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# Correlation of FOXL2 with Inhibin and Calretinin in the Diagnosis of Ovarian Sex Cord Stromal Tumors

**Ruchi RATHORE, Deepshikha ARORA, Sarla AGARWAL, Sonal SHARMA**

*Department of Pathology, University College of Medical Sciences, NEW DELHI, INDIA*

## ABSTRACT

***Objective:*** Alpha-inhibin and calretinin have been traditionally used as immunomarkers for sex cord stromal tumors. However, the variation in their immunoreactivity and their lack of specificity for sex cord stromal tumor makes the search for a more sensitive and specific immunohistochemical marker essential in routine diagnosis of sex cord stromal tumor. This study was conducted to correlate the diagnostic utility of FOXL2 with inhibin and calretinin in the diagnosis of sex cord stromal tumors of ovary.

***Material and Method:*** The study was conducted in the department of pathology. 31 cases of sex cord tumors received in past eight years (2002-2010) were included in this study. Immunostaining for inhibin, calretinin and FOXL2 was performed and compared on the basis of staining intensity and percentage positivity on all the cases.

***Results:*** Calretinin was found to be positive in 29/31 sex cord stromal tumors with variable intensities and was negative in two cases of sex cord stromal tumors, inhibin was positive in 28/31 and only three cases had no cytoplasmic staining. All the 31 cases included in this study were positive for FOXL2 with variable staining intensities and percentage positivity. Ten cases of each surface epithelial and germ cell tumors were also negatively stained with FOXL2.

***Conclusion:*** In contrast to inhibin and calretinin, FOXL2 had a sensitivity and specificity of 100% for all the cases of sex cord stromal tumors included in this study.

***Key Words:*** FOXL2, Inhibin, Calretinin, Ovarian neoplasms, Sex cord stromal tumor

## INTRODUCTION

Sex cord stromal tumors (SCST) constitute 8% of all the ovarian neoplasms comprising those that contain granulosa cells, theca cells, and their luteinized derivatives, Sertoli cells, Leydig cells and fibroblast of gonadal stromal origin singly or in various combinations with varying degrees of differentiation (1). These tumors as known for their hormonal activity are slow growing tumors and have a tendency of late recurrence. Since the patients are often young and most tumors are unilateral, accurate diagnosis is necessary for proper treatment and maintenance of fertility where desirable.

Although the mainstay for diagnosing these SCST remains gross and microscopic features, the presence of diverse morphological variations makes the use of immunohistochemistry essential in their diagnosis. The widely varied appearance of SCST and the fact that some of them are uncommon can lead to difficulties in their diagnosis. Some of them may show atypical or unconventional microscopic patterns and, therefore, morphology alone may not be reliable for an unequivocal classification. In these cases, immunohistochemistry can be of value. Alpha-inhibin (2-6) and calretinin (7,8) have been traditionally used as immunomarkers of SCST. However due to the variation in their immunoreactivity and a lack of specificity for SCST, the search for a more sensitive and specific immunohistochemical marker becomes essential in routine diagnosis of SCST.

In the recent past, FOXL2 mutations have been consistently found in adult granulosa cell tumors (9,10). Currently there are increasing numbers of studies eliciting the role of FOXL2 in the diagnosis of adult granulosa cell tumors but there is paucity of literature on its diagnostic utility in comparison to inhibin and calretinin in the diagnosis of the entire spectrum of SCST of the ovary. The aim of this study was to evaluate the role of FOXL2 and its correlation with inhibin and calretinin in the diagnosis of entire spectrum of SCST.

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**Correspondence: Sonal SHARMA**
University College of Medical Sciences, Department of Pathology,
NEW DELHI, INDIA
E-mail: sonald76@gmail.com Phone: +91 98100 44303

## MATERIAL and METHODS

### Case Selection

The study was conducted in the department of pathology of our institute. Thirty-one cases of SCST received in past eight years (2002-2010) were included in this study. These consisted of 17 adult granulosa cell tumors, 1 juvenile granulosa cell tumor, 7 fibrothecoma, 1 fibroma, 1 fibrosarcoma, 1 SCST of uncertain histogenesis, 1 poorly differentiated tumor possibly granulosa cell tumor and 2 cases of Sertoli-Leydig cell tumor. For all the cases, Hematoxylin and Eosin (H&E) stained slides were retrieved from archives and reviewed by expert pathologists. Ten cases each of surface epithelial and germ cell tumors were also included in the study for FOXL2 staining.

