Genetic and epigenetic alterations of steroidogenic factor-1 in ovarian tumors

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Abstract. Steroidogenic factor-1 (SF-1), the product of the NR5A1 gene, is an essential transcription factor that is known to regulate steroidogenesis in ovarian epithelia, including the synthesis of progesterone, a suppressor of ovarian cancer. Expression of the SF-1 protein, a potential ovarian tumor suppressor, has been demonstrated in normal OSE cells, but is lost in most ovarian tumors and ovarian tumor cell lines. We examined loss of heterozygosity (LOH) and promoter methylation as potential mechanisms that may explain the loss of SF-1 protein in ovarian tumor tissues. Genotyping of three NR5A1 SNPs in matched tumor/normal tissues identified LOH in 16/36 (44%) of the ovarian tumors successfully analyzed, and somatic mutations (gain of allele) in 10% of the tumors. Furthermore, a methylation-sensitive restriction enzyme method was used to demonstrate statistically significant (p<0.0001) increase in the frequency of NR5A1 gene methylation in ovarian tumors (36/46; 78%) versus normal ovaries (1/11; 9%). These data suggest that the SF-1 encoding gene exhibits frequent genetic (LOH/base substitution) and epigenetic (methylation) somatic alterations in ovarian tumors. These data also present novel molecular mechanisms that may explain the loss of SF-1 protein in ovarian tumors, and its potential role in ovarian carcinogenesis.

Introduction

Ovarian cancer is the most frequent cause of death from gynecologic neoplasm in the Western World, mainly due to lack of early detection and understanding of its etiology (1). Most ovarian malignancies have epithelial origin and are often derived from ovarian surface epithelial (OSE) cells (2). Thus, understanding the molecular mechanisms that control OSE cell proliferation and differentiation may lead to the design of novel targeted therapies. Normal human OSE cells are capable of steroidogenesis (3-5) and in epithelial ovarian cancer (EOC), intratumoral steroid biosynthesis is closely linked with carcinogenesis (2,5,6).

Several studies support a role for SF-1 as a suppressor of ovarian cancer: i) ectopic expression of SF-1 inhibits rat ovarian epithelial cell proliferation, causing cell cycle arrest and promoting apoptosis (7); ii) the tumor suppressor Rb1 synergizes with steroid receptor co-activator-2 (SRC-2) to enhance the activity of SF-1 as well as nuclear receptors ERα and ERβ (8); Rb1 may thus promote the transcription of target genes linked to cell differentiation; iii) SF-1 promotes differentiation of human and rat granulosa cells associated with the developing oocytes (9).

It is well established that OSE cell proliferation and ovarian steroidogenesis are closely linked (2,5,6). Specifically, both cell culture and epidemiologic data support a protective role for progesterone against ovarian cancer (10,11). In addition to upregulating steroidogenic enzymes p450scc and 3β-HSD II, SF 1 stimulates expression of the human StAR gene (12). The expression and functional integrity of the StAR protein and enzymes p450scc and 3β-HSD II are particularly important for progesterone biosynthesis (13).

We have previously shown that while human SF-1 and StAR are expressed in normal OSE cells, ovarian cancer cell lines SKOV-3, OVCar3 and BG1 do not show SF-1 or StAR expression (14). We then utilized immunohistochemistry to demonstrate that the vast majority of the human ovarian tumor tissues examined do not express SF-1 protein (unpublished data). In addition, real-time PCR studies on epithelial ovarian cancers suggest that StAR mediated progesterone biosynthesis may inhibit OSE tumor cell proliferation (15). Collectively these

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Abbreviations: SF-1, steroidogenic factor-1; StAR, steroidogenic acute regulatory protein; OSE, ovarian surface epithelial; ERα, estrogen receptor α; ERβ, estrogen receptor β; EOC, epithelial ovarian cancer; Rb1, retinoblastoma 1; SNP, single nucleotide polymorphism

Key words: somatic, mutation, ovarian, carcinogenesis, steroidogenesis
studies support the hypothesis that loss of SF-1 protein may contribute to carcinogenesis in ovarian epithelial cells, in part, through decreased progesterone biosynthesis.

It is noteworthy that the human NR5A1 gene has been mapped to chromosome 9 at position 9q33 (16), a region that shows genetic alterations (LOH, microsatellite instability, and amplification) in more than half of ovarian tumors (17). Particularly significant is the observation that nearly all of the tumors that show genetic alterations at 9q include the subchromosomal region 9q32-34, suggesting that a candidate tumor suppressor gene may reside in this region (17). The location of human SF-1 in the region of 9q33 supports our hypothesis that SF-1 is a candidate tumor suppressor gene in the ovary and that abolished or aberrant SF-1 expression in OSE cells may promote tumor growth. We thus decided to examine the degree of LOH in ovarian tumors, specifically at the NR5A1 locus and report LOH in 44% of the tumors.

Methylation controls the time and cell-type specific NR5A1 gene expression in the endocrine system (18). Thus we examined the methylation status of the NR5A1 gene promoter in ovarian tumors, and report significantly higher prevalence of NR5A1 gene methylation in ovarian tumors compared to normal (i.e. non-tumor) ovaries. These data suggest that LOH and methylation may contribute to the loss of SF-1 protein in ovarian tumors, which in turn may result in ovarian carcinogenesis.

