OVARIAN CELLULAR FIBROMAS LACK FOXL2 MUTATIONS: A USEFUL DIAGNOSTIC ADJUNCT IN THE DISTINCTION FROM DIFFUSE ADULT GRANULOSA CELL TUMOUR

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ABSTRACT
Ovarian cellular fibromas are uncommon neoplasms which may result in considerable diagnostic confusion with diffuse adult granulosa cell tumour. This is an important distinction since the former usually exhibits benign behaviour while the latter is a low grade malignant neoplasm capable of recurrence and metastasis. FOXL2 mutation (402C→G) has been demonstrated in >95% of ovarian adult granulosa cell tumours, only rarely in other ovarian sex cord-stromal neoplasms and never in ovarian fibromas. In this study, we evaluated a series of ovarian cellular fibromas or mitotically active cellular fibromas (n=22), 3 with minor sex cord elements, for FOXL2 mutation. These were mostly received in consultation, often with a differential diagnosis of diffuse adult granulosa cell tumour. Immunohistochemically, 10 of 10 cases tested exhibited nuclear staining with FOXL2. FOXL2 (402C→G) mutation was not demonstrated in any of the 22 cellular or mitotically active cellular fibromas. Three additional neoplasms composed of cellular nodules of epithelioid cells in a background fibrous stroma, raising the possibility of adult granulosa cell tumour with a prominent fibrothecomatous component, were also tested; 2 of these were mutation negative and 1 contained a FOXL2 mutation. FOXL2 mutation analysis is a useful adjunct in distinguishing between diffuse adult granulosa cell tumour (mutation present) and cellular fibroma (mutation absent). Mutation testing should be considered in problematic cases since this will provide prognostic information for the patient.

Key words:- ovary, cellular fibroma, adult granulosa cell tumour, FOXL2 mutation.

INTRODUCTION
Ovarian cellular fibromas are uncommon, but by no means rare, neoplasms which not infrequently result in diagnostic problems for a number of reasons. One problem is that, especially when mitotically active, they may be confused with a fibrosarcoma or some other malignant mesenchymal neoplasm. To this end, it has been shown that morphologically bland ovarian fibromatous neoplasms, even when mitotically active, usually behave in a benign fashion and the term cellular or mitotically active cellular fibroma is appropriate for such
tumours (1). Another problem with ovarian cellular fibromatous neoplasms which has received scant attention in the literature is the distinction from a diffuse adult granulosa cell tumour. The distinction between these 2 neoplasms may be extremely problematic, and to an extent somewhat arbitrary, in a proportion of cases. Recently it has been shown that over 95% of ovarian adult granulosa cell tumours harbour a somatic missence mutation in codon C134W (402C→G) of FOXL2 gene (2-8). This mutation is uncommon in other ovarian sex cord-stromal tumours, although it has been demonstrated in occasional thecomas and juvenile granulosa cell tumours but not, to our knowledge, in fibromas (2-8). Given the significant problems in the morphological distinction between ovarian cellular fibroma and diffuse adult granulosa cell tumour, we have analysed a series of the former neoplasms, chiefly comprising consultation material referred because of difficulties in the distinction between these 2 tumours, looking for FOXL2 mutation. We wished to determine whether the mutation status might be useful in the distinction between these two neoplasms.

MATERIALS AND METHODS

Cases
Twenty two ovarian cellular fibromas or mitotically active cellular fibromas were derived from the pathology archives of the institutions of the authors. Most of the cases were received in consultation where the differential diagnosis was usually a diffuse adult granulosa cell tumour or a fibrosarcoma. In all cases, the available histological sections were reviewed. The cellular fibromas were composed of interlacing fascicles of bland cells, predominantly with ovoid to spindle shaped nuclei exhibiting little in the way of nuclear atypia. In some cases, a proportion of the nuclei were more epithelioid in shape. There was sometimes a low power nodular architecture with alternating hypercellular and hypocellular areas. “Epithelial” arrangements, such as insular, nested, trabecular, cored and follicular, were absent in most of the cases (see next paragraph for exceptions), although in occasional tumours there were small focal ill-defined nested or trabecular areas. Three of the cellular fibromas contained minor sex cord elements comprising “epithelioid” foci resembling adult granulosa cell tumour or Sertoli cell tumour but involving less than 10% of the neoplasm. Necrosis was present in 6 tumours but this was always considered to be of infarct type rather than coagulative tumour cell necrosis, although in occasional cases the distinction was difficult. Mitotic activity was sometimes conspicuous with mitotic counts ranging from 0 to 40 per 10 high power fields. In those neoplasms with mitotic activity of greater than or equal to 4 per
10 high power fields, a diagnosis of mitotically active cellular fibroma was made. Figure 1 illustrates the morphological features of some of the cellular fibromas and figure 2 minor sex cord elements within 1 of these neoplasms.