### Immunohistochemistry

The blocks were selected for immunohistochemical demonstration of inhibin, calretinin and FOXL2. Then 3-4 micrometer thick sections were taken from each block on poly-L-lysine-coated slides. These were stained with monoclonal antibody against α subunit of inhibin, antibody to calretinin and polyclonal antibody to C-terminal hexadecapeptide of the human FOXL2 protein respectively using the avidin biotin method. The primary antibody for FOXL2 used in this study was polyclonal FOXL2-antiserum (1:200; Imgenex, San Diego, CA, USA). For calretinin and inhibin the primary antibodies used were monoclonal anticalretinin (RTU, Spring Biosciences, CA, USA) and monoclonal anti-inhibin (1:50; Spring biosciences, CA, USA) respectively. Serial dilutions of antibody were tested to avoid background staining and to find optimal staining before the entire series was processed. Sections from normal ovary with graffian follicles were used as positive control. For negative controls, primary antibody was replaced by TRIS buffer.

### Immunostaining Analysis

The slides were examined at 40x magnification. Positive reaction was characterized by cytoplasmic (inhibin), nuclear and cytoplasmic (calretinin), nuclear (FOXL2) staining showing variable intensities with the distribution of staining approximated for variable intensity levels. The presence of any nuclear staining of FOXL2, regardless of intensity or focality, was considered to be positive.

### Statistical Analysis

Sensitivity, specificity, negative predictive value, and positive predictive value for inhibin, calretinin and FOXL2 were determined taking histopathology as the gold standard. Ten cases each of malignant surface epithelial tumors and germ cell tumors were included in this study to determine the specificity of antibodies for SCST. The exact binomial method (Clopper-Pearson) was applied to calculate the confidence interval of diagnostic indices. SPSS version 17-statistic software was used for data analysis

### Comparison of Immunostaining for FOXL2, Inhibin and Calretinin

Immunostaining for FOXL2, inhibin and calretinin were compared on the basis of staining intensity and percentage positivity in all the cases. The staining intensity was graded as -weak (1+), moderate (2+), strong (3+). The percentage of tumor cells labeled by FOXL2, inhibin and calretinin were semiquantitatively scored as <5% (negative), 5-25% (1+), 26-50 % (2+), 51-75% (3+), >75% (4+).

## RESULTS

### Inhibin Immunoanalysis

Inhibin was positive in the majority of the SCST i.e. 28/31 and only 3 cases had no cytoplasmic staining (Table I). Of all negatively stained tumors, two were granulosa cell tumors which included a cystic granulosa cell tumor and the other was a granulosa cell tumor with postchemotherapy changes (bizarre cells), and the remaining case was a SCST of uncertain histogenesis (Figure 1A-D) (Table I).

Taking H&E stained sections as the gold standard, the sensitivity of inhibin was 90.3% and the specificity was 100% for SCST. The positive predictive value for inhibin in our study was 100% and the negative predictive value was 87.0%.

### Calretinin Immunoanalysis

Calretinin was found to be positive in 29/31 SCST with variable intensities and was negative in 2 cases of SCST including 1 case of fibrothecoma and the other of fibrosarcoma (Table I). Both the above stated cases were also found to be negative, when the percentage positivity of all the 31 cases was seen. The remaining 29/31 cases had variable staining intensities ranging from 1(+) to 4(+) (Table I).

The sensitivity of calretinin in this study was 93.5% while the specificity was 100%. The negative predictive value of calretinin for this study was 90.9% and the positive predictive value was 100%. H&E stained sections were taken as the gold standard for the analysis. (Figure 1A-D)

### FOXL2 Immunoanalysis

Thirty-one cases of SCST were stained using a polyclonal antibody against FOXL2, which is a nuclear protein. Ten cases each of surface epithelial and germ cell tumors were

