helps to identify LCTs, which in some cases might resemble SCTs\(^21\) and clusters or sheets of Leydig cells in SLCTs. Although StAR protein is expressed in luteinized stromal cells in AGCTs, it is usually stained in individual cells rather than in clusters or sheet patterns in SLCTs. StAR protein also showed high sensitivity in luteinized sex cord cells in ovary. Given the role of StAR protein in steroidogenesis, it is not surprising to find its strong expression in luteinized thecomas and in some individual luteinized stromal cells in other SCSTs. We also observed StAR protein-positive cells in a yolk sac tumor and 5 borderline serous cystadenomas.

StAR protein showed different staining in other sex cord cells in SCSTs. In AGCTs, it was detected focally but with strong sensitivity (96%) in tumor cells regardless of the morphologic variants. It seemed that JGCTs express more StAR protein than AGCTs in this study, indicating that many tumor cells in JGCTs are luteinized.\(^22,23\) StAR
protein was not detected in Sertoli cells and nonluteinized theca cells. In other organs and sites, StAR protein was only stained in adrenocortical adenoma and adrenocortical carcinoma, which are steroidogenic tumors too. With regard to clinical pathology, adrenocortical adenoma and adrenocortical carcinoma rarely enter the differential diagnosis of SCSTs. StAR protein might be positive in scattered luteinized stromal cells in ovarian tumors as in yolk sac tumor and serous adenomas in our cases, but the neoplastic cells were negative, therefore, these individual positive cells usually do not cause difficulty in differential diagnosis. Therefore, StAR protein showed relative specificity in SCSTs.

Some valuable markers, including α-inhibin, calretinin, CD99, have been applied in the differential diagnosis of SCSTs. In this study, we investigated the expression of StAR protein in SCSTs and chose α-inhibin, CD99, and calretinin for comparison. Our

![FIGURE 3. StAR protein staining in testicular LCT. A, Tumor cells show abundant eosinophilic cytoplasm, H&E × 100. B, Tumor cells were strongly stained with StAR protein, × 100. H&E indicates hematoxylin and eosin; LCT, Leydig cell tumor; StAR, steroidogenic acute regulatory.](image)

![FIGURE 4. StAR protein staining in ovarian AGCT. A, Tumor cells show trabecular pattern, H&E × 100. B, StAR protein shows focal or dotlike staining in tumor cells, × 100. AGCT indicates adult granulosa cell tumor; H&E, hematoxylin and eosin; StAR, steroidogenic acute regulatory.](image)

**TABLE 3. Positive StAR Protein Staining Protein in SCSTs**

<table>
<thead>
<tr>
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<th>StAR Positivity (%)</th>
<th>Positive Cell Type</th>
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<tbody>
<tr>
<td>AGCT</td>
<td>30/31 (95)</td>
<td>Granulosa cells, luteinized stromal cells</td>
</tr>
<tr>
<td>JGCT</td>
<td>3/3 (100)</td>
<td>Granulosa cells, luteinized stromal cells</td>
</tr>
<tr>
<td>Fibrothecoma</td>
<td>0/10</td>
<td>None</td>
</tr>
<tr>
<td>Luteinized thecoma</td>
<td>2 (100)</td>
<td>Luteinized theca cells</td>
</tr>
<tr>
<td>SLCT</td>
<td>4 (100)</td>
<td>Leydig cells</td>
</tr>
<tr>
<td>Sclerosing stromal tumor</td>
<td>0/4 (100)</td>
<td>None</td>
</tr>
<tr>
<td>LCT</td>
<td>3/3 (100)</td>
<td>Leydig cells</td>
</tr>
</tbody>
</table>

AGCT indicates adult granulosa cell tumor; JGCT, juvenile granulosa cell tumor; LCT, Leydig cell tumor; SCST, sex-cord stromal tumors; SCT, Sertoli cell tumor; SLCT, Sertoli-Leydig cell tumor; StAR, steroidogenic acute regulatory.
results showed that StAR protein was as sensitive as inhibin and calretinin in LCTs. Compared with the other 3 markers that stained both types of Leydig and Sertoli cells, StAR protein uniquely stained Leydig cells in SLCTs. In AGCTs, StAR protein only showed focal staining in neoplastic cells, but considering that none of inhibin, calretinin, and CD99 are 100% positive in all AGCTs and the positive rate of StAR protein is high in AGCTs (96%), StAR protein is worth entering the panel of markers when AGCT needs to be differentiated from non-SCSTs. Melan-A can be used as a marker for SCSTs. Although the results from different studies are not completely identical, the antibody of melan-A showed an interesting specificity for lipid-containing cells. For instance, it inclined to label Leydig cells and was negative for Sertoli cells in SLCTs, a staining pattern similar to that of StAR protein as observed in this study. Unlike StAR protein, the staining of melan-A on Leydig cells might represent an immunologic cross-reaction. 

In summary, our results indicate that StAR protein is a useful marker for differential diagnosis of SCSTs. It is very sensitive and specific for Leydig cells in tumors containing this component (LCT, SLCT), and can express focally in granulosa cell tumors. It is negative for Sertoli cells and nonluteinized theca cells.

REFERENCES