There were 3 additional cases where multiple cellular nodules composed of cells with an epithelioid appearance were present within a background hypocellular fibrous stroma. The epithelioid cells within the cellular nodules were bland with little in the way of mitotic activity and contained a moderate amount of cytoplasm. The nuclei were vesicular and occasional nuclear grooves were present. The architecture within the nodules was largely diffuse but focal small nested and trabecular areas were present, the nested and trabecular architecture being highlighted by reticulin staining which showed reticulin fibres investing groups of cells within these areas rather than a pericellular pattern. Figures 3 and 4 illustrate the morphological features of these cases which raised the possibility of adult granulosa cell tumours with a prominent fibrothecomatus component. Two of these had originally been diagnosed as adult granulosa cell tumour and 1 as a cellular fibroma.

34 cases of diffuse adult granulosa cell tumour had been previously tested in our laboratory for FOXL2 mutation (2). This diagnosis was made when, in addition to a predominantly diffuse architecture, the nuclei were vesicular and epithelioid in shape and there were significant areas exhibiting “epithelial” arrangements, as described above (figure 5).

**Immunohistochemistry**

Immunohistochemistry was performed on sections of 4 µm-thickness cut from a representative paraffin block of 10 cases (8 cellular fibromas without minor sex cord elements and 2 of the cases composed of cellular nodules with an epithelioid appearance). The other cases did not have tissue available for immunohistochemical analysis. The sections were stained using a semi-automated Ventana Discovery XT or Benchmark XT instrument (Ventana Medical Systems, Tucson, AZ, USA). The primary antibody was polyclonal FOXL2 antiserum (1:25; Imgenex, San Diego, CA, USA). Tissue sections were incubated with the primary antibody for two hours at room temperature followed by 30-minute incubation with the secondary antibody (unconjugated rabbit anti-goat, Jackson ImmunoResearch Labs, West Grove, PA, USA) at 1:500. The tertiary antibody was the pre-diluted Ventana UltraMap Anti-Rabbit HRP that was incubated for 16 minutes. Appropriate
positive and negative controls were stained in parallel with each round of immunohistochemistry.

Immunostaining for FOXL2 was scored as either negative or positive, based on the absence or presence of nuclear staining respectively. The presence of any nuclear staining, regardless of intensity or focality, was considered positive.

**Molecular Testing for FOXL2 (402C→G) Mutation**

In each of the 25 cases (22 cellular fibromas, 3 cases with cellular epithelioid nodules), a representative block was tested for the presence of FOXL2 (402C→G) mutation using real-time polymerase chain reaction (PCR). Briefly, several 5 or 10-µm sections were scrolled from the formalin-fixed paraffin block. The scrolls were deparaffinized and DNA was extracted using the Ambion Recover All Total Nucleic Acid Isolation Kit. A TaqMan reverse transcriptase-PCR (RT-PCR)–based allelic discrimination assay (Applied Biosystems) was used to detect and genotype the FOXL2 (402C → G) mutation. Further details regarding the methodology protocol used in molecular testing for FOXL2 (402C → G) were published previously (2,9).

**RESULTS**

**Immunohistochemistry**

All 10 cases tested exhibited positive nuclear staining with FOXL2. In 7 cases, this was diffuse involving >50% of the cells (5 cellular fibromas and the 2 cases composed of cellular nodules with an epithelioid appearance) and in 3 cases focal involving <50% of the neoplasm (figure 6).

**Mutation Results**

FOXL2 mutation was not present in any of the 22 cellular or mitotically active cellular fibromas tested, including the 3 with minor sex cord elements. Two of the 3 cases with cellular epithelioid nodules were mutation negative while the other contained a heterozygous
mutation. 31 of the 34 diffuse adult granulosa cell tumours previously tested harboured a mutation (2).