**Table I:** Inhibin, calretinin and FOXL2 immunoanalysis for sex cord stromal tumors.

| GRADE / CASES | Staining Intensity | | | | % Positivity of Tumor Cells | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | **Negative** | **Mild** | **Moderate** | **Strong** | **Negative** | **1+** | **2+** | **3+** | **4+** |
| Adult Granulosa Cell Tumors (17) | | | | | | | | | |
| **Inhibin** | 2 | 6 | 8 | 1 | 2 | 2 | 8 | 4 | 1 |
| **Calretinin** | | 6 | 8 | 3 | | 6 | 7 | 1 | 3 |
| **FOXL2** | | 6 | 8 | 3 | | 6 | 7 | 1 | 3 |
| Juvenile Granulosa Cell Tumors (1) | | | | | | | | | |
| **Inhibin** | | | 1 | | | | | 1 | |
| **Calretinin** | | | 1 | | | | | 1 | |
| **FOXL2** | | 1 | | | | | 1 | | |
| Fibrothecomas (7) | | | | | | | | | |
| **Inhibin** | | 2 | 5 | | | 2 | 3 | 2 | |
| **Calretinin** | 1 | 3 | 3 | | 1 | 3 | 2 | 1 | |
| **FOXL2** | | 4 | 2 | 1 | | 2 | 3 | 2 | |
| Fibroma (1) | | | | | | | | | |
| **Inhibin** | | | 1 | | | | 1 | | |
| **Calretinin** | | | 1 | | | | 1 | | |
| **FOXL2** | | 1 | | | | 1 | | | |
| Fibrosarcoma (1) | | | | | | | | | |
| **Inhibin** | | | 1 | | | | 1 | | |
| **Calretinin** | 1 | | | | 1 | | | | |
| **FOXL2** | | | 1 | | | | | 1 | |
| Sex Cord Stromal Tumor of Uncertain Histogenesis (1) | | | | | | | | | |
| **Inhibin** | 1 | | | | 1 | | | | |
| **Calretinin** | | 1 | | | | | 1 | | |
| **FOXL2** | | | 1 | | | 1 | | | |
| Poorly Differentiated Tumor Possibly Granulosa Cell Tumors (1) | | | | | | | | | |
| **Inhibin** | | | 1 | | | | | 1 | |
| **Calretinin** | | | | 1 | | | | 1 | |
| **FOXL2** | | | | 1 | | | | | 1 |
| Sertoli-Leydig Cell Tumors (2) | | | | | | | | | |
| **Inhibin** | | | 1 | 1 | | | 1 | 1 | |
| **Calretinin** | | | 2 | | | | 1 | 1 | |
| **FOXL2** | | 1 | 1 | | | 1 | 1 | | |
| **PC (H&E) (31) : {In,Cl,Fx}** | | | | 31 | | | | | 31 |
| **NC {GCT (10) & S E T (10)} : {In,Cl,Fx}** | 31 | | | | 31 | | | | |

**PC :** Positive Control, **NC :** Negative Control, **H&E :**Hematoxylin and Eosin, **GCT:** Germ Cell Tumor, **SET:** Surface Epithelial Tumor, **In:** Inhibin, **Cl:** Calretinin, **Fx :** FOXL2.

also stained with FOXL2. All negative controls as well as surface epithelial and germ cell tumors were negative. Positive control had a nuclear pattern of staining in the granulosa cells of normal ovary (Figure 1A-D).

FOXL2 was positive with variable staining intensities and percentage positivity in all the cases included in this study. While 16/31 SCST had mild intensity of staining, 6/31 had strong staining intensity and 9/31 had moderate intensity of staining. Tumors, which were strongly positive included 4/17 adult granulosa cell tumors and 1/7 fibrothecomas. One tumor which was signed out as poorly differentiated tumor possibly granulosa cell tumor

