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# Cancer-testis antigen expression is shared between epithelial ovarian cancer tumors

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## HIGHLIGHTS

- Heterogeneous expression of Cancer-testis antigens was found in epithelial ovarian cancer (EOC) tumors.
- Cancer-testis antigen expression is shared between EOC tumors.
- MAGE-A4, MAGE-A1, MAGE-A3, OY-TES-1 and MAGE-C1 being shared among 95% of all specimens analyzed.
- SP17, GAGE, NY-ESO-1 and XAGE-1D expression associated with higher risk of disease progression.
- A multi-antigen vaccination strategy could be a more suitable immunotherapeutic approach in EOC.

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## 1. Introduction

Epithelial ovarian cancer (EOC) is the most lethal gynecologic malignancy. It accounts for approximately of 80% of ovarian cancers. The treatment approach for patients with advanced ovarian cancer consists of aggressive surgical cytoreduction followed by systemic chemotherapy. There is high overall clinical response achieved with platinum-based therapy (up to 80%), including a high proportion of complete responses. Even though the median overall survival for optimally debulked patients has increased to 65.6 months, <30% of patients will remain free of disease [1]. A high proportion of patients with platinum sensitive disease at initial recurrence can achieve a second complete response, but these responses are of short duration. Ultimately, most patients relapse and develop drug-resistant disease [2]. This phenomenon highlights the importance of developing therapeutic modalities that will at least prolong recurrence free intervals and minimize or circumvent the emergence of resistance.

Infiltration of immune cells into EOC tumors has been demonstrated [1–4] and the presence of tumor infiltrating lymphocytes (TILs) has been associated with a more favorable prognosis. However, this tumor specific immune response is not sufficient to eradicate tumors, likely due to the immune suppressive tumor microenvironment. As for most human tumors, no exclusive tumor-specific antigens have been identified for EOC, but antigenic targets for the immune system exist in the form of tumor-associated antigens [5–10]. Cancer-testis (CT) antigens are normally expressed only in testis, the early developing embryo and placenta. These antigens are also expressed in various tumor types [11]. The presence of these antigens is known to be heterogeneous with no single antigen found to be universally expressed. SeveralCT antigens are expressed in EOC, including NY-ESO-1 [5,12], MAGE-1 and MAGE-3 [10], SPAG9 [13], OY-TES-1 [14], SSX [15] and LAGE-1 [5]. Prior knowledge of shared antigens in melanoma, suggests that tumor antigen sharing between allogeneic tumors of the same type may be a generalizable phenomenon. We hypothesize that EOC from different patients will share tumor-associated antigens. Our objective was to

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evaluate the expression of a panel of 20 CT antigens in EOC tumor specimens, and to determine if antigen sharing occurs between tumors.

## 2. Material and methods

### 2.1. Specimens

Fresh tissue specimens were obtained from patients undergoing cytoreductive surgery for EOC at our institution during 2012–2013. The specimens were collected from the primary tumor site by the primary surgeon. All tissues were collected with informed consent under an approved protocol by the Institutional Review Board (IRB). We also queried the tumor registry to identify patients that underwent surgery for epithelial ovarian cancer in our institution since 2009. All pathology was reviewed in our institution and tumors were classified according to WHO criteria [16]. Paraffin embedded blocks from each selected patient were reviewed by a pathologist to identify the block that had the highest tumor content. This was done in attempts to avoid sampling areas of necrosis, hemorrhage or normal stroma. A coil-slice from the selected paraffin embedded blocks was retrieved for each patient. In addition, frozen EOC specimens and benign ovarian tissues were obtained from the Collaboratory Human Tissue Network (CHTN). The tissues obtained from CHTN corresponded to frozen samples collected from 2007 to 2012 at various institutions. All of the specimens were verified by CHTN using H&E. Samples received were accompanied by the corresponding pathology report. Clinical and pathological characteristics were abstracted from pathology records and retrospective review of clinical charts. For those specimens received from CHTN no clinical data was available for review.

### 2.2. Cell lines

Ovarian cancer cell lines were obtained from American Type Culture Collection(ATCC), Dr. Fiona Simpkins (Sylvester Cancer Center, Miami Florida) and Dr. Johnathan Lancaster (Moffit Cancer Center, Tampa Florida) laboratories. Short tandem repeat (STR) profiling was performed in all cell lines by John's Hopkins DNA Services/FAF. They carried out the profiling following the ANSI/ATCC ASN-0002-2011, Authentication of Human Cell Lines: Standardization of STR Profiling. Only those cell lines that were confirmed to be derived from ovarian cancer cell lines were included in our study.

