ORIGINAL ARTICLE

Mutation of *FOXL2* in Granulosa-Cell Tumors of the Ovary

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ABSTRACT

BACKGROUND

Granulosa-cell tumors (GCTs) are the most common type of malignant ovarian sex cord–stromal tumor (SCST). The pathogenesis of these tumors is unknown. Moreover, their histopathological diagnosis can be challenging, and there is no curative treatment beyond surgery.

METHODS

We analyzed four adult-type GCTs using whole-transcriptome paired-end RNA sequencing. We identified putative GCT-specific mutations that were present in at least three of these samples but were absent from the transcriptomes of 11 epithelial ovarian tumors, published human genomes, and databases of single-nucleotide polymorphisms. We confirmed these variants by direct sequencing of complementary DNA and genomic DNA. We then analyzed additional tumors and matched normal genomic DNA, using a combination of direct sequencing, analyses of restriction-fragment–length polymorphisms, and TaqMan assays.

RESULTS

All four index GCTs had a missense point mutation, 402C→G (C134W), in *FOXL2*, a gene encoding a transcription factor known to be critical for granulosa-cell development. The *FOXL2* mutation was present in 86 of 89 additional adult-type GCTs (97%), in 3 of 14 thecomas (21%), and in 1 of 10 juvenile-type GCTs (10%). The mutation was absent in 49 SCSTs of other types and in 329 unrelated ovarian or breast tumors.

CONCLUSIONS

Whole-transcriptome sequencing of four GCTs identified a single, recurrent somatic mutation (402C→G) in *FOXL2* that was present in almost all morphologically identified adult-type GCTs. Mutant *FOXL2* is a potential driver in the pathogenesis of adult-type GCTs.

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Granulosa-cell tumors (GCTs) occur at a frequency of 1 per 100,000 persons and account for less than 5% of all ovarian cancers.[1] For women with advanced-stage or recurrent disease, the effectiveness of traditional chemotherapy has been limited, indicating the need for more effective therapeutic approaches.[2] For stage I and II disease, reported 5-year survival rates vary widely, from 55% to 95%, perhaps indicating inconsistent diagnostic criteria for GCTs.[1] The histopathological features of these tumors (Fig. 1) can be mimicked by a variety of other neoplasms.[3]

The cause and molecular pathogenesis of GCTs are unknown.[4-7] GCTs have morphologic and molecular features that are similar to those of normal granulosa cells, including the expression of follicle-stimulating hormone receptor and inhibin.[8,9] Cytogenetic studies have shown that GCTs have greater genomic stability than the more common epithelial ovarian cancers.[4,10]

Methods for rapidly sequencing whole cancer genomes have recently been developed,[11-14] and initial studies have suggested that hundreds of samples of any cancer type would have to be studied to derive clinically useful information.[15] Because GCTs are genomically stable, we hypothesized that common, GCT-specific molecular abnormalities in the transcriptomes of GCTs could be discovered through the analysis of a small number of samples. We thus subjected four GCTs to whole-transcriptome paired-end RNA sequencing (also called RNA-seq) (see Glossary).

Methods

Patients and Samples

We selected 4 ovarian adult-type GCTs (1 primary and 3 recurrent tumors) obtained from index patients, along with 10 ovarian carcinomas and 1 cell line derived from a serous borderline tumor provided by the OvCaRe (Ovarian Cancer Research) frozen tumor bank. Patients provided written informed consent for research using these tumor samples before undergoing surgery, and the consent form acknowledged that a loss of confidentiality could occur through the use of samples for research. Separate approval from the hospital's institutional review board was obtained to permit the use of these samples for RNA-sequencing experiments.

For primary validation, we obtained 74 formalin-fixed, paraffin-embedded blocks of frozen samples from additional putative GCTs, along with 48 matched samples of normal tissue. We obtained two additional blocks (GCT78 and GCT59) that were recurrences of samples GCT29 and GCT76, respectively, which we also genotyped. In addition, we analyzed frozen tissue samples from 149 epithelial ovarian tumors and 180 breast carcinomas. We obtained another series of formalin-fixed, paraffin-embedded ovarian sex cord–stromal tumors (SCSTs), some of which have been described previously[16] (see Glossary). This second series consisted of 95 tumor samples: 27 adult-type GCTs, 8 juvenile-type GCTs, 23 fibromas, 14 Sertoli–Leydig cell tumors, 13 thecomas, and 10 steroid-cell tumors. (Details regarding the numbers and types of GCTs that were provided by each institution are listed in Table 1 in the Supplementary Appendix, available with the full text of this article at NEJM.org.)