Materials and methods

Samples. Following approval of a research protocol by the Institutional Review Board Committees of Tulane University and Louisiana State University in New Orleans, 66 archival formalin-fixed paraffin-embedded (FFPE) tissue blocks of ovarian tissue were obtained from the Departments of Pathology at the Tulane University Health Sciences Center and from the Interim LSU Hospital. Sixteen samples were representative of normal ovaries from women who had undergone gynecological surgeries for non-ovarian related causes. The rest of the 50 samples consisted of 3 benign ovarian tumors, 7 tumors of low malignancy potential and 40 cases of ovarian carcinoma. Each case of ovarian tumor was matched with corresponding benign tissue control. All FFPE tissue blocks were sectioned and stained with hematoxylin and eosin for histological assessment.

DNA extraction

FFPE tissue blocks. Tissue (1.5-2 mg) was excised from each normal and tumor FFPE tissue block using a sterile scalpel and placed in an autoclaved 1.5 ml centrifuge tube. Samples were deparaffinized with 1 ml of xylene followed by vortexing at top speed for 2 min. The tissue was then centrifuged at 10,000 x g for 3 min using Microcentrifuge 16 from Beckman Coulter Inc. (Brea, CA, USA) and the supernatant was pipetted out. To remove any residual xylene and facilitate pelleting, 1 ml 100% ethanol was added to the tissue sample, followed by spinning at 10,000 x g. The supernatant was decanted and tissue pellets were allowed to air-dry at room temperature. Subsequently, pellets were subjected to protease digestion by 100 µl/ml proteinase K in Digestion Buffer [10 mM Tris-HCl (pH 8.0), 1 mM ethylenediaminetetraacetic acid (EDTA)] at 52˚C for 16 h. Following the digestion, DNA was isolated using the QiaGen (Valencia, CA, USA) DNeasy for FFPE kit, following the manufacturer’s recommended protocol.

Normal ovarian tissue samples. Ovarian surface epithelial cells from normal ovarian FFPE tissue samples were dissected using the PALM® Robot Microbeam laser microdissection system (PALM GmbH, Bernried, Germany) at the Louisiana State University Morphology and Imaging core facility. DNA was then extracted from the epithelial cells using the proteinase K DNA extraction Solution and incubation at 65˚C for 16 h, as suggested by the manufacturer (Arcturus®, Applied Biosystems, Life Technologies Corporation, Carlsbad, CA, USA).

Genotyping. Both tumor and normal DNA samples were genotyped for SNPs: rs2279605, rs10120967, rs7851737 using Applied Biosystems TaqMan probes, with IQ power mix (Bio-Rad, Hercules, CA, USA) or AmpliTag Gold, 25 mM MgCl2, and 10X PCR Gold buffer from Applied Biosystems and dNTPs from VWR International (Radnor, PA, USA). Applied Biosystems Taq Man probes are labeled with Fam and Vic dyes. A total of 20 µl PCR reactions were set up in a 96-well plate which was covered with Microseal ‘B’ film from Bio-Rad. Bio-Rad Thermal cycler IQ5 was used to run the real-time PCR and Image Quant 5 software from Bio-Rad used for plate read document and analysis of the real-time data post PCR. All genotyping assays were done in triplicates and when the three independent assays yielded ambiguous results, were repeated again.

Digestion of genomic DNA for methylation study. DNA (0.5 µg) from each sample (tumor and normal from the same patient) was digested using 5 units of AfeI enzyme (New England Bioscience, Ipswich, MA, USA) in a total reaction volume of 50 µl. The digestion was performed in 1X NEBuffer (New England Bioscience); 1X NE buffer contains: 20 mM Tris-acetate, 50 mM potassium acetate, 10 mM magnesium acetate, 1 mM dithiothreitol (pH 7.9 at 25˚C). The samples were incubated with the enzyme for 1 h at 37˚C to allow digestion of DNA, following which AfeI was inactivated by incubating the samples at 65˚C for 20 min. Alternatively, digestion was performed overnight.

PCR amplification for methylation study. All samples were simultaneously PCR-amplified for the promoter region of the β-actin gene and NR5A1 gene in 200 µl tubes. For each 50 µl reaction, 2 µl of DNA was used and reagent concentrations were optimized at: 5 mM for MgCl2, from Applied Biosystems, 2 mM for each dNTP from VWR; 5 mM for primers (β-actin, forward primer: 5’-TGC AAA GAA CAC GGC TAA GTG TGC-3’, β-actin, reverse primer: 5’TCT AAG ACA GTG TTG TGG GTG TAG GT-3’, NR5A1 gene, forward primer: 5’-AAC ACC AAC AAA GAA GGC GAG AGG-3’, NR5A1 gene, reverse primer: 5’-TCA CTT ACG AAG CCG AAG CAGC-3’) from IDT DNA (Coralville, IA, USA) in 10X PCR buffer II [final concentration: 50 mM potassium chloride and 10 mM Tris-HCl (pH 8.3 at room temperature) from Applied Biosystems] along with 1.25 units AmpliTaq® DNA polymerase per 50 µl of reaction. PCR amplification was performed on a PTC-100™ programmable thermocycler from MJ Research Inc. (Quebec, Canada) allowing initial denaturation at 95˚C for 20 min followed by 40 cycles of 95˚C for 1 min, 62˚C for
1 min, 72°C for 1 min and completing the terminal extension with 10 min at 72°C.