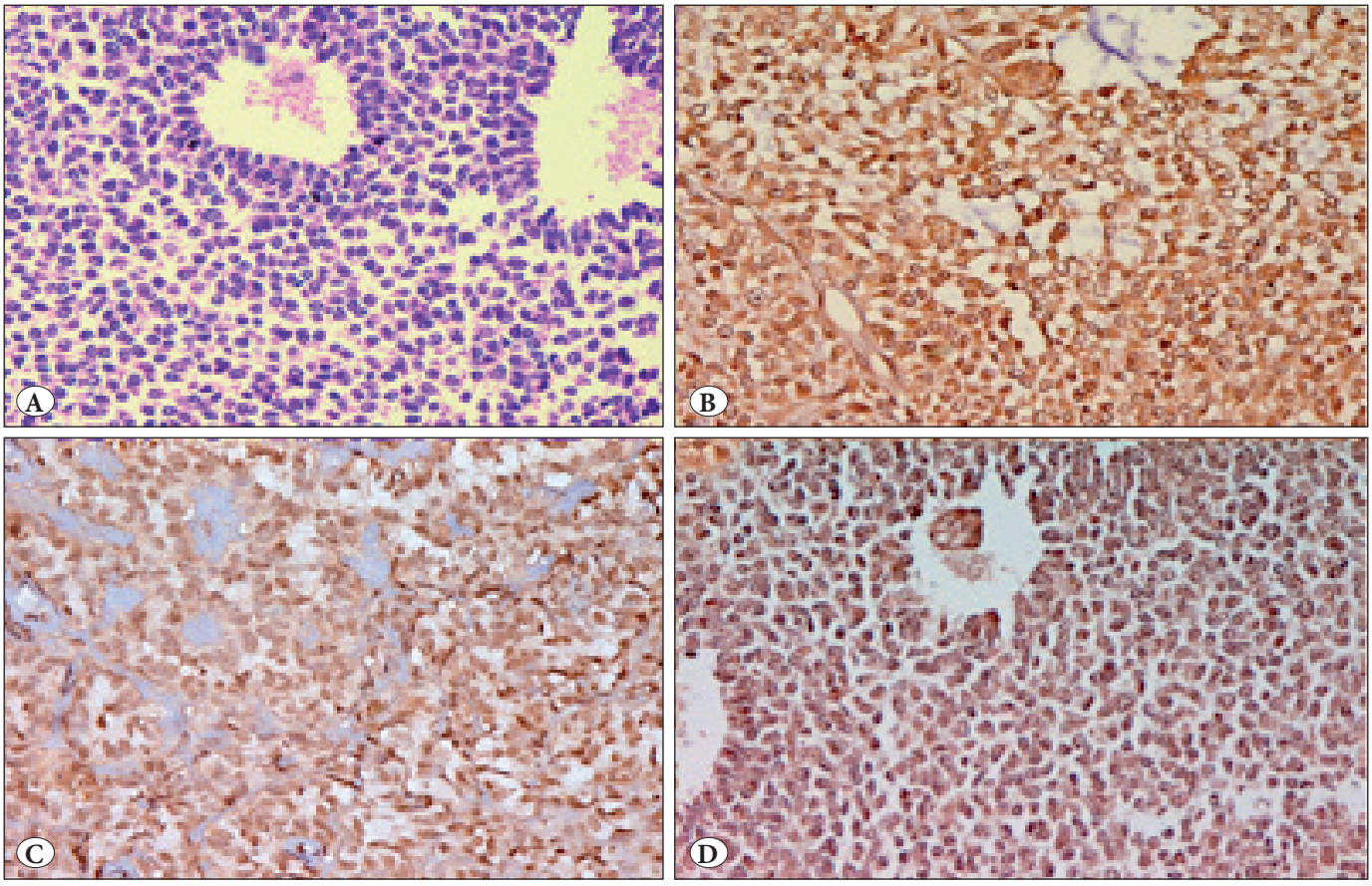


**Figure 1:** Granulosa cell tumor: **A)** H&E staining section (H&E; x400) **B)** Cytoplasmic positivity for inhibin (Inhibin; x400) **C)** Nuclear and cytoplasmic positivity for calretinin (Calretinin; x400). **D)** Nuclear positivity for FOXL2 (FOXL2; x400).

and was positive for inhibin and calretinin also had strong intensity of staining with FOXL2 (Figure 2A,B). Tumors showing moderate intensity of staining included 4/17 adult granulosa cell tumor, 2/7 cases of fibrothecoma, 1/1 of fibrosarcoma and 1/2 Setoli-Leydig cell tumor. 1/1 SCST of uncertain histogenesis included in this study was also moderately positive for FOXL2 (Table I). The remaining cases, including 9/17 adult granulosa cell tumors, 1 /1 case of juvenile granulosa cell tumor, 4/7 fibrothecomas, 1/1 fibroma and 1/2 Sertoli-Leydig cell tumors, were mildly positive for FOXL2 (Table I).