### 2.3. Real time (RT)-PCR

Total RNA from normal tissues, including normal testis, uterus and ovaries, was obtained commercially from Zyagen and Science cell. Total RNA was isolated from EOC samples, ovarian cancer cell lines and benign specimens, using Quiagen's RNeasy Mini Kit and RNeasy FFPE kit. Quality and concentration of extracted RNA were measured using a 2100 Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). cDNA was made with Quiagen QuantiTect Reverse Trascription kit, using one microgram of total RNA per 20 μl per reaction. Quantitative real time-PCR (qRT-PCR) analysis was performed to determine the expression level of 20 CT antigens. The CT antigens selected as part of the panel evaluated in this study were chosen based on those that either have been previously described as expressed in ovarian cancer and/or have been shown to be immunogenic by prior studies. Also, we chose CT antigens for which a PCR primer sequence has been previously validated. Bio-Rad's iTaq Universal SYBR Green super mix was used to perform the real time-PCR in 96 well-plates. The cDNA corresponding to 10 ng of total RNA was used per 10 μl real time-PCR reaction. Duplicates were performed for all PCR amplifications. After one cycle of 2 min at 95°C, forty cycles of amplification were undertaken, each consisting of 15 s at 95°C and 1 min at 65°C. Data acquisition was done during all the elongation steps at 65°C. The primer sequences used for each CT antigen were obtained from previously published studies (Supplemental Table 1). Control amplification for housekeeping gene 18S was performed in all samples. qRT-PCR efficiency of each sample was compared using BioRad PrimePCR control assays. The following conditions had to be fulfilled before a sample was considered positive for expression of each CT antigen: [1] absence of PCR contamination using water submitted to real-time PCR steps as negative control, [2] Prime PCR control assay Ct < 30, [3] Prime PCR DNA contamination assay with Ct ≥ 35 and [4] only one dissociation peak noted to be similar to that seen with positive control.

Following the amplification, the same threshold was set for analyzing all experiments to compare Ct values derived from different experiments. The mean Ct values from each sample were normalized against the corresponding 18S Ct values, calculated as ($\Delta Ct = Ct_{Experimental\ gene} - Ct_{18S}$). The normalized data from tumor samples were compared with the normalized testicular value ($\Delta\Delta\ Ct = \Delta Ct_{sample} - \Delta Ct_{testis}$), and the level of expression of each CT antigen in the samples was expressed as fold expression relative to testicular expression, calculated as $2^{-\Delta\Delta Ct}$ [17].

### 2.4. Statistical analysis

All statistical analyses were performed with SAS v9.4 and GraphPad Prism software. Associations between categorical groups (i.e. Ct antigen expression and clinico-pathological parameters) were examined by Fisher's exact test. Kruskal-Wallis test was used to evaluate the association between non-categorical groups (i.e. rank sum score of antigen expression level of each antigen) and categorical clinic-pathological parameters. Orthogonal contrasts of one-way ANOVA was used to test the differences of number of shared antigens between different groups. Logistic regression models were fitted to dichotomous antigen expression outcomes: positive or negative for each CT antigen. Odds ratio (OR), its 95% confidence interval (95%CI) along with p-value were calculated. Overall survival (OS) was calculated as the elapsed time between the dates of diagnosis and death or last follow-up for living patients. Progression free survival (PFS) was calculated as the elapsed time between the dates of diagnosis and earliest progression (local recurrence or distant metastasis or death) or last follow-up for patients without recurrence. Two patients with less than one month PFS time were excluded from survival analysis. Unadjusted hazard ratio (HR), its 95%CI along with p-value were calculated from fitting Cox proportional hazard regression models to identify significant predicting antigens of the clinical outcomes such as OS and PFS. Kaplan-Meier [18] survival analyses were used for OS and PFS where median survival and survival rates at 1, 3, and 5 years were calculated respectively. Log-rank test was used to test the differences in survival between the groups. Two-tailed $p \leq 0.05$ was considered statistically significant. Bonferroni method gave the type I error rate for significance of pairwise comparison.

## 3. Results

### 3.1. CT antigen expression in epithelial ovarian cancer (EOC)

CT antigen expression was analyzed in 62 EOC tumors (12 fresh specimens, 21 frozen specimens from CHTN and 29 paraffin embedded tumors), 9 ovarian cancer cell lines (A2780, IGROV-1, OVCAR-5, OVCAR-8, TYNKu-cis-R, TYNKu, SKOV-3, TOV21G, TOV112D) and 3 benign ovarian specimens (2 fresh specimen and 1 frozen specimen from CHTN). The clinico-pathologic characteristics for the samples of EOC are shown in Table 1. Fifty-nine (95%) EOC tumors analyzed expressed at least one of the 20 CT antigens evaluated (Supplemental Fig. 1). The 3 (5%) EOC tumors that did not express any of the 20 CT antigens were paraffin embedded specimens, that passed the quality control, but still had inferior RNA quality compared to other samples. Expression of at least one of the CT antigens was noted in all the cell lines evaluated. The mean number of CT antigens expressed by EOC tumors was 4.3 (0–17) and 6.9 (4–10) for cell lines As shown in Table 2 the most

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