Increased Notch1 Expression Is Associated With Poor Overall Survival in Patients With Ovarian Cancer

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Objective: Despite improvements in surgery and chemotherapy, ovarian cancer remains a deadly disease in need of improved therapies. We have previously shown that Notch1 intracellular domain (NICD) is highly expressed in ovarian cancer. We have also shown that NICD inhibition can lead to growth arrest in ovarian cancer cells. The objective of the current study was to delineate whether NICD expression correlates with prognosis of women with ovarian cancer.

Methods: After the institutional review board approval, patients with a diagnosis of primary ovarian cancer between the years 2001 and 2007 who underwent surgery at our institution were identified. Paraffin blocks from the primary ovarian tumor were analyzed, and core samples were obtained to build a tissue microarray. Cytoplasmic NICD expression was assessed by quantitative immunofluorescent morphometry using the automated quantitative analysis system. These results were correlated with clinical and pathology data retrieved from the patient records.

Results: We identified 328 patients with primary ovarian cancer during this period. Seventeen percent of patients had stage I, 11% had stage II, 59% had stage III, and 13% had stage IV disease. Most patients (70%) had papillary serous histology, and most (86%) underwent optimal debulking to less than 1 cm of residual disease. High NICD expression was found to correlate strongly with low overall survival ($P = 0.001$). This effect remained in multivariate analysis ($P = 0.023$).

Conclusions: High expression of NICD in the primary tumor of women with ovarian cancer is an independently poor prognostic factor for overall survival. Further research into the therapeutic inhibition of the Notch1 pathway is warranted.

Key Words: Ovarian cancer, Survival, Notch receptor

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With an estimated 22,240 new cases in 2013, ovarian cancer is the second most common gynecologic malignancy in the United States, resulting in an estimated 14,030 deaths annually.[1] Due to nonspecific symptoms and lack of reliable biomarkers, the majority of patients present at an advanced stage (60%–70% present at stage III or stage IV).[2] An optimal surgical resection and adjuvant platinum-based chemotherapy are the most important determinants of patient prognosis.[3] However, despite these conventional treatments, 5-year survival is only 44%, and cure rates remain a dismal 15% to 20%.[1] Two years after initiation of treatment, 15% to 40% of patients will have cancer recurrence with an estimated average progression-free survival (PFS) from

20.8 to 28 months.[4,5] It is evident that improved therapies for advanced ovarian cancer are greatly needed.

There are 4 Notch proteins commonly found in mammals. Each one is a large transmembrane protein, which consists of an extracellular domain involved in ligand binding and a transmembrane domain with a nuclear localization sequence. Ligand binding occurs with different transmembrane ligands of the Delta and Jagged families, which then initiates a cleavage event releasing the extracellular domain. This allows the gamma secretase enzyme to cleave the transmembrane domain releasing the active Notch1 intracellular domain (NICD), which is then translocated to the nucleus where it activates the CBF-1 transcription complex.

Notch1 functions as an integral component of a cell signaling pathway and has been found to be an oncogene in a variety of cancer types, including pancreatic, breast, cervical, and renal cancers, as well as some human T-cell acute lymphomas.[6–10] We have previously reported that 3 common ovarian cancer cell lines all express NICD and that NICD was overexpressed in 76% of a small set of primary ovarian tumors samples. In addition, we found that depletion of NICD by siRNA correlated with growth reduction in ovarian cancer cell lines.[11] These findings encouraged us to further investigate the role of Notch1, as this pathway may serve as a therapeutic target in advanced ovarian cancer.

To better understand the role that Notch1 plays in ovarian cancer, we wanted to explore NICD expression in a large set of ovarian tumor samples and correlate expression with clinical and pathologic characteristics. Given our previous findings, we hypothesized that increased expression of NICD would be a poor prognostic factor in patients with ovarian cancer.

MATERIALS AND METHODS

Approval from our institutional review board was obtained. We used our state's cancer registry to identify all the consecutive patients with ovarian cancer who had surgery in our hospital for 7 consecutive years from January 1, 2001 to December 31, 2007. These years were chosen to ensure that adequate data were available and that an adequate follow-up period was obtained.