**Gel electrophoresis.** A total of 8 µl of PCR product was added to 2 µl of loading dye (2% xylene cyanol, 40% glycerol in DDI H2O) from Boston Bioproducts (Ashland, MA, USA). For sizing 1 kb plus DNA ladder (Invitrogen, Life Technologies Corporation, Carlsbad, CA, USA) was loaded on a 2% agarose gel containing 1X Tris-acetate-EDTA buffer (40 mM Tris acetate and 1 mM EDTA) and 5 µg of ethidium bromide for staining. The gel was run on a horizontal system for Gel electrophoresis from Bethesda Research Laboratories Inc. (Gaithersburg, MD, USA) at 100 V for 60 min. Following the gel electrophoresis the amplified fragments were visualized on Molecular Imager® Gel DocTM using Image Lab™ software from Bio-Rad.

**Methylation analysis.** Electrophoretic images were analyzed for relative (NR5A1/β-actin) band intensity using Image Quant 5.1 software from GE Healthcare (Piscataway, NJ, USA). Relative intensities were categorized in quartiles as follows: - 1st; + 2nd; ++ 3rd; +++ 4th quartile. All experiments were done at least twice and the relative intensities were averaged.

**Clinical application.** A retrospective chart review was performed gathering clinical data on the patients for whom we had malignant ovarian tissue. Characteristics examined were: age, race/ethnicity, date of diagnosis, years survived since diagnosis, stage of ovarian cancer, histologic grade, date of debulking surgery and treatment with chemotherapy or radiation.

**Statistics.** For most statistical calculations, two-tailed p-values were obtained using Fisher's exact test. The log-rank test was used for calculating p-values for potential differences in survival.

**Results**

**Samples.** The clinical characteristics of the ovarian tumor samples examined are shown on Table I.

**LOH at the NR5A1 locus in ovarian tumors.** In the current study, we considered two molecular hypotheses of SF-1 protein loss in ovarian tumors: LOH and increased methylation. To probe for the prevalence of LOH at the NR5A1 locus, we genotyped matched ovarian tumor and normal FFPE tissue DNA samples from the same ovarian cancer patient, for three NR5A1 gene SNPs: rs2279605, rs10120967 and rs7851737. SNPs were selected based on the following criteria: i) high (>30%) frequency of heterozygosity in the racial/ethnic groups present in our study population (based on available data at dbSNP: http://www.ncbi.nlm.nih.gov/projects/SNP/); ii) availability of a preoptimized Taqman SNP Genotyping Assay for each SNP. Genotyping was performed by Taqman SNP Genotyping Assays using real-time PCR. Assays were performed in triplicates and repeated again, if the genotyping results were ambiguous. All three genotyped SNPs were in Hardy-Weinberg equilibrium in normal samples (data not shown).

The genotyping results for the ovarian tumors and the LOH data for each sample, are shown in Fig. 1A. These data show that out of the 36 ovarian tumor tissues that were heterozygous for at least one of the three NR5A1 gene SNPs, 16 (44%) had LOH (Figs. 1A and 2). The majority of the ovarian tumors had a single LOH event at the NR5A1 locus (out of maximum three possible), but 5 tumors (14%) showed multiple LOH events (Fig. 1A). Also, each SNP showed LOH in multiple tumors, with rs7851737 showing most losses (Fig. 1B). Thus, LOH occurs frequently at the NR5A1 locus in ovarian tumors.

With regards to the type of observed loss, LOH events at rs2297605 were equally distributed between the two alleles, while the other two SNPs showed bias in the LOH events towards one of the two alleles (Fig. 1B). The significance of this finding is unclear, since these are non-coding SNPs. Interestingly, the genotyping results (Fig. 1A) also uncovered somatic mutations other than LOH in the tumors, manifested as allele gains; base substitutions turning a homozygous genotype (normal DNA) into a heterozygote genotype in the tumor, hence called 'gain of allele'; Fig. 1B. These somatic NR5A1 substitutions were present in 10% of ovarian tumors (Fig. 2). Thus the genotyping data share frequent genetic (LOH/substitution) events at the NR5A1 locus in ovarian tumors.

**Methylation of the NR5A1 gene in ovarian tumors.** A methylation-sensitive restriction enzyme (Afe1) method (e.g. 19) was used to quantify site-specific methylation at -30 bp (compared to translation start) of the NR5A1 gene promoter in ovarian tumor tissue from patients with ovarian cancer and in matched normal tissue from the same patients (when available).

