Sox2 Gene Amplification Significantly Impacts Overall Survival in Serous Epithelial Ovarian Cancer

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Abstract

Epithelial ovarian cancer (EOC) is the deadliest gynecologic cancer. Recently, the existence of ovarian cancer stem cells has been reported. Sox2, Nanog and Oct4 are key markers of “stemness”. The objective of this study was to determine whether Sox2, Nanog, and Oct4 are associated with EOC and poor outcome. The expression of these markers was assessed by immunofluorescence staining and real-time RT-PCR in human EOC cell lines MDAH-2774 and SKOV-3, while the cancer genome atlas (TCGA) dataset was analyzed for associations with survival. Sox2, Nanog and Oct4 (POU5F1) were all detected by immunofluorescence staining and these results were confirmed by real-time RT-PCR. The TCGA dataset revealed a 26%, 9%, and 6% amplification of Sox2, Nanog and POU5F1, respectively. Additionally, K-M survival analyses showed a significant median overall survival difference (41 versus 48.3 months, $P = .01$) for Sox2 amplification, but not for Nanog (44.1 versus 36.2 months, $P > .05$) and POU5F1 (43.5 versus 45.0 months, $P > .05$). Our results suggest that Sox2 gene amplification significantly influences overall survival.

Keywords

Sox2 amplification, overall survival, serous epithelial ovarian cancer

Introduction

Ovarian cancer is the principal cause of death of all gynecologic cancers with an estimated 22 240 new cases and 14 030 deaths expected in 2013 in the United States alone.¹ Although the exact etiology of epithelial ovarian cancer (EOC) in the general population remains elusive, breast cancer genes 1/2 (BRCA 1/2) mutation carriers and those affected with Lynch II syndrome are considered at high risk.^{2,3} Indeed, a strong family history of EOC is the single most important risk factor.^{2,4} Nulliparity seems to be another risk factor; however, oral contraceptive use, tubal ligation, pregnancy, and lactation confer protection against the disease.⁴⁻⁶ Among different proposed theories for the pathogenesis of EOC, the “incessant ovulation” model seems to be the most widely accepted.⁷ Accordingly, cyclical ovulations without intervening pregnancy will, overtime, increase the likelihood of chronic inflammation and generation of reactive oxygen species that may induce various mutations, and concomitant with impairment of repair mechanisms will initiate tumorigenesis.⁸⁻¹⁰ Recently, increasing evidence suggests that factors involved in cell pluripotency and self-renewal, key characteristics of stem cells, are implicated in cancer biology.¹¹⁻²³ Specifically, the existence of human ovarian cancer-initiating cells with stemness properties and

enhanced resistance to cisplatin and paclitaxel has been reported.¹⁷ Indeed, since the isolation of human embryonic stem cells (ESCs),²⁴ significant interests have been generated in science and medicine because of their therapeutic potential. Self-renewal and pluripotency are complex processes that demand the cooperation of both intrinsic and extrinsic pathways to maintain the state of stemness and undergo clonal expansion.²⁵⁻²⁹ More recently, the discovery of induced pluripotent stem cells (iPSCs) has furthered the field by providing an alternative tool for research and cell-based therapy.^{30,31} Induced PSCs are adult human somatic cells that can revert to embryonic-like state following transfection by 4 transcription

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Table 1. Oligonucleotide Primers.

Accession Number	Gene	Sense (5'-3')	Antisense (3'-5')	Amplicon (bp)	Initial PCR Cycle, seconds	Annealing Temperature, °C
NM_024865	Nanog	ACTCTCCAACATCCTGAA	TTCTGCCACCTCTTAGAT	84	1000	55
NM_001173531.2	POU5F1	CGCTGGCTTATAGAAGGT	ACAGGTGTCATAAGAATGGATA	155	1000	54
NM_003106	Sox2	GGATGGTTGTCTATTAACCT	TCAAACCTCTCTCCCTTT	153	1400	53

Abbreviations: PCR, polymerase chain reaction; Sox2, sex determining region Y-related HMG box 2.

factors (Nanog, sex determining region Y-related HMG box 2 [Sox2], c-Myc, and Klf4).³⁰

