FOXL2 mutation and large-scale genomic imbalances in adult granulosa cell tumors of the ovary

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Adult granulosa cell tumors (AGCTs) are a rare class of ovarian tumors with recurrent cytogenetic abnormalities including trisomy 12, trisomy 14, monosomy 16/deletion 16q, and monosomy 22. Over 90% contain a missense point mutation (C134W) in the FOXL2 gene at 3q22.3. The relationship between FOXL2 mutation and cytogenetic abnormalities is unclear, although both are presumably early events in tumorigenesis. In addition, FOXL2 C134W mutant allele imbalance has been noted in a minority of AGCTs, but the mechanism for allelic imbalance has not yet been described. We used a microarray platform designed for formalin-fixed, paraffin-embedded (FFPE) tissue specimens, the Affymetrix OncoScan FFPE Express 330K Molecular Inversion Probe (MIP) array, to explore the correlation between genomic imbalances detected by microarray and FOXL2 mutation status detected by pyrosequencing in a series of 21 archived AGCTs. Tumors were characterized by histopathologic features, stage, and alpha-inhibin expression by immunohistochemistry. All tumors were positive for inhibin, and 18/21 tumors contained a FOXL2 mutation. The most common genomic imbalances were a gain of 14q, a loss of 16q, and a loss of 22q. Three tumors showed evidence of FOXL2 mutant allele imbalance by pyrosequencing; microarray revealed a 32.5 Mb deletion encompassing FOXL2 in 1 case and a 70.9 Mb stretch of homozygosity encompassing FOXL2 in the other case. The third case, with a FOXL2 mutant allele imbalance, showed a diminished mutant allele population (32%) despite high estimated tumor content (>90%), suggesting tumor heterogeneity for the mutation. This study provides the first correlation of FOXL2 mutation status and genomic imbalances in AGCTs, and it further elucidates the mechanisms for mutant allele imbalance in cancer.

Keywords Granulosa cell tumor, ovary, FOXL2, microarray, mutant allele imbalance

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Adult granulosa cell tumor (AGCT) is the most common type of ovarian sex cord stromal tumor (SCST) and is usually diagnosed in the childbearing or postmenopausal years, often in the setting of excessive uterine bleeding due to estrogen production by the tumor. Most AGCTs are stage I and are cured by surgical resection; however, late recurrences and metastatic disease are observed in a minority of cases. The histologic features are quite distinctive, and therefore the diagnosis can be rendered based on histology alone in most cases. These tumors are characteristically comprised of a monomorphic population of cells with vesicular chromatin, prominent nuclear grooves, and high nuclear-to-cytoplasmic ratios. They exhibit a variety of growth patterns (microfollicular with Call-Exner bodies, macrofollicular, insular, trabecular, watered-silk, diffuse), which are
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commonly admixed in any given tumor. Marked nuclear atypia is occasionally observed and does not appear to be of prognostic significance. Mitotic activity varies but is generally low. By immunohistochemistry, expression of alpha-inhibin and calretinin and lack of expression of epithelial membrane antigen (EMA) are useful distinguishing features of AGCT in diagnostically challenging cases (1–3). AGCT is biologically distinct from juvenile granulosa cell tumor (JGCT), but both share similar histologic features and both are characterized by hyperestrinism.

The most frequently reported cytogenetic abnormalities in AGCT are trisomy 12, trisomy 14, monosomy 16/deletion 16q, and monosomy 22 (4–6). More recently, a missense mutation in the FOXL2 gene (c.402C>G, p.Cys134Trp or C134W) was identified in AGCTs by paired-end whole transcriptome RNA sequencing and was subsequently confirmed to be present in 86 of 89 (97%) of AGCTs tested (7). The FOXL2 gene resides on chromosome band 3q22.3 and encodes forkhead box protein L2, a forkhead-winged helix family transcription factor that is expressed in the eyelid and gonad during embryogenesis and actively maintains ovarian follicles during adulthood (8–10). Germ-line mutations associated with loss of function of the gene are a cause of blepharophimosis, ptosis, and epicanthus inversus syndrome (BPES) type II, a disorder often associated with premature ovarian failure. The newly reported C134W mutation has not been reported as a germline mutation (11), and in contrast to the loss of function mutations in BPES type II, the C134W mutation is thought to result in a gain of function. The FOXL2 mutation is presumed to be an initiating event in AGCT formation (7,12), and the C134W mutation has been shown to result in decreased apoptosis in granulosa cells (13). Subsequent studies on a variety of tumors have demonstrated the high specificity of the C134W mutation for ovarian SCSTs, because the mutation has not been described in other classes of ovarian tumors or tumors of other organs (14,15).