Pathological Review

All tumor samples that are included in this study were independently reviewed by a gynecologic pathologist before mutational analysis. In cases in which the review diagnosis differed from the source diagnosis, the samples were further reviewed by another gynecologic pathologist, who acted as arbiter. Both pathologists were unaware of the results of genomics studies. Immunohistochemical staining for calretinin, epithelial membrane antigen, and inhibin were performed. (For details, see the Methods section in the Supplementary Appendix.) Histologic images of all GCTs were obtained with the use of a ScanScope XT digital scanning system (Aperio Technologies) and are available on request.

Paired-End RNA Sequencing and Analysis

A detailed description of RNA sequencing and subsequent data analysis are provided in the Methods section of the Supplementary Appendix. Briefly, double-stranded complementary DNA (cDNA) was synthesized from polyadenylated RNA and sheared. The fraction from 190 to 210 bp was isolated and amplified with 10 cycles of a polymerase-chain-reaction (PCR) assay, according to the paired-end protocol for the Genome Analyzer II (GAII) (Illumina). The resulting libraries were then sequenced on the GAII. Short DNA sequences

(reads) obtained from the GAII were mapped to the reference human genome (National Center for Biotechnology Information build 36.1, hg18) and a database of known exon junctions[13] with the use of MAQ software[17] in paired-end mode. Putative point mutations and small insertions and deletions were identified (for details, see the Methods section in the Supplementary Appendix). These mutations were cross-referenced against human genome databases to eliminate previously described germ-line variants. Genome instability of the index samples was determined with the use of Affymetrix 6.0 genotyping arrays interpreted for copy number.

MUTATION VALIDATION

We selected variants present in at least three of the four GCT RNA-sequencing libraries and examined RNA-sequencing libraries from 10 non-GCT ovarian tumors and from a serous borderline-tumor–derived cell line for the presence of the GCT-derived variants. Transcriptome variants that were absent in the 11 non-GCT ovarian tumors were classified as GCT-specific variants and subjected to further analysis. We confirmed the identification of the GCT-specific variants using Sanger sequencing of PCR amplicons of cDNA and genomic DNA (gDNA) obtained from the index samples (both tumor and matched normal tissue when available).

We used a combination of direct sequencing, analyses of restriction-fragment–length polymorphisms (RFLPs), and a TaqMan real-time PCR–based allelic discrimination assay (Applied Biosystems) to genotype the *FOXL2* 402C→G mutation in additional cancers (see Glossary). In the primary validation studies, samples were scored as positive or negative for this mutation only if there were clear and concordant results from at least two assays. The use of assays with different primers was intended to minimize PCR artifacts caused by amplifying poor-quality DNA templates from formalin-fixed, paraffin-embedded tissue blocks.[18] The TaqMan and RFLP assays produced clear and concordant results for all 149 non-GCT tumors, as well as for the 4 index GCT samples, and these results matched the results from sequencing of the 74 samples with interpretable results from all assays. In cases in which the PCR–RFLP analysis did not produce a PCR product of sufficient quality and quantity for further analysis (presumably because of poor-quality template DNA), it was replaced by the TaqMan assay, which was also used in primary screening for the additional series of SCSTs studied to determine the frequency of mutations in related tumors.

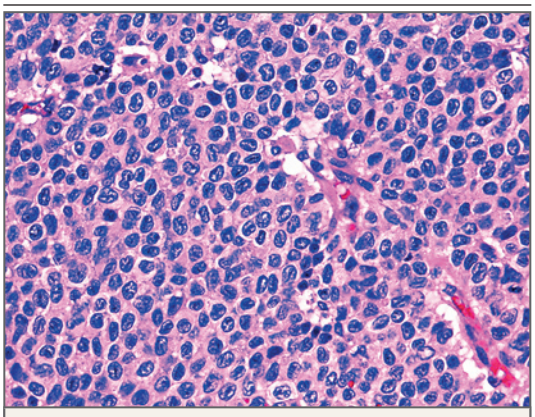


Figure 1. Histopathological Features of a Granulosa-Cell Tumor (GCT).

The typical histopathological features of GCTs, including uniform nuclei with variable nuclear grooves, are visible in a sample obtained from one of four tumors that were subjected to whole-transcriptome paired-end RNA sequencing (hematoxylin and eosin).

RESULTS

PAIRED-END WHOLE-TRANSCRIPTOME RNA SEQUENCING

The genomic stability of the four index GCTs was confirmed by inferring DNA copy-number alterations from high-density genotyping arrays (Fig. 1 in the Supplementary Appendix). The samples were then subjected to RNA sequencing. The total number of mapped DNA sequence reads and the total number of reads (which included the fraction of exonic, intronic, and intergenic sequences), the number of base pairs covered, and details regarding the variants detected in the 4 GCT samples and in the other 11 ovarian neoplasm-derived comparator libraries are shown in Table 1. Sequence reads from these samples showed expected subtype-specific differential gene expression (Fig. 2 in the Supplementary Appendix).