Comparing the percentage positivity for all the 31 cases of SCST, it was seen that of the three cases having 4(+) percentage positivity, 2 were granulosa cell tumors with diffuse microscopic patterns and one was a poorly differentiated tumor. 8/31 cases that had 3(+) positivity, which included 5/17 adult granulosa cell tumors followed by 1/7 fibrothecomas, 1/1 fibrosarcoma and 1 case of Sertoli-Leydig cell tumors. The only case of juvenile granulosa cell tumor included in the study had 2(+) percentage positivity while SCST of uncertain histogenesis had 1(+) percentage positivity. 3/7 fibrothecomas had 1(+) and 3/7 had 2(+) percentage positivity respectively (Table I).

Taking H&E sections as the gold standard, the sensitivity and specificity of FOXL2 was 100% and negative predictive values as well as positive predictive value were also 100% in our study. Moreover, ten cases each of surface epithelial tumors and germ cell tumors were negative for FOXL2.

### Comparison of FOXL2, Inhibin and Calretinin

In contrast to inhibin and calretinin, FOXL2 had a sensitivity and specificity of 100% for all the cases of SCST included in the study (Table II).

## DISCUSSION

Inhibin is a dimeric 32 KDa peptide hormone composed of an α subunit and a β subunit. Apart from being produced normally by granulosa cells and testicular Sertoli cells, its extragonadal expression in the placenta, adrenal gland, pituitary gland and liver has also been demonstrated in various studies (11-16). Most studies have shown that inhibin is expressed in nearly all granulosa cell tumors,