Afe1 cleaves genomic DNA at 5'-AGC/GCT-3', but cleavage is blocked by methylation (http://www.neb.com/nebecomm/products/productr0652.asp). Since the Afe1 enzyme cleaves the unmethylated CpG's, only methylated CpG's can be amplified and quantified following gel electrophoresis. Complete DNA digestion was confirmed by prolonged (overnight) Afe1 digestion, which yielded similar results (data not shown). To control for differences in DNA level and/or quality between tumor samples, we also amplified β-actin as an internal control. The primers used for the β-actin gene were selected to amplify a region that lacks an Afe1 cleavage site. Therefore, β-actin is amplified regardless of methylation status, and relative band intensity (NR5A1/β-actin) was used as a function of NR5A1 gene methylation (see Materials and methods for details). This analysis indicated that 36 out of 46 (78%) ovarian tumors had appreciable NR5A1 methylation (2nd, 3rd and 4th quartile of methylation levels), and 17/46 (37%) had high levels of NR5A1 methylation (3rd and 4th quartile of methylation levels; Table II). Thus the NR5A1 gene is methylated in most ovarian tumors. Furthermore, we detected both a high level of NR5A1 gene methylation and LOH in 21% of the ovarian tumors that we analyzed (Fig. 1 and Table II). The cumulative data also demonstrate that 62% of the ovarian tumors have LOH, high level of methylation, or both (Fig. 2) at the NR5A1 locus.

As indicated by Table I, most ovarian tumors are diagnosed at an advanced stage, reducing the ability to obtain normal ovarian tissue from most patients. In the absence of an adequate number of matched normal ovaries available for study, 16 non-tumor ovaries were analyzed (from unrelated individuals) to evaluate the relative methylation of the promoter region of the NR5A1 gene in normal ovarian tissue, with the same methylation-sensitive restriction enzyme method used above. Since human ovarian tumors have epithelial origin (2,20), we obtained OSE cells from these ovarian tissues (by...
laser-capture microdissection) and analyzed them following DNA extraction. β-actin was again amplified from each sample as an internal control. These data show that only one out of the 11 (9%) normal ovaries that were successfully evaluated (i.e. that had β-actin amplification) showed appreciable NR5A1 methylation (Table III). This difference between the prevalence of NR5A1 methylation in tumor versus normal ovaries is statistically significant (p<0.0001). Thus, ovarian tumor tissues display significantly more frequent NR5A1 gene methylation than normal ovarian epithelial tissues.

Clinical correlation. Retrospective analysis of the clinical data suggest that presenting stage and histologic grade of ovarian tumors are not significantly affected by the presence of somatic NR5A1 gene alterations (Table I and data not shown). Furthermore, Kaplan-Meier survival curves were similar for both ovarian tumors with and without NR5A1 gene alteration (LOH/methylation; data not shown). Likewise, the presence

Figure 1. Genotyping reveals somatic NR5A1 gene alterations in ovarian tumors. (A) Genotype distribution by sample. Somatic mutations were identified by genotyping matched ovarian tumors and normal tissue from the same individuals by Taqman SNP genotyping assays for the indicated SNPs. Only tumor genotypes are shown. (B) Genotyping distribution by SNP. Key: LOH, loss of heterozygosity (e.g. AG>AA); gain, gain of heterozygosity (e.g. GG>AG). Unknown, unclear genotype, for either tumor or normal sample.