VALIDATION OF IMPLICATED VARIANTS

The RNA-sequencing analysis generates short reads that map to a reference sequence. Figure 2 depicts typical RNA-sequencing data for a portion of the

Glossary

Bit: A measure of the purity of the distribution of alleles at each nucleotide position, with 2 representing a homozygous position.

Grantham distance: A measure of amino acid dissimilarity developed by Grantham in 1974 on the basis of composition, polarity, and molecular volume of amino acids.

Paired-end sequencing: Sequencing of fragmented and size-selected complementary DNA from both ends, resulting in two short sequences with an unsequenced insertion between the sequences.

Restriction-fragment–length polymorphism: A variation in the DNA sequence that can be determined by an assay that takes advantage of a restriction site that is either introduced or removed by a polymorphism; the assay verifies the presence of the polymorphism with the use of restriction-enzyme digestion, followed by gel electrophoresis.

RNA sequencing: A standard term for whole-transcriptome sequencing with the use of high-throughput parallel processing technology to sequence randomly fragmented, size-selected DNA (see the Supplementary Appendix for details).

Sequencing read: A short string of decoded nucleotides that is obtained from a single fragment of DNA or RNA. Millions of reads are compiled through analytic software to determine allelic frequency at each position.

Sex cord–stromal tumor: Any tumor that is derived from ovarian stroma, including sex cords, forerunners of the endocrine apparatus of the postnatal ovary.

TaqMan: A rapid fluorophore-based real-time polymerase-chain-reaction assay that uses specific primers and labeled probes to detect sequence variants.

Transcriptome: The set of all messenger-RNA transcripts that are present in a cell or population of cells.

FOXL2 gene and shows how nucleotides for expressed genes can be represented by multiple reads, as opposed to an average read generated by standard sequencing. We predicted the identification of mutations using SNVmix, a probabilistic model that is used to infer such a result on the basis of a binomial mixture model (see the Supplementary Appendix for details). Two putative point mutations that were implicated by SNVmix and a base-pair insertion met our criteria for candidate tumorigenic mutations, since they were present in three or more GCTs and absent in the comparator sequence libraries (i.e., sequence libraries generated from non-GCT tumors, in addition to sequences in existing data banks). All four GCTs contained a C→G change in the *FOXL2* gene at position 402 (genomic position 140147853 on chromosome 3; National Center for Biotechnology Information [NCBI] human genome build 36.1), which is predicted to result in the substitution of a tryptophan residue for a highly conserved cysteine residue at amino acid position 134 (C134W) (Fig. 2). The Grantham distance (see Glossary) of this amino acid change was 215, the highest value we observed in the 6410 nonsynonymous predicted variants derived from the analysis of all 15 ovarian cancers (Table 1). Of the 6410 mutations, only 15 had a Grantham distance of 215. All predicted variants from the four index GCTs are available from the European Genotyping Archive (accession number, EGA00000000040).

Using two additional independent methods, we validated the *FOXL2* 402C→G variant in the cDNA and gDNA of the four index samples and determined that it was somatic in the two samples from patients for whom normal tissue was available. The two other variants that met our criteria were one at genomic position 357452 on chromosome 4 in *ZNF141* and a base-pair insertion at position 24488600 on chromosome 16 in *RBBP6*. We could not confirm either of these variants by PCR validation and considered them to be systematic artifacts generated by misalignment of the reads. We therefore focused on the *FOXL2* 402C→G variant (Fig. 3A, and Fig. 3 in the Supplementary Appendix).

PREVALENCE OF *FOXL2* 402C→G

We used a combination of RFLP, TaqMan (Fig. 4A), and direct-sequencing (Fig. 4B) analyses to determine whether the *FOXL2* variant was present in 69 samples of pathologically confirmed GCTs (67 adult-type and 2 juvenile-type GCTs) and three other SCSTs (Fig. 3B). We obtained unequivocal results in 64 of 69 GCTs and in all 3 SCSTs (Fig. 4 in the Supplementary Appendix). *FOXL2* 402C→G was present in 59 of 64 GCTs. Two additional paired recurrences also carried the 402C→G variant. The mutation was absent in DNA extracted from paired normal tissue from 48 mutation-positive patients. Five GCTs were mutation-negative, including the two juvenile-type GCTs (GCT24 and GCT45) (Fig. 5A and 5B in the Supplementary Appendix). Two mutation-negative tumors (GCT11 and GCT22) did not express inhibin or calretinin, which were expressed in 100% and 86% of the mutation-positive GCTs, respectively (Fig. 5C and 5D in the Supplementary Appendix). The fifth mutation-negative tumor (GCT33) had a prominent fibrothecomatous component, also seen in two mutation-positive samples (GCT44 and GCT71) (Fig. 5E in the Supplementary Appendix). The *FOXL2* mutation was absent in 149 other epithelial ovarian tumors and in 180 breast cancers (Fig. 3D).