The transcription factors Sox2, Nanog, and octamer-binding transcription factor 4 (Oct4) form the core regulatory positive-feedback loop essential to sustain the stem cells capacity for self-renewal and pluripotency. Several pathways are known to affect the expression of these genes such as leukemia inhibiting factor, Notch, Sonic hedgehog, and Wnt.³²⁻⁴⁰ Together, the genes and signaling pathways that regulate the stem cell state are referred to as the “stemness” signature.⁴¹ The hypothesis of this study is that core regulatory stemness genes (Sox2, Nanog, and Oct4) impact survival outcome in EOC. To test this hypothesis, we studied the expression of pluripotency markers, Sox2, Nanog, and Oct4 (POU5F1) in human EOC cell lines MDAH-2774 and SKOV-3, utilizing immunostaining and real-time reverse transcription-polymerase chain reaction (RT-PCR). Additionally, we used large-scale Ovarian Serous Cystadenocarcinoma genomics data obtained from The Cancer Genome Atlas (TCGA).

Materials and Methods

Cell Lines, Media, and Cell Culture Conditions

The human EOC cell lines, MDAH-2774 (CRL-10303) and SKOV-3 (HTB-77), were obtained from American Type Culture Collection (ATCC, Manassas, Virginia). Cells were cultured in 100-cm² cell culture dishes (Corning Incorporated, Corning, New York) with McCoy's 5A medium (Invitrogen, Carlsbad, California) supplemented with 100 U/mL penicillin and 100 µg/mL streptomycin and 10% heat-inactivated fetal bovine serum at 37°C in 5% CO₂. Culture medium was replaced every 2 days. Once confluent, 5.0 × 10⁶ cells were seeded in a 100-mm dishes and were collected after 24 hours.

Immunostaining

Cells (125 000) were seeded on 170-µm coverslips (Thomas Scientific, Swedesboro, New Jersey) in a 24-well dish for 24 hours. Cells were fixed with 4% paraformaldehyde for 10 minutes followed by washing in 0.05% tween in phosphate buffered saline (PBST) 2 × 2 minutes each. Cells were permeabilized in 0.2% Triton X-100 for 10 minutes followed by washing with PBST 3 × 5 minutes each. Antigen retrieval was done in 10 mmol/L sodium citrate buffer at 95°C for 20

minutes followed by washing 3 × 5 minutes each. Cells were blocked in 10% donkey serum (Sigma Aldrich, St Louis, Missouri) for 1 hour at room temperature. Cells were incubated overnight at 4°C in primary antibody (1:50 rabbit-anti-Sox2 or 1:50 rabbit-anti-Oct4 or 1:100 goat-anti-Nanog; Sigma Aldrich) in 10% donkey serum. Cells were washed in PBST 3 × 5 minutes each followed by incubation with the secondary antibodies (1:800 donkey-antigoat Alexa Fluor 488 or 1:800 donkey-antirabbit Alexa Fluor 488; AbCam, Cambridge, Massachusetts) for 30 minutes at room temperature. Cells were washed 3 × 5 minutes each, and nuclei were counterstained with ProLong Gold Antifade reagent with 4',6-diamidino-2-phenylindole (DAPI; Life Technologies, Grand Island, New York). Cells were visualized using an Axiovert 25 inverted microscope (Zeiss, Thornwood, New York) using DAPI (blue) and Alexa Fluor 488 (green), fluorescent filters with excitation and emission wavelengths of 365 and 445 nm and of 470 and 525 nm, respectively. Images were taken using the Axiovision software (Zeiss) and a microscope-mounted camera.

Real-Time RT-PCR for Sox2, Nanog, and Oct4 (POU5F1)

RNA isolation. Total RNA was extracted from cells using the RNeasy Mini Kit (Qiagen, Valencia, California) according to the manufacturer's protocol.

Reverse transcription. A 20-µL complementary DNA (cDNA) reaction volume, utilizing 1 µg RNA, was prepared using the QuantiTect Reverse Transcription Kit (Qiagen), according to the manufacturer's protocol.