Figure 2. NR5A1 gene alterations in ovarian tumors. Key: High methylation = 3rd and 4th quartile of methylation levels (see Materials and methods). Substitution, a somatic mutation other than LOH (e.g. AA>AG). LOH/High methylation, LOH or high methylation.
| Age | Race       | Date of Dx | Study no. | Years survived since Dx | Stage | Histological grade | Surgery | Chemo | Radiation |
|-----|------------|------------|-----------|-------------------------|-------|--------------------|---------|-------|-----------|
| 53  | Black      | 6/13/07    | T23       | Unknown                 | 3C    | 3                  | 6/13/07 | Yes   | No        |
| 62  | White      | 6/19/07    | T24       | <1 year                 | 3C    | 3                  | 6/19/07 | No    | No        |
| 32  | White      | 4/19/07    | T25       | <1 year                 | 4     | 3                  | 8/23/07 | No    | No        |
| 54  | Black      | 12/12/07   | T26       | Living                  | 0     | Borderline         | 12/12/07| No    | No        |
| 31  | White      | 1/15/08    | T27       | Living                  | 0     | Borderline         | 1/15/08 | No    | No        |
| 26  | Black      | 2/18/08    | T28       | Living                  | 3B    | 1                  | 2/18/08 | No    | No        |
| 45  | Hispanic   | 4/28/08    | T29       | Living                  | 0     | 2                  | 4/29/08 | No    | No        |
| 64  | White      | 5/8/08     | T30       | Living                  | 0     | Borderline         | 5/18/08 | No    | No        |
| 60  | White      | 8/13/08    | T31       | Living                  | 3C    | 3                  | 8/13/08 | Yes   | No        |
| 47  | Black      | 10/8/08    | T32       | Living                  | 0     | Borderline         | 10/8/08 | No    | No        |
| 55  | White      | 10/9/08    | T33       | 1 year                  | 3C    | 2                  | 10/9/08 | No    | No        |
| 72  | Unknown    | 10/27/08   | T34       | Living                  | 3C    | 3                  | 10/27/08| Yes   | No        |
| 57  | White      | 12/7/08    | T35       | <1 year                 | 3C    | Borderline         | 12/7/08 | Unknown| Unknown   |
| 74  | Hispanic   | 12/11/08   | T36       | Unknown                 | 3C    | 3                  | 12/11/08| Yes   | No        |
| 50  | Black      | 1/29/09    | T37       | <1 year                 | 4     | 2                  | 1/29/09 | No    | No        |
| 49  | Black      | 5/11/09    | T38       | Living                  | 0     | Borderline         | 5/11/09 | No    | No        |
| 56  | Black      | 9/28/09    | T39       | Living                  | 3A    | 2                  | 9/28/09 | Yes   | Yes       |
| 70  | Unknown    | 10/1/09    | T40       | Living                  | 3C    | 3                  | 10/1/09 | Yes   | No        |
| 46  | Black      | 3/2/07     | T21       | Unknown                 | 3C    | 3                  | 3/2/07  | Yes   | No        |
| 41  | Black      | 6/12/07    | T22       | 1 year                  | 3C    | 3                  | 6/12/07 | Yes   | No        |
| 73  | White      | 4/24/97    | T10       | 1 month                 | 3B    | 3                  | 11/24/97| No    | No        |
| 41  | Black      | 7/21/98    | T8        | 2 years                 | 4     | 3                  | 7/21/98 | Yes   | No        |
| 69  | White      | 4/5/99     | T13       | 6 years                 | 4     | 3                  | 4/5/99  | Yes   | No        |
| 63  | Black      | 3/5/00     | T9        | 2 years                 | 3C    | 2                  | 3/5/00  | Yes   | No        |
| 47  | White      | 5/31/00    | T14       | Living                  | 3B    | 3                  | 5/31/00 | Yes   | No        |
| 26  | White      | 5/21/99    | T15       | Living                  | 3C    | 3                  | 5/21/99 | Yes   | Yes       |
| 61  | Black      | 3/5/02     | T49       | <1 year                 | 4B    | 2                  | 3/5/02  | Yes   | No        |
| 36  | White      | 5/29/02    | T50       | Living                  | 0     | Borderline         | 5/29/02 | No    | No        |
| 60  | White      | 6/28/02    | T11       | Unknown                 | 3C    | 2                  | 6/28/02 | Unknown| Unknown   |
| 83  | White      | 8/9/02     | T51       | <1 year                 | 3C    | 3                  | 8/9/02  | No    | Yes       |
| 77  | Hispanic   | 3/28/03    | T52       | <1 year                 | 4B    | 3                  | 6/13/03 | Yes   | No        |
| 67  | Black      | 9/24/03    | T53       | 3 years                 | 3C    | 3                  | 9/26/03 | Yes   | No        |
| 43  | White      | 6/18/01    | T54       | 2 years                 | NA    | 3\textsuperscript{a} colon primary | 6/18/01 | Yes   | No        |
| 47  | White      | 12/19/03   | T17       | Living                  | 3A    | 3                  | 12/19/03| Yes   | No        |
| 68  | Black      | 2/16/04    | T55       | <1 year                 | 4     | 3                  | 2/16/04 | No    | No        |
| 75  | White      | 6/20/03    | T1        | 2 years                 | 4     | 3                  | 6/20/03 | Yes   | No        |
| 35  | Hispanic   | 5/25/05    | T41       | <1 year                 | 3C    | 2                  | 5/25/05 | Yes   | No        |
| 76  | White      | 7/20/06    | T42       | 2 years                 | 3C    | 3                  | 10/24/06| Yes   | No        |
| 45  | White      | 10/21/02   | T12       | 4 years                 | 2A    | 10/21/02           | No      | No    | No        |
| 60  | NA         | 2/10/02    | T4        | <1 year                 | 4     | 3                  | 3/5/02  | Yes   | No        |
| 51  | White      | 3/13/07    | T3        | Living                  | 3C    | 3                  | 3/13/07 | Yes   | No        |
| 70  | Indian     | 3/16/07    | T2        | Unknown                 | 4     | 3                  | 3/16/07 | Unknown| Unknown   |
| 65  | White      | 6/8/07     | T44       | <1 year                 | 4     | 3                  | 6/12/07 | No    | No        |
| 68  | Black      | 8/17/07    | T45       | Living                  | 4     | 3\textsuperscript{a} endometrial cancer | 8/17/07 | No    | Yes       |
| 64  | White      | 6/23/08    | T56       | Living                  | 3C    | 3                  | 7/9/08  | Yes   | No        |
| 49  | White      | 6/25/09    | T46       | Living                  | 2B    | Unknown            | 6/25/09 | No    | No        |
| 66  | Hispanic   | 7/16/09    | T47       | Living                  | 3C    | 3                  | 7/16/09 | Yes   | No        |
| 51  | White      | 8/8/09     | T48       | Living                  | 1A    | 1                  | 8/5/09  | No    | No        |
| 30  | White      | 8/5/09     | T57       | Living                  | 1A    | Borderline         | 8/5/09  | No    | No        |

Key: Borderline/stage 0 tumors, indicate atypical changes in the ovary that have low malignancy potential. Dx, diagnosis.
of NR5A1 gene alteration did not correlate with race/ethnicity or treatment, such as radiation and chemotherapy (Table I and data not shown).