Sample GCT18, a thecoma, was the only non-GCT tumor that was positive for the *FOXL2* variant in the first validation series. Further analysis of this sample revealed a minor granulosa-cell component (Fig. 6 in the Supplementary Appen-

Table 1. Summary of Data Generation from Whole-Transcriptome Paired-End RNA Sequencing.*

Sample Number	Mapped Sequence Reads	Total Sequence Reads	Total Mapped Mbp	Novel Variants		Known SNPs		Genes Expressed	Sequence Reads		
				All	Nonsynonymous	All	Coding		Exonic	Intronic	Intergenic
	number								*percent*		
GCT26	52,685,618	47,705,386	1929	700	431	15,591	6510	22,083	49	37	14
GCT28	18,511,140	53,165,674	2016	679	417	17,544	6659	22,199	56	29	15
GCT77	94,561,966	83,557,468	3275	784	495	22,065	8797	23,782	56	29	15
GCT78	49,287,588	45,548,714	1843	456	289	14,750	5151	21,955	44	38	17
CCC1	28,607,068	25,250,674	1026	582	397	9,744	3184	19,672	51	34	16
CCC2	28,669,500	25,216,874	1026	801	540	11,524	3640	20,246	63	25	12
CCC3	21,936,504	30,544,726	1237	755	505	13,442	4914	20,923	62	26	12
CCC4	73,009,800	44,922,322	1762	1301	929	16,181	6057	22,376	59	29	12
CCC5	46,218,944	33,329,608	1345	556	369	13,802	5453	20,820	54	34	12
EOC1	14,748,476	13,608,092	680	352	213	10,999	4307	19,009	65	25	10
EOC2	9,145,424	39,026,730	1884	682	429	17,082	7173	21,962	57	31	12
EOC3	52,059,038	45,901,264	1703	543	352	17,748	7567	22,382	51	39	11
EOC4	31,163,258	27,913,210	1300	505	306	14,843	6049	21,076	55	33	13
HGS1	41,968,374	43,212,348	1788	568	369	16,740	6701	22,151	62	25	13
SBOT3.1	25,430,324	30,336,686	1199	556	369	12,931	5303	19,239	76	14	10

* CCC denotes clear-cell carcinoma, EOC endometrioid ovarian carcinoma, GCT granulosa-cell tumor, HGS high-grade serous carcinoma, SBOT3.1 serous borderline-tumor cell line, and SNP single-nucleotide polymorphism.

dix). Three tumors (GCT9, GCT35, and GCT38) were either homozygous for the 402C→G (C134W) mutation, as determined by sequencing and the TaqMan assay profile, or had a loss of heterozygosity of the normal allele. A single GCT (GCT44) had both the 402C→G (C134W) mutation and a second *FOXL2* somatic variant, 404A→G (E135G) (Fig. 7 in the Supplementary Appendix). By sequencing cloned PCR products, we observed that these variants were in *cis*. This sample tested positive for the 402C→G variant on RFLP analysis, but repeated TaqMan assays did not produce results. We did not detect the 404A→G variant in any of the 4 index samples, in 37 other GCTs, or in 21 non-GCTs for which we had sequencing data.

We carried out fluorescence in situ hybridization to assess potential amplification of *FOXL2* on 32 GCTs and 5 epithelial ovarian cancers and obtained negative results (Fig. 8 and Table 2 in the Supplementary Appendix). Immunohistochemical analysis showed that *FOXL2* was expressed in the nuclei of normal granulosa cells as well as in GCTs that were heterozygous or appeared to be hemizygous or homozygous for the 402C→G mutation (Fig. 4C, 4D, and 4E).

TEST FOR REPLICATION

We obtained a second series of 95 clinical samples to determine the specificity of this variant within SCSTs of the ovary (Fig. 3C). We found the *FOXL2* mutation in all 27 adult-type GCTs that we tested. Among the other ovarian SCSTs, 1 of 8 juvenile-type GCTs and 2 of 13 thecomas carried the *FOXL2* variant; all other tumors (14 Sertoli–Leydig cell tumors, 23 fibromas, and 10 steroid-cell tumors) were negative for the mutation.