Real-time RT-PCR primer design and controls. Optimal oligonucleotide primer pairs for real-time RT-PCR amplification of reverse-transcribed cDNA were selected with the aid of the software program, Beacon Designer (Premier Biosoft Int., Palo Alto, California). Human oligonucleotide primers, which amplify variable portions of the protein coding regions, are listed in Table 1. Standards with known concentrations were designed specifically for these primers using Beacon Designer software, allowing for construction of a standard curve using a 10-fold dilution series. A specific standard for each gene allows for absolute quantification of the gene in copy numbers, which can then be expressed as nanogram per microgram of RNA.

Real-time RT-PCR was performed with the QuantiTect SYBR Green RT-PCR kit (Qiagen) and a Cepheid 1.2f

Detection System (Cepheid, Sunnyvale, California). Each 25 μ L reaction consisted of 12.5 μ L of 2 $\times$ QuantiTect SYBR Green RT-PCR master mix, 1 μ L of cDNA template, and 0.2 μ mol/L each of target-specific primer that was designed to amplify a part of the gene of interest. To quantify each target transcript, a standard curve was constructed using a 10-fold dilution series of the standard for the specific gene of interest. The PCR conditions for the primers are summarized subsequently and in Table 1. An initial cycle was performed at 95°C as indicated in Table 1, followed by 35 cycles of denaturation at 95°C for 15 seconds, annealing for 30 seconds as described in Table 1, and a final cycle at 72°C for 30 seconds to allow completion of product synthesis. Following real-time RT-PCR, a melting curve analysis was performed to demonstrate the specificity of the PCR product as a single peak. A control containing all the reaction components except for the template was included in all experiments.

Validating of the Cell Line Findings and Analyzing the Impact of Sox2, Nanog, and POU5F1 on Survival Outcomes Using “TCGA” Data set

The TCGA is a joint effort of the National Cancer Institute and the National Human Genome Research Institute. Patient information, tumor samples, analysis of microarray data for many cancers, as well as treatment characteristics, and outcome measures have been previously published.^{42–46} The database was queried using the Cancer Genomics Portal Web site (<http://www.cbioportal.org/public-portal/>) to visualize, analyze, and download large-scale Ovarian Serous Cystadenocarcinoma genomics provisional data following previously published methods.⁴⁷ A gene set consisting of Sox2, Nanog, and POU5F1, which codes for Oct4, has been analyzed for mutations, copy number alterations (CNAs), and association with survival outcome measures of ovarian cancer. Copy number alterations or variations (CNAs or CNVs) are genomic structural polymorphisms, ranging from kb to Mb, identifiable by genome-deanalysis tools such as comparative genomic hybridization.^{48–50} They consist of changes in the number of copies of chromosome segments and genes consisting of deletions, amplifications, insertions, and translocations.^{48–51} This experiment was conducted in 2 consecutive steps. First, testing of the presence, frequency, and survival outcome associated with CNAs and mutations of each of the selected genes. Second, the data were reconstructed to an excel spreadsheet containing dichotomous CNAs and mutations data for Sox2, Nanog, and POU5F1 for further analysis. Finally, all the clinical and CNAs data were merged per “Case Id” as the linking variable after the separate files were uploaded to SPSS statistical package (SPSS for Mac V.21).

Exploration of the presence, frequency, and survival outcome associated with CNAs and mutations of Sox2, Nanog, and POU5F1. In the Cancer Genomics Portal (Home page), using

the query tab, Ovarian Serous Cystadenocarcinoma (TCGA provisional) was selected, followed by mutations and copy number alterations from GISTIC in the “Genomic Profiles” section. A user-defined case list was created using all pertinent tumor and treatment characteristics data ($n = 570$). The selected genes were entered using *Human Genome Organisation* (HUGO) gene symbols and/or aliases.

Cox regression analysis of Sox2, Nanog, and POU5F1 as a predictor of survival outcomes.