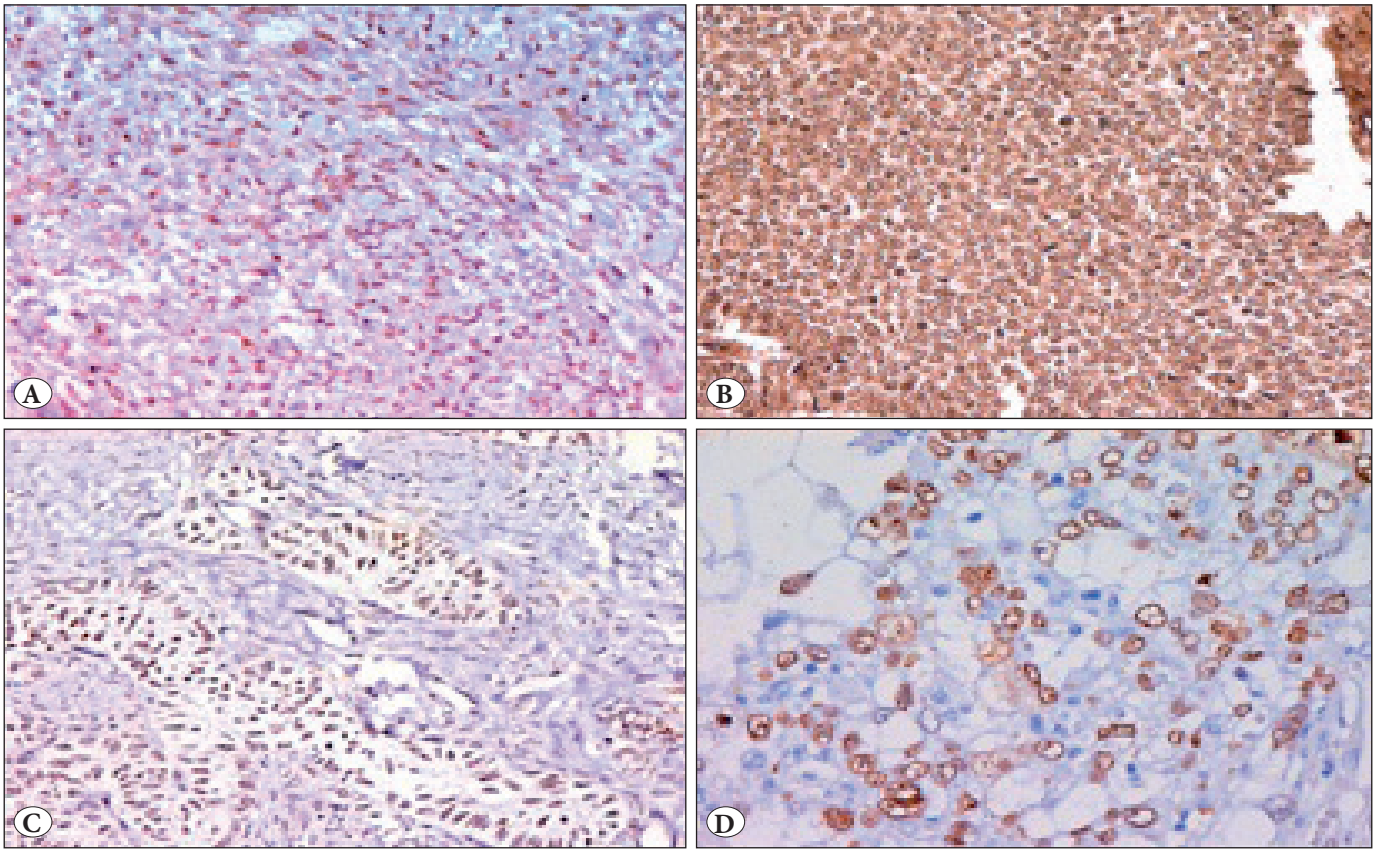


**Figure 2: A)** Nuclear FOXL2 staining in fibrothecoma (Immunohistochemistry; x400). **B)** Adult Granulosa Cell Tumor (FOXL2; x400). **C)** Sertoli-Leydig cell tumor (FOXL2; x400). **D)** Juvenile granulosa cell tumor (FOXL2; x400).

**Table II:** Comparison of immunohistochemical staining sensitivity, specificity, negative predictive value and positive predictive value of inhibin, calretinin and FOXL2

| Comparison of Inhibin, Calretinin and FOXL2 | | | | | |
|---|---|---|---|---|---|
| **Immunostain** | +ve Cases | Sensitivity (%) | Specificity (%) | NPV (%) | PPV (%) |
| **Inhibin (CI)** | 28/31 | 90.3 (74.2-98) | 100 (83.2-100) | 87.0 (66.4-97.2) | 100 (87.7-100) |
| **Calretinin (CI)** | 29/31 | 93.5 (78.6-99.2) | 100 (83.2-100) | 90.9 (70.8-98.9) | 100 (88.1-100) |
| **FOXL2 (CI)** | 31/31 | 100 (88.8-100) | 100 (83.2-100) | 100 (83.2-100) | 100 (88.8-100) |

**NPV:** Negative Predictive Value, **PPV:** Positive Predictive Value, **CI:** Confidence Interval

juvenile and adult types, most Sertoli and Leydig cell tumors, SCST with annular tubules, gynandroblastomas, and steroid cell tumors (17,18-21). However, Matias-Guiu et al. demonstrated that it is less frequently expressed in fibromas, fibrothecomas and unclassified SCST and is absent in fibrosarcoma (17). In our study it was seen that inhibin was positive in the majority of the SCST i.e. 28/31 (90.3%) and only 3 cases had no cytoplasmic staining. As opposed to the previous studies, all 7/7 cases of fibrothecoma and 1/1 cases of fibroma (moderate intensity) were positive for inhibin. In fact, a single case of fibrosarcoma included in our study demonstrated moderate intensity of staining with inhibin. However, in accordance with the described literature, the only case of SCST of uncertain histogenesis included in our study was negative for inhibin immunoreactivity.

Calretinin, a 29-kd calcium-binding protein, has been shown in human ovarian surface epithelial cells, theca interna cells, stromal cells, hilar cells, and certain types of ovarian SCST by various research groups (22-25). Calretinin, initially used for the diagnosis of mesothelioma, has been shown to be highly sensitive but a less specific marker than inhibin for SCST of the ovary. In studies conducted by Movahedi et al. and Shah et al., it was seen that calretinin has a sensitivity of 97% and specificity of 85% (except in granulosa cell tumors with extensive luteinization, which less frequently express calretinin)

compared with inhibin having 71% sensitivity and 97% specificity (26,27). However, the sensitivity of calretinin in this study was 93.5% while the specificity was 100%. The negative predictive value of calretinin for this study was 90.9% and the positive predictive value was 100%.

In contrast to inhibin, calretinin is more frequently expressed in epithelial ovarian tumors including serous, mucinous, clear cell, and endometroid carcinomas (as many as 22% of cases in one series) and is therefore less reliable in the differential diagnosis of SCST and endometroid carcinoma (20,26). It has been noted in various studies that fibromas are frequently positive for calretinin and negative for inhibin and the sole use of calretinin in this setting is more discriminatory (26, 28,29). In our study it was seen that in the single case of SCST of uncertain histogenesis where inhibin was found to be negative, calretinin staining gave a 1(+) percentage positivity in cells whereas the only case of a poorly differentiated tumor possibly granulosa cell tumor on morphology had strong positivity for both inhibin as well as calretinin. Moreover, it was seen that in the case of cystic granulosa cell tumor and granulosa cell tumor with postchemotherapy changes where inhibin was negative, calretinin was strongly positive. Our study supports that calretinin is more sensitive than inhibin for the diagnosis of SCST as described in the literature.