DISCUSSION

We found a recurrent somatic mutation (402C→G) in *FOXL2* in tumor samples from four patients with GCTs, using RNA sequencing to study the transcriptomes of the samples. *FOXL2* was not expressed in the other RNA-sequencing libraries derived from ovarian cancers, and the mutation was absent in the gDNA of these cancers. We analyzed two additional series of GCTs for this mutation. The combined results from both series showed that the mutation was present in 86 of 89 morphologically identified adult-type GCTs (97%), in 1 of 10 juvenile-type GCTs (10%), and in 3 of 14

thecomas (21%); the mutation was absent in 49 other ovarian SCSTs, in 149 epithelial ovarian tumors, and in 180 breast cancers.

Although juvenile-type GCTs share some features with adult-type GCTs and have a similar biomarker-expression profile, they differ with respect to clinical presentation (in prepubertal children vs. young adults) and histopathological features.[1] These features, along with those of a naturally occurring mouse model of juvenile-type GCT,[20] suggest that juvenile-type GCT is a distinct disease from the adult type; our data support this hypothesis. The finding of a mutation that is characteristic of adult-type GCTs in thecomas is not surprising because the distinction between thecomas and GCTs can be arbitrary and mixtures of the two types of tumor cells may be seen in individual tumors. The diagnostic category of granulosa–theca-cell tumor has been used for such cases in the past.[21] The presence of the *FOXL2* mutation in a subgroup of thecomas suggests a cut point on this morphologic continuum between thecoma and GCT. Studies of additional patients with follow-up will be required to determine whether thecomas with a *FOXL2* mutation are biologically more similar to GCTs than to mutation-negative thecomas.

The three mutation-negative adult-type GCTs were diagnostically challenging. Two of these tumors had immunohistochemical profiles that were distinct from those of most GCTs, suggesting that they were not true GCTs but, rather, morphologic mimics. We selected GCTs for this study on the basis of light microscopy (reflecting current diagnostic practice), not on the basis of immunostaining profiles; had we taken immunostaining into consideration, we would have excluded these two tumors.

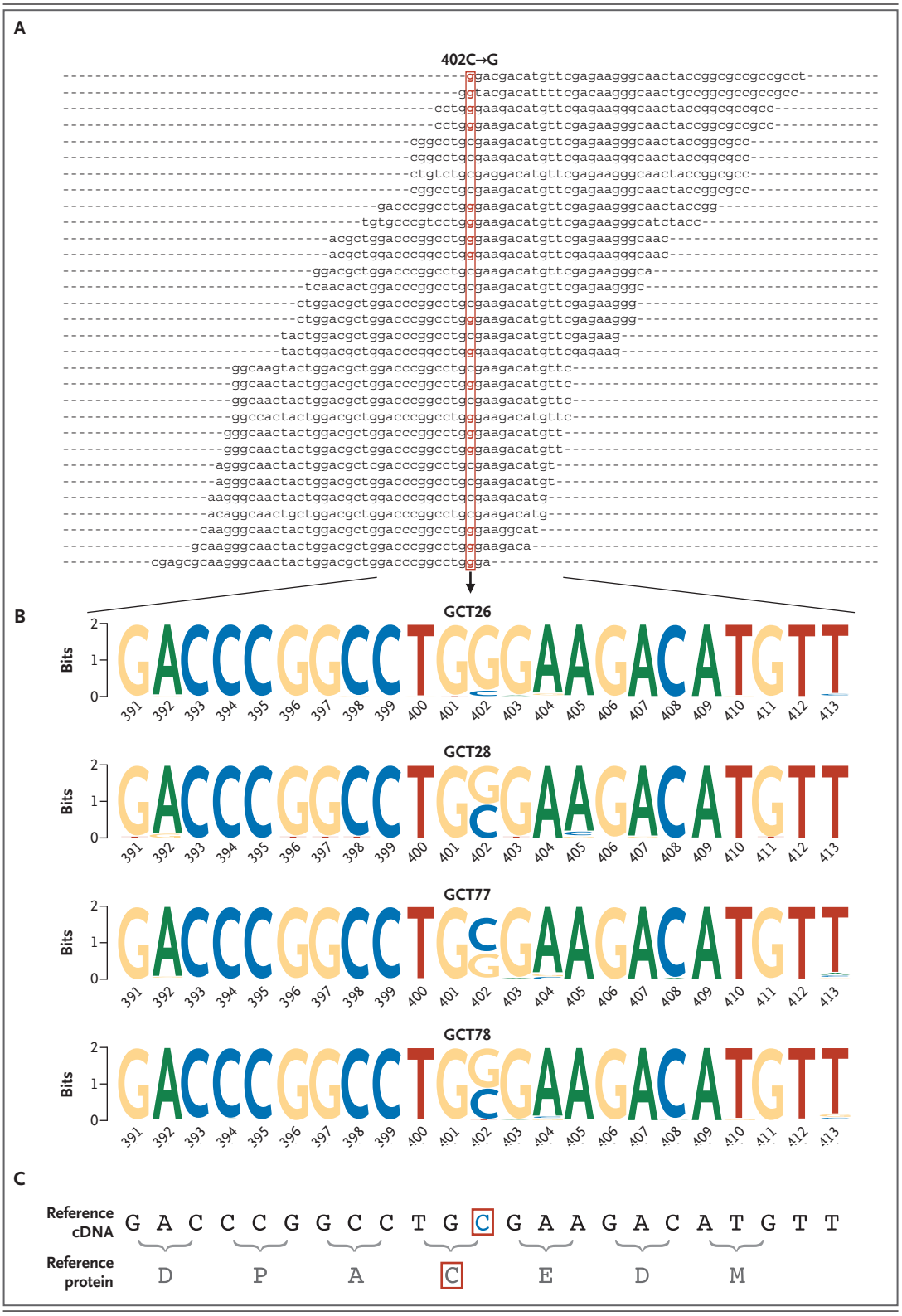
FOXL2 is a member of the forkhead–winged-helix family of transcription factors containing a highly conserved DNA-binding forkhead domain. It is one of the earliest markers of ovarian differentiation, and its expression persists into adulthood. FOXL2 is required for the normal development of granulosa cells[22,23] and shows strong expression in granulosa cells and moderate expression in stromal cells; no expression has been detected in oocytes.[22,24] Few targets of FOXL2 have been described; it has been shown to have a role, as part of an AP1–SMAD3–SMAD4 complex, in activating the transcription of *GNRHR* (encoding the gonadotropin-releasing hormone receptor) in pituitary cells and repressing the transcription of *STAR* (encoding steroidogenic acute regulatory protein) in the adult ovary.[25,26] Granulosa-cell proliferation and differentiation are governed, at least in part, by transforming growth factor β–receptor signaling through SMAD2 and SMAD3.[26]