**Discussion**

A common feature of many tumor suppressor genes is their inactivation in cancer tissue through LOH and other somatic mutations. In ovarian tumors, LOH and somatic mutations have been documented for tumor suppressors such as TP53, BRCA1, BRCA2 and PTEN (21). The data presented herein support such a role for human SF-1, and may provide a molecular mechanism to partially explain the loss of SF-1 protein reported in both ovarian tumors and ovarian cancer cell lines. Specifically, the data demonstrate that most ovarian tumors contain genetic and/or epigenetic alterations at the NR5A1 locus, significantly more frequently compared to normal ovaries. These somatic alterations include LOH, base substitution, and methylation of the NR5A1 gene promoter. Specifically, the data demonstrate that most ovarian tumors contain genetic and/or epigenetic alterations at the NR5A1 locus, significantly more frequently compared to normal ovaries. These somatic alterations include LOH, base substitution, and methylation of the NR5A1 gene promoter.

**Table II. NR5A1 gene methylation in ovarian tumors.**

| Samples | Relative intensity | Methylation level |
|---------|-------------------|------------------|
| T1      | 0.24              | -                |
| T2      | 0.81              | ++               |
| T3      | 1.14              | +++              |
| T4      | 0.78              | ++               |
| T8      | 0.35              | +                |
| T9      | 0.72              | ++               |
| T10     | 0.02              | -                |
| T11     | 0.34              | +                |
| T12     | 0.40              | +                |
| T13     | 0.77              | ++               |
| T14     | 0.48              | +                |
| T15     | 0.54              | +                |
| T17     | 0.40              | +                |
| T21     | 0.45              | +                |
| T22     | 0.62              | ++               |
| T23     | 0.58              | ++               |
| T24     | 0.56              | +                |
| T25     | 0.68              | ++               |
| T26     | 0.37              | +                |
| T27     | 0.87              | +++              |
| T28     | 0.55              | +                |
| T29     | 0.78              | ++               |
| T30     | 0.83              | ++               |
| T31     | 0.58              | ++               |
| T32     | 0.09              | -                |
| T33     | 0.57              | +                |
| T34     | 0.32              | +                |
| T35     | 0.17              | -                |
| T36     | 0.53              | +                |
| T37     | 0.43              | +                |
| T38     | 0.54              | +                |
| T39     | 0.57              | ++               |
| T40     | 0.42              | +                |
| T41     | NA                | NA               |
| T42     | 0.75              | ++               |
| T43     | NA                | NA               |
| T44     | 0.14              | -                |
| T45     | 0.00              | -                |
| T46     | 1.14              | +++              |
| T47     | NA                | NA               |
| T48     | NA                | NA               |
| T49     | 0.10              | -                |
| T50     | 0.11              | -                |
| T51     | 0.79              | ++               |
| T52     | 0.53              | +                |
| T53     | 1.07              | +++              |
| T54     | 0.00              | -                |
| T55     | 0.00              | -                |
| T56     | 0.54              | +                |
| T57     | 0.30              | +                |

Methylation was quantified by the methylation-sensitive restriction enzyme method. Relative intensity, NR5A1/β-actin band intensity. Methylation level is indicated by quartiles of relative intensity: -, 1st; +, 2nd; ++, 3rd; and ++++, 4th quartile.

**Table III. NR5A1 gene methylation in normal ovaries.**

| Samples | Relative intensity | Methylation level |
|---------|-------------------|------------------|
| N41     | 0                 | -                |
| N61     | 1.5               | +++              |
| N44     | 0.1               | -                |
| N34     | NA                | NA               |
| N45     | 0.16              | -                |
| N60     | 0                 | -                |
| N42     | 0                 | -                |
| N59     | 0                 | -                |
| N52     | 0                 | -                |
| N29     | NA                | NA               |
| N51     | 0                 | -                |
| N38     | 0                 | -                |
| N54     | NA                | NA               |
| N53     | 0.21              | -                |
| N57     | NA                | NA               |
| N27     | NA                | NA               |

Bold indicates samples with available tumor. The numbers for normal (N) tissue are scrambled, and do not correspond to the tumor (T) tissue numbers. For key, see Table II.
carcinogenesis. Analysis of the early stage/grade tumors in our dataset suggests a similar prevalence of somatic NR5A1 alterations in advanced and non-advanced tumors (data not shown). However, this interpretation is tempered by the existence of low numbers of non-advanced tumors in our dataset (Table I). Examination of larger numbers of non-advanced and/or benign tumors for somatic NR5A1 alterations, may help confirming this concept.