DISCUSSION

The distinction between an ovarian cellular fibroma and a diffuse adult granulosa cell tumour may be extremely problematic because of significant morphological overlap. There has been little attention with regard to this in the literature, although it is stated in the Armed Forces Institute of Pathology fascicle on Tumors of the Ovary, Maldeveloped Gonads, Fallopian Tube, and Broad Ligament that “adult granulosa cell tumors with a diffuse pattern may be difficult to distinguish from pure stromal tumors, particularly highly cellular fibromas and thecomas” (10). Although there is generally no difference in initial management between these two tumour types, the distinction is extremely important since adult granulosa cell tumour is a low grade malignant neoplasm with a long natural history and a distinct tendency to recurrence or metastasis which may be many years later while cellular fibroma usually exhibits a benign behaviour, although occasional neoplasms exhibit extraovarian spread or recur locally secondary to adherence (1,11). These 2 types of neoplasm generally occur in the same age group and there may be significant overlap in the gross features in that both can have a solid or solid-cystic yellow or white cut surface. Useful microscopic features in suggesting a diagnosis of diffuse adult granulosa cell tumour rather than cellular fibroma include the presence in the former of more round or epithelioid rather than spindle shaped nuclei together with focal “epithelial” formations with an insular, nested, corded, trabecular or follicular architecture, grooved nuclei and a focal pattern of reticulin surrounding nests of cells rather than enveloping individual cells (10). However, with regard to these parameters there may be significant overlap between these two tumour types. For example, nuclear grooves are not identified in all adult granulosa cell tumours, are not prominent in many and may be present in cellular fibromas. Moreover, some fibromas have cells with a rather epithelioid appearance and diffuse adult granulosa cell tumours may contain spindle shaped nuclei and exhibit a pericellular reticulin pattern similar to that characteristic of cellular fibromas or the reticulin pattern may be “intermediate” between that expected in a granulosa cell tumour and a fibroma. The immunophenotype is similar in that both tumour types stain with sex cord markers such as inhibin, calretinin, CD56 and steroidogenic factor 1. It has been suggested that inhibin staining is useful in the distinction between a cellular fibroma and
a diffuse adult granulosa cell tumour with widespread immunoreactivity favouring the latter. However, it is the experience of 1 of the authors (WGM) that many cellular fibromas are diffusely positive with inhibin while it is well known that adult granulosa cell tumours may be only focally immunoreactive or even negative. Thus, alternative methods which are of use in distinguishing between these two tumour types are important. The hypothesis of this study was that FOXL2 mutation status might prove useful in this diagnostic scenario.

A missense point mutation 402C→G (C134W) in FOXL2, a gene encoding a transcription factor known to be critical for granulosa-cell development, was first identified in 90 of 93 (97%) adult granulosa cell tumours in a study in 2009 (2). In that study, 3 of 14 thecomas (21%) and 1 of 10 juvenile granulosa cell tumours (10%) also harboured the mutation. The mutation was not demonstrated in 49 ovarian sex cord-stromal tumours of other types or in an additional 329 neoplasms comprising ovarian tumours of non sex cord-stromal lineage or breast carcinomas. Another study examined 1353 tumour tissues from various origins, including ovarian neoplasms and other common cancers, by single-strand conformation polymorphism analysis (6). FOXL2 codon 134 missense mutations were identified in 53 of 56 adult granulosa cell tumours (94.6%) and two of 16 thecomas (12.5%), but not in other neoplasms. In another study, FOXL2 mutation was demonstrated in 70% of adult granulosa cell tumours and in 0 of 18 juvenile granulosa cell tumours (5). In a further study, 52 of 56 (93%) adult granulosa cell tumours harboured FOXL2 mutation; morphological reappraisal of the 4 cases exhibiting wild-type FOXL2 sequences suggested that they may have been misclassified at diagnosis (4). Kim et al found FOXL2 mutations in 18 of 20 (90%) adult granulosa cell tumours but in 0 of 3 juvenile granulosa cell tumours (3). Al-Agha et al found 93% of adult granulosa cell tumours, 8% of Sertoli Leydig cell tumours and 40% of thecomas to contain mutations (8). FOXL2 mutations have not been demonstrated in ovarian fibromas; for example, in 1 study all 9 tested cases were mutation negative (8).

Additional studies have looked for FOXL2 mutation in other ovarian sex cord-stromal tumours. For example, in one study of 6 gynandroblastomas, including 3 with a component of adult granulosa cell tumour, FOXL2 mutations were not identified, even in the component of adult granulosa cell tumour (12). A small number of testicular adult granulosa cell tumours have been evaluated for FOXL2 mutation (13,14). In one study, all 4 testicular adult granulosa cell tumours were mutation negative (13) while in another FOXL2 mutation was identified in 2 of 5 (40%) of these neoplasms but not in the small number of testicular juvenile granulosa cell tumours, Leydig cell tumours and Sertoli-Leydig cell tumours tested.
Taken together, the results of the various studies suggest that FOXL2 mutation is highly sensitive and quite specific for adult granulosa cell tumour and may be useful in the categorisation of problematic ovarian sex cord-stromal tumours which can sometimes be extremely difficult due to overlapping morphology between the various tumour types and between sex cord-stromal and non sex cord-stromal tumours. The presence of mutations also opens the possibility for the development of targeted therapies in the future. While mutation analysis is not widely available, it is straightforward using standard methodology (9).

In the current study, all the cellular and mitotically active cellular fibromas were mutation negative. Three cellular fibromas with minor sex cord elements were also mutation negative, suggesting that these do not represent diffuse adult granulosa cell tumours with an abundant fibrothecomatous component.