The data set was reconstructed in SPSS, and the data were recoded to a modified categorical and continuous variable scheme as compared to the one used in the original files from the Cancer Genomics Portal. Specifically, we consolidated the following tumor and clinical variables into binary categorical schemes: International Federation of Gynecology and Obstetrics (FIGO) stages into early (IIA-IIIB) and advanced (IIIC-IV); FIGO grades (G2) and (G3); platinum status sensitive (sensitive), resistant (resistant), and other (too early and missing). The “other” category was excluded in the analyses. Similarly, “Primary Therapy outcome success” has been recoded as “Prim. Tx. Out. Succ.” collapsing “complete response with stable disease (CR/SD)” and “partial response with progressive disease.” Ultimately, “tumor residual disease” was recoded as “TRD” with optimal disease (≤ 10 mm) and suboptimal (> 10 mm). Unadjusted and adjusted hazard ratios for each variable were conducted using the Cox predictive model.

Statistical analysis. Data were analyzed using SPSS 19.0 and Microsoft Excel for Mac 2011. *P* values are expressed at $< .05$ and 95% confidence interval was used for statistical significance.

Results

Sox2, Nanog, and Oct4 (POU5F1) Are Expressed in EOC Cell Lines

Immunostaining showed positive signals for all 3 pluripotency markers tested with the strongest expression for Sox2. Additionally, Sox2 was found more in the nuclear region as compared to Nanog and Oct4 (cytoplasmic and nuclear; Figure 1).

The immunostaining results were confirmed by real-time RT-PCR, which demonstrated the strongest expression for Nanog, followed by Sox2 and POU5F1. No statistically significant difference was found for the expression levels of Nanog and Sox2 between MDAH-2774 and SKOV-3 ($t = .36$, $P = .75$, 2-tailed; $t = 1.73$, $P = .22$, 2-tailed), respectively (Figure 2). However, the mRNA level of POU5F1 was significantly lower in SKOV-3 than MDAH-2774 ($t = 12.6$, $P = .006$, 2-tailed; Figure 2). In MDAH-2774 cells, the mean $\pm$ standard deviation was 280.40 ± 71 fg/ μ g RNA for Nanog, 125 ± 26 fg/ μ g RNA for Sox2, and 83 ± 6.9 fg/ μ g RNA for POU5F1. Similarly, in SKOV-3 cells, the mean $\pm$ standard deviation was 304.45 ± 61 fg/ μ g RNA for Nanog, 92 ± 7 fg/ μ g RNA for Sox2, and 19.5 ± 1.8 fg/ μ g RNA for POU5F1.

Figure 1. Representative sample of immunostaining for (A) sex determining region Y-related HMG box 2 (Sox2), (B) Nanog, and (C) octamer-binding transcription factor 4 (Oct4). ** indicates positive and * negative control in SKOV-3 human epithelial ovarian cancer cell line.

Figure 2. Real-time RT-PCR for sex determining region Y-related HMG box 2 (Sox2), Nanog, and POU5F1 in EOC cells (*P < .05) EOC indicates epithelial ovarian cancer.

Table 2. Frequency of Copy Number Variations Per Gene.

	Amplification, %	Deletion, %	Mutation, %	Other, %
Sox2	26	0	0	0
Nanog	9	0	0	0
POU5F1	6	0	0	0
Total	41	0	0	0

Abbreviation: Sox2, sex determining region Y-related HMG box 2.

Validating of the Cell Line Findings and Analyzing the Impact of Sox2, Nanog, and POU5F1 on Survival Outcomes Using “TCGA” Data set

Exploration of the presence, frequency, and survival outcome associated with CNA and mutations of Sox2, Nanog, and POU5F1. Of the 570 samples available for the Ovarian Serous Cystadenocarcinoma provisional data set, 488 (85.6%) were selected for further analyses due to incompleteness of the data.

Figure 3. Kaplan-Meier survival overall survival (OS) curves. The red curves in the Kaplan-Meier plots include all tumors with (A) sex determining region Y-related HMG box 2 (Sox2), (B) Nanog, and (C) POU5F1 amplifications as compared to the blue curves, which include all samples without amplifications.

Table 3. Unadjusted Hazard Ratio for the Variables Included in the Model.