The finding of somatic NR5A1 gene mutations (gain of allele substitutions) in 10% of the ovarian tumors (Fig. 2) is striking, given the fact that we interrogated only three base pairs of the NR5A1 gene in these assays (the three SNP positions). This fact together with the finding of LOH at this locus in 44% of the ovarian tumors (Fig. 2), strongly suggest a high somatic mutation frequency of the NR5A1 gene in ovarian cancer. Thus, the NR5A1 gene should be screened for somatic mutations by a more comprehensive method (such as sequencing) in both advanced and benign ovarian tumors, especially tumors that show LOH. This analysis should include the NR5A1 gene promoter, since SF-1 protein expression is lost in both human ovarian tumors and tumor cell lines. Identification of a somatic mutation and/or methylation together with LOH in the same tumor, may explain the loss of SF-1 protein reported in ovarian tumor tissue. To that effect, the detection of both a high level of NR5A1 gene methylation and LOH in 21% of the ovarian tumors that we analyzed (Fig. 1 and Table II), may partially explain this loss.

LOH can be caused by two different mechanisms in tumor cells: i) deletion (loss of allele/gene) or ii) base substitution (which includes gene conversion). Although 14% of the tumors had multiple LOH events (Fig. 1) suggesting a deletion at the NR5A1 locus, the majority of LOH events involved only one (out of three possible) SNPs at the NR5A1 locus (Fig. 1), suggesting no extensive NR5A1 deletion, at least around the three interrogated SNPs. However, even a microdeletion involving only the genomic area around a single NR5A1 SNP can affect SF-1 protein expression. Also, gene conversion involves recombination (22), which may cause deletions, rearrangements and other functionally important (for SF-1 expression) genetic events upstream or downstream from the interrogated SNPs (undetected by our assay). Furthermore, both molecular heterogeneity within the same tumor and contamination with normal cells can result in underestimation of the extent of LOH, or confinement of the observed LOH in a smaller genetic region. In addition, the high somatic mutation frequency observed at the NR5A1 locus may have functional effects. Therefore, the frequently observed genetic (LOH/substitution) events at the NR5A1 locus may significantly contribute to the reported loss of SF-1 protein in ovarian tumor tissue.

A limitation of the methylation-sensitive restriction enzyme method utilized here is that the use of PCR, which exponentially amplifies the target DNA, makes the method less quantitative. For this reason, we included β-actin as an internal control, and also focused our methylation analysis on quartiles of methylation rather than absolute levels. The significant difference observed in the frequency of appreciable methylation (2nd quartile or higher) between tumor and normal ovarian tissue (Tables II and III) suggests that NR5A1 methylation is much more prevalent in tumors. The exact degree of methylation, and the subsequent reduction of SF-1 protein, is hard to estimate from these data. However, the fact that the 37% of the ovarian tumors that show high methylation (++ or higher methylation level; Fig. 2) display >57% of the band intensity of β-actin (Table II), suggests that a significant proportion of the NR5A1 gene is methylated in these ovarian tumors in vivo. Thus methylation may be a major molecular mechanism leading to the reported loss of SF-1 protein in ovarian tumors.

Interestingly, hypomethylation and subsequent transcriptional activation of SF-1 has been reported in endometriosis, an estrogen dependent disease (23). In contrast, hypermethylation leading to silencing of gene expression has been reported in ovarian tumors for multiple key tumor suppressor genes including BRCA1, BRCA2, WT1, APC, CDDN2A and MLH1 (24,25).

In conclusion, we report frequent somatic alterations of the NR5A1 locus in ovarian tumors, including LOH, base substitution, and methylation of the NR5A1 gene promoter. These molecular abnormalities may partially explain the loss of SF-1 protein, and contribute to the model of SF-1 as an ovarian tumor suppressor. The existence of both genetic and epigenetic NR5A1 gene abnormalities in ovarian tumors further suggest that SF-1 is a common and important target in ovarian carcinogenesis.