Using our electronic medical record system and the department of pathology database, we included the following: patients with primary epithelial ovarian cancer who underwent a primary surgical debulking and had adequate pathology slides and ovarian tumor volume for microarray and staining studies; and those who had consistent longitudinal clinical follow-up. The exclusion criteria were patients whose only tissue was from a surgery for recurrent ovarian cancer, patients who had undergone neoadjuvant chemotherapy, patients whose pathology slides had insufficient tumor to sample for the microarray, and those who lacked clinical longitudinal follow-up.

The following clinical data were collected from each patient chart: patient's age and body mass index (BMI), medical comorbidities (diabetes mellitus, hypertension, chronic lung disease, renal or liver disease, or on immunosuppressant medications), ovarian cancer histology (grade and stage) using the FIGO system,[12] operative surgical debulking status (microscopic, ≤1 cm, and ≤2 cm), chemotherapy treatment (type, route, and number of cycles), date of recurrence, and date of last follow-up. Recurrence was defined as either clinical, biochemical (defined as a CA 125 ≥ twice the upper limit of normal on 2 occasions more than 2 weeks apart), or radiologic recurrence. Progression-free survival is defined as the time from surgery to recurrence, whereas overall survival (OS) was defined as the time from surgery to the time of death. All outcomes data were retrospectively reviewed and analyzed with data current up to March 1, 2011.

Tissue Microarray Construction

Using formalin-fixed paraffin-embedded specimens from the pathology archive, tissue microarray (TMA) was constructed using areas of cancer selected from the primary ovarian carcinoma rather than from sites of metastatic disease. Two investigators carefully reviewed each patient's slides, and areas of interest were marked on a representative hematoxylin and eosin stained section. Three cores of 0.6 mm from the corresponding paraffin block were punched out for the TMA. We used a positive control (from parathyroid cancer) and a negative control from normal ovarian tissue.

Automated Quantitative Analysis System Analysis

Staining quality on the tissue TMA was ensured by incorporation and evaluation of positive control cores and a negative control TMA slide. Cores with poor staining quality due to section folding, loss of tissue, or excess trapping of fluorochrome were excluded from analysis. Image acquisition and algorithmic analysis of TMA were carried out using the automated quantitative analysis (AQUA) system (HistoRx, New Haven, CT) as previously described extensively.[13] Briefly, tumor cells within the tissue core were identified by antipancytokeratin antibody (PCK) tagged with Alexa Fluor 555. This PCK antibody was also used to delineate the membrane/cytoplasmic compartment within the mask created through pixel-based locale assignment for compartmentalization of expression (PLACE) algorithm by subtracting out the nontumor background. DAPI (4′,6-diamidino-2-phenylindole) was used to identify the nuclear compartment. To clearly delineate the nuclear and cytoplasmic compartments, 2 images (1 in focus and 1 out of focus) were taken of the compartment-specific tags and the target protein tag. A rapid exponential subtraction algorithm was used to subtract the out-of-focus information in a uniform fashion from the entire microarray. The target protein Notch1 (Abcam ab27526 at 1:200 dilution) was visualized with Alexa Fluor 647, and pixels within the cytoplasmic compartment in the tumor mask were determined via PLACE. The signal was calculated and expressed as the average signal intensity per unit of compartment area expressed on a scale of 0 to 33333 as the AQUA score.

Validation of Target Protein 1 Versus Control

We assessed the ability of TMA-AQUA to quantify the target protein expression in the tumor cells by comparing AQUA scores for the target protein in tumor cells with AQUA scores for the target protein in control cells.