	Sig	HR	95.0% CI	
			Lower	Upper
Sox2 amplification	.012	0.692	0.520	0.922
FIGO early stage	.009	0.559	0.361	0.865
FIGO low grade	.089	0.733	0.513	1.049
TRD (optimal)	.043	0.758	0.580	0.992
PR/PD	.000	3.680	2.737	4.948

Abbreviations: CI, confidence interval; FIGO, International Federation of Gynecology and Obstetrics; HR, hazard ratio; PR/PD, partial response with progressive disease; TRD, tumor residual disease; sig, significance; Sox2, sex determining region Y-related HMG box 2. Boldface values in Tables 3 and 4 denote statistical significance with p-values < 0.05.

Copy number alteration were noted in 41% of the cases. The most commonly observed was Sox2 amplification (26%), followed by Nanog amplification (9%) and POU5F1 amplification and mutation (6%; Table 2). Utilizing the Cancer Genomics Portal, Kaplan-Meier survival curves were generated for the genes listed. The Sox2 amplification demonstrated statistically significant median overall survival differences (41 vs 48.3 months, $P = .01$) compared to Nanog (44.1 vs 36.2 months, $P > .05$) and POU5F1 (43.5 vs 45.0 months, $P > .05$) and therefore was selected for additional analysis (Figure 3A-C).

Cox regression analysis of Sox2 as a predictor of survival outcomes. The variables selected in the analysis include Sox2 amplification, FIGO stage, FIGO grade, TRD, and primary treatment outcome successes. The “platinum status variable” was dropped from the analysis because the data were either missing or too early to be reported in over 39% of the cases. Interestingly, when the Cox predictive model was used Sox2 amplification, FIGO grade, TRD, Prim. Treat. Out. Succ. (unadjusted), and Sox2 amplification, FIGO grade, “Prim. Treat. Out. Succ.” (adjusted) were identified to be significant predictors of death (Tables 3 and 4).

Table 4. Adjusted Hazard Ratio for the Variables Included in the Model.

	Sig	HR	95.0% CI	
			Lower	Upper
Sox2 amplification	.023	0.670	0.474	0.947
FIGO early stage	.730	0.900	0.493	1.640
FIGO low grade	.021	0.592	0.380	0.923
TRD optimal	.694	1.067	0.773	1.471
PR/PD	.000	3.463	2.511	4.774

Abbreviations: CI, confidence interval; FIGO, International Federation of Gynecology and Obstetrics; HR, hazard ratio; PR/PD, partial response with progressive disease; TRD, tumor residual disease; sig, significance; Sox2, sex determining region Y-related HMG box 2.

Boldface values in Tables 3 and 4 denote statistical significance with p-values < 0.05.

Discussion

Cancer stem cells (CSCs) are thought to be involved in tumor aggressiveness and resistance to chemotherapy in various cancers representing a subset of cells that can be reactivated and induce tumor growth.^{13-15,52,53} More recently, Sox2 has been implicated in many cancers including lungs, colorectal, and stomach.^{12,14,15,54} Particularly, in gynecologic cancers, alterations of pathways involved in cell fate determination such as Hedgehog (Hh) and Notch were frequently observed; however, mutations in those maintaining potency (Nanog, Sox2, and Oct4) were not found as often.^{13,55-59} Therefore, identification of cells within the tumor capable of pluripotency and self-renewal properties is a relevant approach that may add to our current understanding of the pathophysiology of EOC. In this work, we demonstrated the expression of Sox2, Nanog, and Oct4 (POU5F1) in EOC cell lines. Moreover, we found an association between Sox2 amplification and improved overall survival in patients with serous EOC. It is plausible that both expression levels of Sox2 and POU5F1/Oct4 mRNAs and proteins are adequate and correlated as compared to Nanog. Biologically, Nanog expression pattern reflects a loss of feedback inhibition by the protein, therefore, allowing the gene transcription to go on at a higher level.

Interestingly, contrary to Sox2, Nanog and Oct4 amplifications were not significantly detected in both cell lines, nor did they influence survival outcome in the TCGA data analysis. The exact mechanisms underlying these observations are beyond the scope of this work and warrants further study.