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References

1. Ozols RF: Treatment goals in ovarian cancer. Int J Gynecol Cancer 15 (Suppl 1): 3-11, 2005.
2. Leung PC and Choi JH: Endocrine signaling in ovarian surface epithelium and cancer. Hum Reprod Update 13: 143-167, 2007.
3. Rae MT, Niven D, Ross A, Forster T, Lathe R, Critchley HOD, Ghazal P and Hillier SG: Steroid signalling in human ovarian surface epithelial cells: the response to interleukin-1alpha determined by microarray analysis. J Endocrinol 183: 19-28, 2004.
4. Papacleovoulou G, Hogg K, Fegan KS, Critchley HO, Hillier SG and Mason JF: Regulation of 3β-hydroxysteroid dehydrogenase type 1 and type 2 gene expression and function in the human ovarian surface epithelium by cytokines. Mol Hum Reprod 15: 379-392, 2009.
5. Ivarsson K, Sundfeldt K, Brannstrom M and Janson PO: Production of steroids by human ovarian surface epithelial cells in culture: possible role of progesterone as growth inhibitor. J Endocrinol Oncol 82: 116-121, 2001.
6. Okamura H, Katabuchi H and Ohba T; What we have learned from isolated cells from human ovary? Mol Cell Endocrinol 202: 37-45, 2003.
7. Nash DM, Hess SA, White BA and Peluso JJ: Steriogenic factor-1 regulates the rate of proliferation of normal and neoplastic rat ovarian surface epithelial cells in vitro. Endocrinology 139: 4663-4671, 1998.
8. Batsche E, Desroches J, Bilodeau S, Gauthier Y and Drouin J: Rb enhances p160/SRC coactivator-dependent activity of nuclear receptors and hormone responsiveness. J Biol Chem 280: 19746-19756, 2005.
9. Tran PV, Akana SF, Malkovska I, Dallman MF, Parada LF and Ingraham HA: Diminished hypothalamic bdnf expression and impaired VMH function are associated with reduced SF-1 gene dosage. J Comp Neurol 498: 637-648, 2006.
10. Syed V, Ulinski G, Mok SC, Yu G and Ho SM: Expression of gonadotropin receptor and growth responses to key reprodutive hormones in normal and malignant human ovarian surface epithelial cells. Cancer Res 61: 6768-6776, 2001.
11. Hinkula M, Pukkala E, Kyyronen P and Kauppinen A: Incidence of ovarian cancer of grand multiparous women - A population-based study in Finland. Gynecol Oncol 103: 207-211, 2006.
12. Sugawara T, Kiriakidou M, McAllister JM, Holt JA, Arakane F and Strauss JF III: Regulation of expression of the steroidogenic acute regulatory protein (StAR) gene: a central role for steroidalogenic factor 1. Steroids 62: 5-9, 1997.

13. Fujieda K, Tajima T, Nakae J, Sageshima S, Tachibana K, Suwa S, Sugawara T and Strauss JF III: Spontaneous puberty in 46,XX subjects with congenital lipoid adrenal hyperplasia. Ovarian steroidogenesis is spared to some extent despite inactivating mutations in the steroidogenic acute regulatory protein (StAR) gene. J Clin Invest 99: 1265-1271, 1997.

14. Ramayya MS, Sheng M, Moroz K, Hill SM and Rowan BG: Human steroidalogenic factor-1 (hSF-1) regulates progesterone biosynthesis and growth of ovarian surface epithelial cancer cells. J Steroid Biochem Mol Biol 119: 14-25, 2010.

15. Abd-Elaziz M, Moriya T, Akahira J, Suzuki T and Sasano H: StAR and progesterone producing enzymes (3beta-hydroxysteroid dehydrogenase and cholesterol side-chain cleavage cytochromes P450) in human epithelial ovarian carcinoma: immunohistochemical and real-time PCR studies. Cancer Sci 96: 232-239, 2005.

16. Taketo M, Parker KL, Howard TA, Tsukiyama T, Wong M, Niwa O, Morton CC, Miron PM and Seldin MF: Homologs of Drosophila Fushi-Tarazu factor 1 map to mouse chromosome 2 and human chromosome 9q33. Genomics 25: 565-567, 1995.

17. Schultz DC, Vanderveer L, Buetow KH, Boenke MP, Ozols RF, Hamilton TC and Godwin AK: Characterization of chromosome 9 in human ovarian neoplasia identifies frequent genetic imbalance on 9q and rare alterations involving 9p, including CDKN2. Cancer Res 55: 2150-2157, 1995.

18. Hoivik EA, Aumo L, Aesoy R, Lillefosse H, Lewis AE, Perrett RM, Stallings NR, Hanley NA and Bakke M: Deoxyribonucleic acid methylation controls cell type-specific expression of steroidalogenic factor 1. Endocrinology 149: 5599-5609, 2008.

19. Nakamura J, Kitajima Y, Kai K, Hashiguchi K, Hiraki M, Noshiro H and Miyazaki K: Expression of hypoxic marker CA IX is regulated by site-specific DNA methylation and is associated with the histology of gastric cancer. Am J Pathol 178: 515-524, 2011.

20. Seidman JD, Russell P and Kurman RJ: Surface epithelial tumors of the ovary. In: Blausten's pathology of the female genital tract. Kurman RJ (ed). 5th ed. Springer, New York, pp791-904, 2002.

21. Despierre E, Lambrechts D, Neven P, Amant F, Lambrechts S and Vergote I: The molecular genetic basis of ovarian cancer and its roadmap towards a better treatment. Gynecol Oncol 117: 358-365, 2010.

22. Chen JM, Cooper DN, Chuzhanova N, Férec C and Patrinos GP: Gene conversion: mechanisms, evolution and human disease. Nat Rev Genet 8: 762-775, 2007.

23. Xue Q, Lin Z, Yin P, Milad MP, Cheng YH, Confino E, Reierstad S and Bulun SE: Transcriptional activation of steroidalogenic factor-1 by hypomethylation of the 5' CpG island in endometriosis. J Clin Endocrinol Metab 92: 3261-3267, 2007.

24. Balch C, Fang F, Matei DE, Huang TH and Nephew KP: Minireview: epigenetic changes in ovarian cancer. Endocrinology 150: 4003-4011, 2009.

25. Barton CA, Hacker NF, Clark SJ and O'Brien PM: DNA methylation changes in ovarian cancer: implications for early diagnosis, prognosis and treatment. Gynecol Oncol 109: 129-139, 2008.