Figure 2 (facing page). The *FOXL2* 402C→G Missense Mutation.

Panel A shows the mapped sequence reads from one of four granulosa-cell tumors (GCT28) on chromosome 3 for genomic positions 140147803 to 140147903. The complementary DNA (cDNA) position for *FOXL2* 402 is outlined in red, along with the nonreference G alleles. Panel B shows sequence logos[19] representing the allele distribution of the position of the mutation and surrounding nucleotides. A measure of 2 bits represents the homozygous position (see Glossary). The variant 402C→G is clearly visible in each logo. Panel C shows reference cDNA and protein sequences, with the mutated residues indicated by red boxes.

To date, all mutations that have been described in *FOXL2* are germ-line loss-of-function mutations and are associated with the blepharophimosis–ptosis–epicanthus inversus syndrome[27,28] with primary ovarian failure (in particular, granulosa-cell failure[29]). Like *JAK2* mutations in the case of polycythemia vera,[30] mutations in *FOXL2* are present in a large majority of adult-type granulosa-cell tumors and involve a single base substitution. Cys 134 is located on the surface of the forkhead DNA-binding domain; modeling suggests that the substitution of tryptophan for cysteine does not disrupt the folding of this domain or its interactions with DNA (Fig. 9 in the Supplementary Appendix). We therefore speculate that its pathogenicity is imparted through other mechanisms, such as the altering of one or more interactions between FOXL2 and other proteins. We have ruled out mislocalization of the mutant protein as a probable cause of its pathogenicity by showing that FOXL2 is present in the nuclei of GCTs (Fig. 4) that are either heterozygous or appear to be hemizygous or homozygous for the 402C→G mutation (Table 2 in the Supplementary Appendix).

Next-generation sequencing has been used to characterize mutations and gene expression in cell lines and to identify mutations in patients with acute myeloid leukemia.[12,13,31] On the basis of these studies and others, it has been suggested that the genomic complexity of cancers is so extreme that whole-genome–sequencing studies must be performed at great depth and must include several hundred cancers of any type to yield data that can be interpreted clinically or biologically.[11,14,15] How-



ever, our data suggest that for some tumors (most likely the cytogenetically simple and clinicopathologically homogeneous ones, such as GCTs), the pattern of somatic mutations is recurrent and constrained and thus can be analyzed by studying a small number of samples. Since transcriptomes