FOXL2, a forkhead transcription factor is a key factor in proper differentiation of granulosa cells during folliculogenesis and its expression persists in the ovary after birth (30). Shah et al. identified a single recurrent somatic, missense mutation in FOXL2 (C402G), which was present in almost all morphologically identified adult type granulosa cell tumours (9). Kalfa et al. studied the underexpression of FOXL2 in juvenile ovarian granulosa cell tumors with an aggressive pattern of progression and it thus may be a prognostic factor for these tumors (31). Rosario et al. concluded that FOXL2 mutations target the deregulation of TGF-β signaling genes, a key antiproliferative pathway in the pathogenesis of adult-type GCTs (32). However, its exact role in the pathogenesis of these tumors is still under evaluation. FOXL2 immunostaining gives a nuclear pattern of staining due to the nuclear location of FOXL2 protein, a transcription factor antibody.

Recently a study conducted by Osama et al. demonstrated that FOXL2 is a robust immunohistochemical marker for SCST that works in formalin-fixed, paraffin-embedded tissue sections and is highly expressed in both FOXL2-mutant SCST and a subset of SCST without a (402C-G) mutation in FOXL2. FOXL2 immunostaining is both sensitive and highly specific for SCST, performing better than α inhibin and calretinin. A subset of SCST is typically negative for FOXL2 on immunostaining (retiform or poorly differentiated SLCT), but these tumors usually express α inhibin and/or calretinin. In most of the FOXL2 positive cases in this study, FOXL2 demonstrated a nuclear pattern of staining and stained a high proportion of tumor cells leading to easy interpretable staining (33).

A single study conducted by McCluggage et al. on the expression of FOXL2 in fibromas and its diagnostic utility as an adjunct in the differential diagnosis of diffuse granulosa cell tumors concluded that FOXL2 mutations are absent in ovarian fibromas in contrast to granulosa cell tumors and are thus a useful diagnostic adjunct in distinction from diffuse adult granulosa cell tumors (34).

In our study it was seen that all the cases including 100% of adult granulosa cell tumors, 100% of juvenile granulosa cell tumors, all the cases of fibrothecoma, fibrosarcoma, fibroma, SCST of uncertain histogenesis and poorly differentiated tumor possibly granulosa cell tumor on morphology were positive for FOXL2. McCluggage et al. detected no FOXL2 mutation in ovarian fibroma. The importance of detection of FOXL2 expression in ovarian fibroma is not clear because FOXL2 expression was detected in only one case in our study. This finding should be investigated in large case series and compared with FOXL2 mutation and expressions in the tumor (34). All the cases that were negative for inhibin or calretinin were positive for FOXL2 in our study. Taking H&E sections as the gold standard, the sensitivity and specificity of FOXL2 was 100% and negative predictive values as well as positive predictive value were also 100% in our study. Though the pattern of immunoreactivity in our study was in accordance with the literature, the limited number of cases included in our study was a major setback in studying the role of FOXL2 in the whole range of SCST. No case of steroid cell tumor, Leydig cell tumor or female adnexal tumor of probable Wolffian origin and only a single case of fibroma in which FOXL2 has been shown to be negative in earlier studies was included in our study.

However, it was demonstrated that most cases of SCSTs were positive for FOXL2 as compared to inhibin or calretinin in this study. Our results also suggested that FOXL2 had a better percentage positivity of cells as compared to earlier well-established markers such as calretinin and inhibin. As only a limited number of cases were included in this study, the whole range of sex cord stromal tumors could not be evaluated for immunohistochemical analysis and further studies with a wider range of these tumors are required for the validation of our study.

In conclusion, testing for FOXL2 immunoexpression can serve to distinguish between SCST and non-SCST. However, we acknowledge that the smaller number of cases included in our study was a major limitation and further validation with a larger number of cases is still required.

## CONFLICT of INTEREST

No financial support of any form has been taken from any agency for this study.

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