There were 3 cases in our study where multiple cellular nodules with an epithelioid appearance were present within a background fibromatous stroma (illustrated in figures 3 and 4). These raised the possibility of a diffuse adult granulosa cell tumour with a prominent background fibrothecomatous stroma. Two of these cases were mutation negative, suggesting that they do not represent adult granulosa cell tumour and we feel they are best regarded as cellular fibromas with an unusual focal epithelioid morphology. The third contained a mutation, suggesting that this represents a diffuse adult granulosa cell tumour with a prominent background fibrothecomatous component. Testing for FOXL2 mutation may be useful in such cases since at a morphological level it was virtually impossible to distinguish between the mutation positive and negative cases due to significant morphological overlap. In this regard, it is possible that the occasional cases of ovarian sex cord-stromal tumour other than adult granulosa cell tumour which have been shown to contain a FOXL2 mutation have been misclassified and that molecular classification is superior to morphology in problematic sex cord-stromal neoplasms. To this end, Al-Agha et al reported an abdominal tumour that morphologically was a typical adult granulosa cell tumour in a woman who 2 years previously had an ovarian “thecoma” removed, a diagnosis confirmed by gynaecological pathological consultation at the time of initial presentation (8). On molecular testing, the FOXL2 (402C→G) mutation was present in both the original and the recurrent tumour, supporting the conclusion that the original neoplasm was misclassified and should have been diagnosed as a luteinized adult granulosa cell tumour with a diffuse pattern. It is also possible that the 3 diffuse adult granulosa cell tumours which were mutation negative represent examples of cellular fibroma or some other sex cord neoplasm (2).
There are uncommon neoplasms which have typical areas of adult granulosa cell tumour together with a prominent fibrothecomatous stroma, so-called granulosa-theca cell tumours. It would be interesting to test some examples of these for FOXL2 mutation to determine whether they do indeed represent examples of adult granulosa cell tumour. We have not included any such cases in this study.

We also stained a proportion of the cases with an antibody against FOXL2 and all exhibited positive nuclear staining. This is in keeping with the results of a study showing that FOXL2 immunoreactivity is present in almost all ovarian sex cord-stromal tumours with a FOXL2 mutation but also in a majority of sex cord-stromal tumours without mutation (8). In that study, FOXL2 nuclear immunoreactivity was present in 95 of 119 (80%) sex cord-stromal tumours, including >95% of adult granulosa cell tumours, juvenile granulosa cell tumours, fibromas and sclerosing stromal tumours. Only 50% of Sertoli-Leydig cell tumours expressed FOXL2 as did 1 of 11 steroid cell tumours and 3 of 3 female adnexal tumours of probable Wolffian origin. All other non-sex cord-stromal tumours tested (n=368) were negative for FOXL2 expression (8). This demonstrates that FOXL2 is a useful immunohistochemical marker of ovarian sex cord-stromal tumours and of use in confirming an ovarian neoplasm of this lineage but of little value in distinguishing between the various tumour types within this group of neoplasms. The FOXL2 antibody used in the present study and others (8) is a polyclonal antibody and it would be preferable if a monoclonal antibody was available.

In summary, in this study FOXL2 mutation was not demonstrated in any ovarian cellular fibroma. The demonstration of FOXL2 mutation is a useful adjunct in distinguishing between diffuse adult granulosa cell tumour (mutation present) and cellular fibroma (mutation absent). Since this is a clinically important distinction, mutation testing should be considered in problematic cases.

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FIGURE LEGENDS

Figure 1 Ovarian cellular fibroma with population of morphologically bland spindle shaped cells with a fascicular pattern (a). Some cases exhibit foci with a somewhat epithelioid appearance (b). In some cases, mitotic activity is prominent (c) and areas of necrosis are present in some tumours (d).

Figure 2 Ovarian fibroma (cellular areas were present in other sections) with minor sex cord elements.

Figure 3 Ovarian tumour composed of nodules of cells with an epithelioid appearance within a background fibromatous stroma (a). Intermediate (b) and high power (c) view of epithelioid areas where trabecular areas are seen. The morphological features raise the possibility of an adult granulosa cell tumour with a prominent background fibrothecomatous component but there was no FOXL2 mutation. Given the FOXL2 mutation result, this case was classified as a cellular fibroma with an unusual epithelioid morphology.

Figure 4 Ovarian tumour with low power nodular appearance (a). At high power, the cells have a rather epithelioid appearance with a vague nested architecture (b). FOXL2 mutation was present in this case. Given the FOXL2 mutation result, this case was classified as a diffuse adult granulosa cell tumour with a prominent background fibrothecomatous component.
Figure 5  Diffuse adult granulosa cell tumour with focal “epithelial” formations in the form of nested and trabecular arrangements of cells. This case was not from the current study set but is shown for comparative purposes.

Figure 6  Ovarian cellular fibroma which exhibits diffuse nuclear immunoreactivity with FOXL2. No FOXL2 mutation was present.