In both human and animal models, Sox2 overexpression has been associated with malignant tumor formation and poor outcomes in several cancers.^{12,14,53,55-58,60} First discovered in 1990,⁶¹ the SOX family of genes comprises 10 different groups (A, B₁₋₂; C-I) based on a fairly conserved homologous sequence called *High Mobility Group* (HMG).⁶² They function as transcription factors through their low affinity DNA-binding domain and have been linked in cell fate decisions in various developmental processes.^{63,64} They have various tissue-specific expression patterns in early development.⁶³⁻⁶⁵ They have been described as necessary factors for maintaining pluripotency and self-renewal and also as repressors of genes encoding for cell lineage-specific regulators.^{52,66,67} Our results indicate the

presence and association of Sox2 amplification in EOC. This is in agreement with previously reported evidence implicating Sox2 in CSCs, although the exact mechanisms remain unclear. The overexpression of Sox2 has been linked to several cancers including ovarian and is generally associated with poor outcomes with the exception of lung cancer.^{16,53,55,57,68} For lung cancer, the proposed mechanism involves squamous maturation and thus improved response to chemotherapy.^{69,70} The current study is the first report linking Sox2 amplification to improved outcome in serous ovarian cancer. In this model, we proposed a novel p53-dependent mechanism involving Sox2, Nanog, and Oct4 to explain the improved outcome noted in serous ovarian cancer.

It is well established that Nanog acts as a cofactor of Sox2. Under certain pathological conditions, Nanog is suppressed by p53, a well-known tumor suppressor gene. Mutations of the p53 tumor suppressor gene are overwhelmingly present in the TCGA Serous Ovarian Adenocarcinoma data set, suggesting a high level of genomic instability. Normally, both ESCs and iPSCs require an intact p53 to maintain genomic stability and reprogramming efficiency, respectively. In the event of significant DNA damage, p53 is activated, binds to Nanog promoter, and represses its expression. This results in the induction of differentiation of ESCs into cell types subjected to senescence and apoptosis, therefore, protecting the genome from deleterious mutations.⁷¹⁻⁷³

When aberrations of these stemness regulatory pathways ensue as a consequence of p53 mutations, massive clonal expansion of a malignant phenotype with self-renewal potential becomes possible. Surprisingly, our findings are in contrast with previous evidence linking Sox2 overexpression with poor outcomes.^{53,55,56} In our analysis, both the K-M and the Cox predictive models confer a significant survival advantage with Sox2 amplification. Although the exact meaning of the association between Sox2 amplification and improved outcome is unclear, such relationships are not uncommon. A similar observation was made with BRCA1/2 mutations and their association with better survival outcomes. These reports suggest earlier age at initial diagnosis, better response to standard chemotherapy, and longer survival with BRCA1/2 mutations.⁷⁴⁻⁷⁷ The findings were attributed to the role of BRCA1/2 in DNA homologous recombination repair mechanisms.⁷⁸

The identification of new genes and molecular pathways will advance our understanding of the pathophysiology of EOC. Although, the SKOV-3 cell line has been previously characterized to express the pluripotency markers, it is not the case for MDAH-2774. Indeed, several cell lines such as OVCAR3, A2780, SKOV-3, and IGROV-1; ovarian teratocarcinoma PA-1 cells; and breast carcinoma cells MDA-MB-468, T47D; and MCF-7 express Sox2 and other pluripotency markers.⁶⁸ Our results provide additional evidence of the pluripotency markers in MDAH-2774 cell line.

Moreover, the lack of improvement in overall survival in EOC raises the possibility that other mechanistic pathways, not currently targeted, are involved in the biology of the tumor. In this study, we did not investigate phenotypic correlations. As a separate study, we are currently isolating ovarian cancer cells with stemness signatures with the objective to investigate mitotic activity, apoptosis, response to chemotherapy, and capacity to undergo DNA repair. We hope that our future findings will answer this important question.

The emergence of CSC subpopulations in a tumor poses a real challenge since these cells can be slow growing and are not affected by standard chemotherapy. After initial response to chemotherapy, the repopulation of the tumor bulk can occur resulting in progression and recurrence. Here, we present compelling evidence to support the role of markers involving stemness in EOC, with resultant impact in survival outcomes. Our results suggest that Sox2 amplification may be a potential biomarker related to tumor initiation, progression, and risk stratification in EOC.

Declaration of Conflicting Interests

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