The FOXL2 C134W mutation has been found in over 90% of AGCT and about 10–20% of ovarian thecomas (another class of SCSTs) in multiple case series (7,16–18). The association of this mutation with JGCT is now in doubt, because the one reported JGCT case with a FOXL2 mutation was considered a misclassified AGCT upon retrospective analysis (19). By immunohistochemistry, FOXL2 expression is similar in wild-type and mutated granulosa cells (7,18), and subcellular localization is unaltered (20). Even though FOXL2 expression is unchanged by the C134W mutation, FOXL2 immunohistochemistry has been suggested as a useful cell-of-origin marker for SCSTs because it is positive in 80% of SCSTs (including over 95% of AGCTs) and the staining is typically more intense than alpha inhibin or calretinin (18).

The relationship between the FOXL2 mutation and large-scale genomic imbalances in AGCTs has not yet been described. Presumably, chromosomal abnormalities act in combination with the FOXL2 mutation to drive tumorigenesis. In the current study, we have correlated FOXL2 mutation status with genomic imbalances in a series of archived formalin-fixed, paraffin-embedded (FFPE) AGCTs using a molecular inversion probe (MIP) microarray technology (OncoScan™ FFPE Express, Affymetrix, Santa Clara, CA) designed specifically for FFPE tissue samples.

Materials and methods

The 21 FFPE tumors diagnosed as AGCT were obtained from the University of Utah Department of Pathology archives. Only tumors with sufficient remaining material were used, and all case materials were de-identified in accordance with the University of Utah Institutional Review Board exemption for use of de-identified, paraffin-embedded human tissues. Cases were reviewed by a gynecologic pathologist (E.J.), and tumors were staged according to International Federation of Gynaecologists and Obstetricians (FIGO) criteria based on clinical information available from the pathology report. Immunohistochemistry for alpha inhibin (clone R1, Serotec Inc., Raleigh, NC) was performed on those cases for which it was not already available. Histopathologic features, tumor stage, and alpha inhibin immunohistochemistry are summarized in Table 1. Case 10 lacked the classic histologic features of AGCT but was focally positive for alpha inhibin. Case 12 showed a classic AGCT with a discrete area exhibiting markedly atypical spindle cells, consistent with sarcomatous transformation. The remaining cases showed typical histologic features of AGCT.

DNA for sequencing was obtained by manual macrodissection of aniline blue stained tissue sections mounted on glass slides, using selected areas from an adjacent hematoxylin and eosin (H&E)-stained section to guide dissection. Dissected tissue was incubated at 65°C overnight with 200μL of TE Buffer containing TWEEN and 10 μL Proteinase K (Roche, Indianapolis, IN), followed by heat inactivation of the enzyme at 100°C for 10 minutes. For the OncoScan™ FFPE Express MIP array, DNA was extracted from four 20-micron thick ribbons of paraffin-embedded tumor from FFPE tissue blocks using the Ambion RecoverAll total nucleic acid isolation kit for FFPE tissues (Ambion, Applied Biosystems, Austin, TX). The percentage of tumor in microdissected areas (for sequencing) and thick sections from the same case (for microarray) were estimated by pathologist review (Table 2).

A 96 base pair region containing codon 134 of FOXL2 was amplified using biotinylated forward (5’-GGCGAAGGCAAATCTAGT-3’) and unlabeled reverse (5’-CGGAGGAGCTCTTCAATG-3’) primers for pyrosequencing; the same primers were used for bidirectional Sanger sequencing for independent confirmation of the pyrosequencing results. A unidirectional primer targeting the coding strand (5’-CCCTTCTCGAACATGTC-3’) was used to detect the c.402C>G, p.Cys134Trp mutation by pyrosequencing using the PyroMarkQ24™, and all steps were performed according to the manufacturer’s instructions (Qiagen, Germantown, MD).