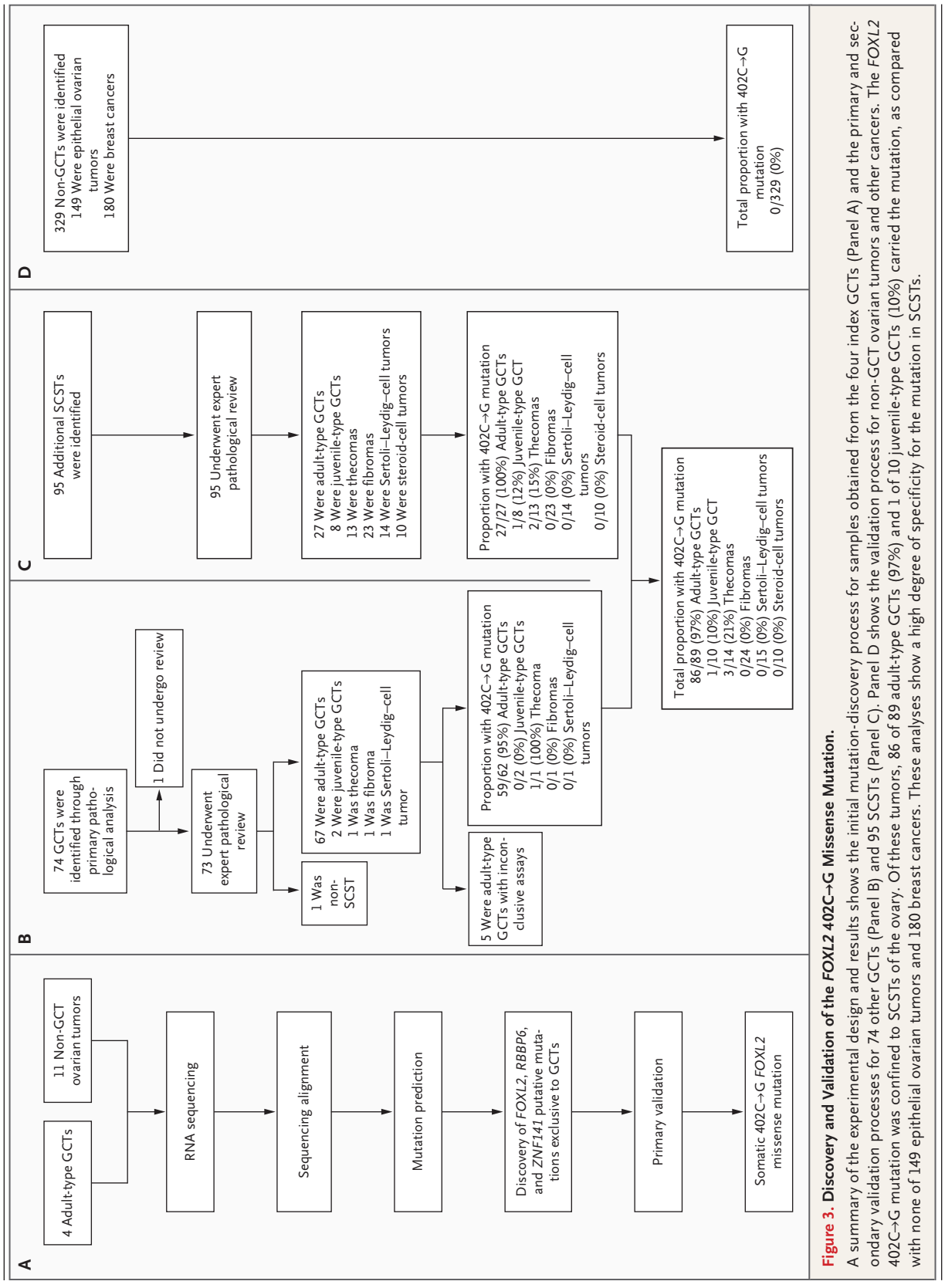


Figure 3. Discovery and Validation of the *FOXL2* 402C→G Missense Mutation.

A summary of the experimental design and results shows the initial mutation-discovery process for samples obtained from the four index GCTs (Panel A) and the primary and secondary validation processes for 74 other GCTs (Panel B) and 95 SCSTs (Panel C). Panel D shows the validation process for non-GCT ovarian tumors and other cancers. The *FOXL2* 402C→G mutation was confined to SCSTs of the ovary. Of these tumors, 86 of 89 adult-type GCTs (97%) and 1 of 10 juvenile-type GCTs (10%) carried the mutation, as compared with none of 149 epithelial ovarian tumors and 180 breast cancers. These analyses show a high degree of specificity for the mutation in SCSTs.