Microarray analysis was performed using the OncoScan™ FFPE Express 330K MIP platform (Affymetrix). The OncoScan™ assay contains more than 300,000 copy number and single nucleotide polymorphism (SNP) oligonucleotide probes with a median probe spacing of 4,200 kilobases (kb), with much denser coverage within known cancer genes. Results were analyzed with Nexus Copy Number 5.1 software (BioDiscovery, El Segundo, CA). Segmentation was performed using the SNP-FASST2 algorithm and quadratic wave correction. Threshold settings for copy number gain and loss were +0.4 and −0.4, respectively (with normal copy number at 0), and a minimum of five consecutive probes outside of the threshold was required to make a copy number call. Only
probes with call rates of 90% or greater and a relative standard deviation of less than 20% were used in the analysis.

For ploidy confirmation on selected cases, whole nuclei were extracted from 50-micron thick sections of tumor and labeled with propidium iodide to determine cellular DNA content. Ploidy was determined using flow cytometric analysis with an internal human diploid standard for comparison as described previously (21).

### Results

Eighteen of 21 (86%) AGCTs were positive for the FOXL2 c.402C>G mutation by both pyrosequencing and Sanger sequencing. Histologic features and alpha inhibin immuno-histochemistry were consistent with the diagnosis of AGCT in all cases except one (case 10), which stained weakly with inhibin but lacked classic histologic features of AGCT. All mutation-positive samples showed allele quantification correlating with the high tumor content observed by histology. The majority of mutated tumors showed a heterozygous pattern with a mutant allele population of approximately 50%. Non-heterozygous allele frequencies were seen in three cases: two (cases 11 and 13) showed an excess of mutant alleles (67–85%) and one showed a reduced proportion of mutant alleles (32%). The latter (case 12) showed classic granulosa cell tumor histology and areas of sarcomatous transformation, both with a 32% mutant allele fraction; all samples tested for the mutation contained at least 90% tumor by histologic evaluation.

Microarray analysis clarified the mutant allele imbalances observed by pyrosequencing in both cases with an excess of mutant allele. In case 11, pyrosequencing showed a mutant allele population of 67%, and in this case, microarray showed a 32.5 Mb deletion of 3q overlapping the FOXL2 gene (Figure 1A). In case 13, pyrosequencing showed a mutant allele population of 85%, and microarray showed a 70.9 Mb segment of loss of heterozygosity of 3q overlapping the FOXL2 gene (Figure 1B).

The most frequent abnormalities detected by microarray were gain of 14q, losses of chromosome 16 or 16q, and loss of 22q. Two samples (cases 2 and 4) showed a complex genome by microarray analysis and results were further clarified by DNA ploidy analysis, which showed a tetraploid population in case 2 and a near-diploid population in case 4.
Case 2 also showed evidence of clonal heterogeneity with losses of chromosomes 13 and X as the primary abnormalities, loss of chromosome 2 as the next most frequent abnormality, and loss of chromosome 14 as the lowest-frequency abnormality (Figure 2). In general, microarray analysis yielded results consistent with previously reported abnormalities in AGCTs (Table 3).

Discussion

Pyrosequencing has become a popular method for targeted mutation detection in FFPE tumor samples because it is more sensitive than traditional Sanger sequencing for low-frequency mutant allele populations (22). Because pyrosequencing provides quantitative data, it can suggest genomic alterations in addition to the mutation itself (23), although interpretation must be performed cautiously in the context of FFPE samples that often contain extensive contamination with non-tumor cells. Suspected genomic imbalances can be further investigated with microarray analysis, but this can be technically challenging in FFPE samples because of the high degree of DNA fragmentation and the presence of interfering substances that inhibit PCR amplification. The OncoScan™ FFPE Express 330K MIP platform has been used successfully for detecting genomic imbalances in archived FFPE tumor samples. The MIP technology is beneficial in FFPE tissue because it allows for amplification of the probe instead of the target DNA. Inclusion of copy number and SNP probes on this platform allows for detection of both copy number change and copy neutral allelic imbalance.

Mutant allelic imbalance has been noted in a variety of tumors with oncogenic mutations, and quantitative PCR and SNP microarray have shown these imbalances to be due most frequently to copy number gain or copy neutral loss of heterozygosity (24). Mutant allelic imbalance has been
shown to affect levels of mutant allele transcription, and increased oncogene function in tumors with an excess mutant allele population has been implicated as a possible factor in tumor progression (24). The presence of a greater-than-heterozygous mutant allele fraction is likely to be less clinically significant than the presence or absence of the mutation. Additionally, mutant allelic imbalance due to low-level copy number gain is likely to have a lesser impact on tumor progression than the high-level amplification of non-mutated oncogenes observed in other cancers (e.g., ERBB2 amplification in breast cancer).

In our series, the FOXL2 mutation was detected in 18 of 21 AGCTs, and the most frequent genomic imbalances were gain of 14q and losses of 16q and 22q. Only two tumors with genomic imbalances detectable by array contained genomic imbalances not including +14q, −16q, or −22q, and in general, whole chromosome or whole chromosome arm gains and losses were the most frequently observed abnormalities. Surprisingly, gain of chromosome 12 was observed in only one case. Gain of 14q and loss of 22q were frequently observed together, while loss of 16q was frequently accompanied by less common abnormalities. Losses of 22q and 16q are recurrent abnormalities in a variety of cancers (25) and are likely to be associated with the loss of tumor suppressor genes yet to be identified. The loss of 16q21-qter in one case (case 15) suggested that the critical region for the deletion might be limited to this segment of chromosome 16.

Three tumors showed a mutant allele imbalance, suggesting additional genetic events affecting FOXL2. Microarray analyses revealed abnormalities overlapping FOXL2 in two cases with a greater-than-heterozygous mutant allele fraction: a 32.5 Mb deletion overlapping FOXL2 in one case and a 70.9 Mb stretch of homozygosity overlapping FOXL2 in the other case. The third case which exhibited a possible mutant allelic imbalance (case 12) had an excess of wild-type alleles in multiple areas of the tumor with high tumor content (>90% tumor by histologic evaluation), suggesting that other genetic abnormalities may have preceded the FOXL2 mutation during tumorigenesis. Alternatively, the samples tested could have contained a higher level of non-tumor DNA than suspected based on histologic evaluation, which was a distinct possibility given the subjective nature of histologic interpretation. This latter case demonstrates the technical
challenge of detecting genetic heterogeneity in samples containing both tumor and non-tumor tissue.

FOXL2 wild-type tumors showed no obvious difference in genomic imbalances in contrast to mutant tumors, although this analysis was limited to three cases: case 4 showed a gain of two copies of chromosome 12 in the context of multiple other gains; case 5 showed a loss of X and a gain of 14; and case 10 showed no genomic imbalances by microarray. Gains of chromosomes 12 and 14 and loss of X have been reported as recurrent abnormalities in AGCT, and similar to case 10, three AGCTs bearing the C134W mutation showed no genomic imbalances by microarray. Overall, genomic imbalances affecting the 3q22.3 region containing FOXL2 were infrequent, indicating that most AGCTs contain heterozygous mutations of FOXL2 along with copy number changes elsewhere in the genome. This is in agreement with a previous study in which comparative genomic hybridization (CGH) showed copy number changes affecting 3q in only 2 of 17 AGCTs: one case with trisomy 3 and one case with a deletion in the long arm of chromosome 3 (6). Use of a combined copy number and SNP array platform in our study allowed detection of a case of copy neutral loss of heterozygosity that would have gone undetected by CGH.

In our series, there was no significant correlation among tumor characteristics (size, mitotic index, or stage) and FOXL2 mutation status or genomic imbalances detected by microarray; however, our series was limited by lack of follow-up data. Historically, tumor stage and presence of residual tumor following surgery appear to be the most reliable predictors of survival in AGCT (26,27), and further studies are necessary to determine whether a correlation exists between genetic abnormalities and long-term outcome in AGCT.

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