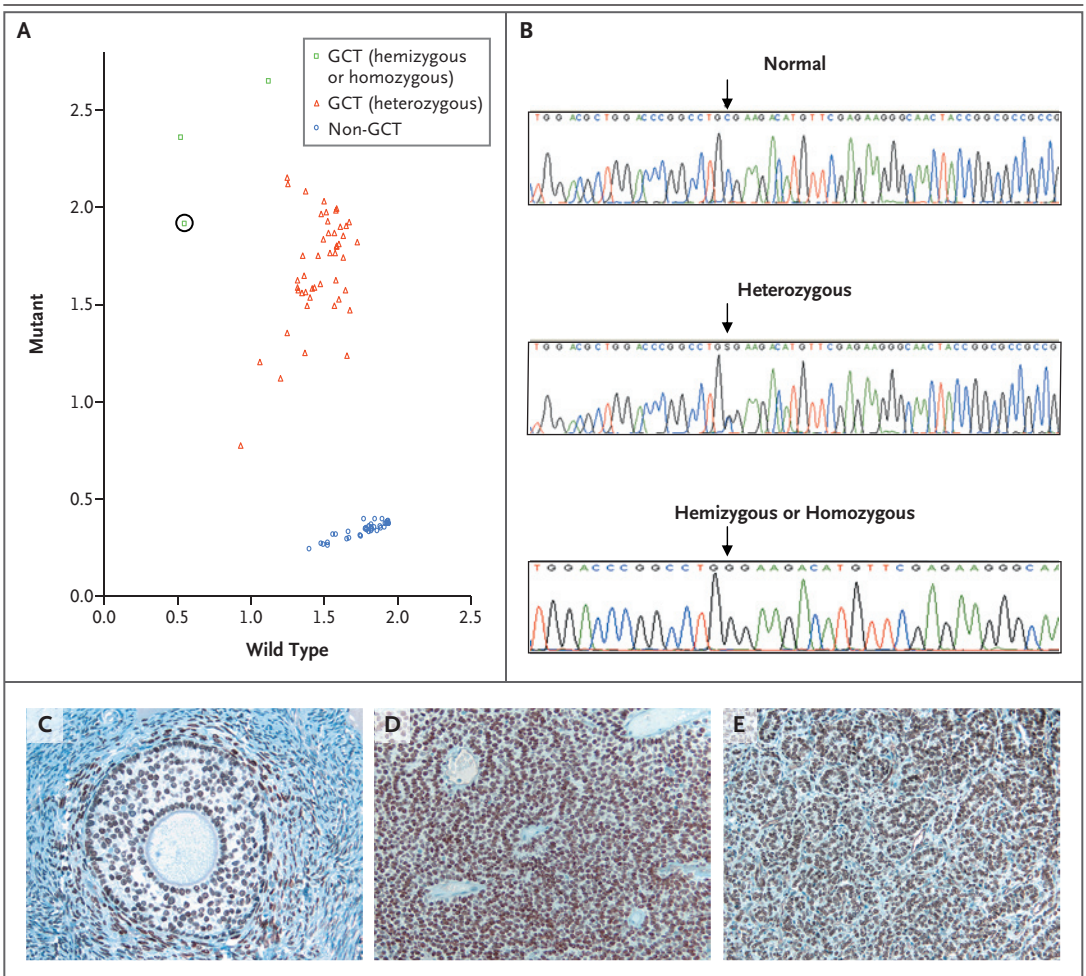


Figure 4. Results of TaqMan Assay, Direct Sequencing, and Immunohistochemical Analysis.

In Panel A, validation of the *FOXL2* 402C→G mutation with the use of a TaqMan allelic discrimination assay shows a clear division between samples that were hemizygous or homozygous for the mutation (presumably through chromosome-based loss of heterozygosity), samples that were heterozygous for the mutation, and samples that were not granulosa-cell tumors (GCTs). The GCT9 sample, which was hemizygous or homozygous for the mutation, is circled within the plot. Direct-sequencing results are shown in Panel B for matched normal tissue, a GCT that was heterozygous for the *FOXL2* mutation, and a sample (GCT9) that appeared to be hemizygous or homozygous for the mutation. The site of the 402C→G mutation is indicated with an arrow. Immunohistochemical analysis shows that *FOXL2* is expressed in the nuclei of normal granulosa cells (Panel C), as well as in GCTs that are either heterozygous (Panel D) or presumed to be hemizygous or homozygous (Panel E) for the 402C→G mutation. The staining pattern is normal in all cases, and the mutation does not appear to have affected nuclear localization of the FOXL2 protein.

are less complex and 50 to 100 times smaller than genomes, sequencing transcriptomes, as opposed to genomes, has been proposed as a more efficient method of finding mutations expressed through RNA.[12] Our study supports this proposal. This approach cannot detect certain types of mutations, such as noncoding changes and mutations that are subject to nonsense-mediated decay.

The diagnosis of GCTs can be difficult, and treatment for this cancer is nonspecific and often unsuccessful. We speculate that testing for the presence of the *FOXL2* 402C→G mutation may improve diagnosis in problematic cases and lead to more targeted therapies. In addition, new insights into the cause of this disease may be derived from future studies of the function of this mutation.

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APPENDIX

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