Quantification of Target Protein Expression (NICD) in Subcellular Compartments (Cytoplasm and Nucleus)

The TMA-AQUA has previously been shown to be a good screening tool for diagnostic biomarkers that correlate well with the chromagenic staining and assessments made by pathologists.[14] Expression of the target antigens in the tumor cells, derived from PLACE-based colocalization of target Notch1 fluorescence (Alexa Fluor 647) with DAPI fluorescence, was quantified as an AQUA score. To evaluate the subcellular localization of the target protein in our TMA, we looked at fluorescent staining patterns of the target protein, NICD, in each compartment and found a significantly higher nuclear localization compared with cytoplasmic expression.

Statistical Methods

For exploratory purposes, AQUA NICD expression was categorized in quartiles, and the association with well-known prognostic factors (covariates), such as stage and grade, was examined by Fisher exact test. We distributed AQUA NICD expression into quartiles and deciles of the primary predictor instead of grouping patients according to arbitrary cutoffs, as this approach more objectively explores the data. The Kaplan-Meier (product limit) method was used to estimate the survival function for OS. The Cox proportional hazard model was also performed to examine the association between survival and NICD expression on a continuous scale with and without multiple covariates.

RESULTS

We identified 347 consecutive ovarian cancer patients who met clinical inclusion criteria. Of these patients, 19 were further excluded due to a lack of adequate pathological material on their paraffin blocks, which gave us a total of 328 patients.

Table 1 details the patient characteristics. The mean patient age was 59 years, and the majority of them were white. The mean BMI of patients was 28.5. Most tumors were papillary serous (70%) and grade 3 (80%). At the time of diagnosis, over two thirds of the patients had stage III or IV disease (71.6%). Most patients were optimally debulked (283/328, 86.3%) to either microscopic disease (55.5%) or 1 cm or smaller (30.8%), and most (298/328, 91%) subsequently received adjuvant platinum-based chemotherapy.

Ovarian tumor samples from the 328 patients were shown to have high levels of mean expression of NICD, with a mean AQUA score of 2761 (range, 0–10,717.1). The distribution of AQUA NICD staining scores was divided into quartiles of 82 patients each, with quartile 4 representing the highest NICD expression (Table 2). The distribution of AQUA NICD staining on each quartiles (Table 1, Supplemental Digital Content, http://links.lww.com/IGC/A247) and the association of this staining to different clinically relevant data that are also known prognostic factors in ovarian cancer is seen in Table 3. There was no statistically significant difference in the distribution of NICD staining quartiles with relation to known prognostic factors.

The median longitudinal clinical follow-up in this study was 76.2 months (6.35 years). The range of follow-up for OS in censored patients was 3.15 to 10.21 years. The median overall PFS is 15.5 months (95% confidence interval [CI], 13.8–20.7 months), whereas the median OS is 45.7 months (38.3–54.3 months).

Kaplan-Meier survival curves revealed that patients with the highest NICD expression (quartile 4) had the worst OS ($P = 0.001$; Fig. 1) and PFS ($P = 0.045$; Fig. 2, Supplemental Digital Content, http://links.lww.com/IGC/A249). A multivariate analysis showed that OS was significantly decreased by each of the following: a 500-unit increment of

TABLE 1. Patients' characteristics

Characteristics	Value
Age, y	59.1 (12–85)
Race	
White	306 (93.3%)
Others	22 (6.7%)
BMI	28.5 (15.5–56.2)
Comorbidities (total)	
0	166 (50.6%)
1	129 (39.3%)
2	30 (9.2%)
3	3 (0.9%)
Stage	
I	56 (17.1%)
II	37 (11.2%)
III	192 (58.5%)
IV	43 (13.1%)
Histology	
Papillary serous	231 (70.4%)
Endometrioid	38 (11.6%)
Mucinous	13 (3.9%)
Clear	27 (8.3%)
Others	19 (5.8%)
Pathology grade	
1	37 (11.3%)
2	28 (8.5%)
3	263 (80.2)
Debulking status	
Microscopic	182 (55.5%)
≤1 cm	101 (30.8%)
≤2 cm	7 (2.1%)
>2 cm	38 (11.6%)
Chemotherapy	
No chemo	21 (6.4%)
IV carbo-taxol	272 (82.9%)
IP carbo-taxol	3 (0.9%)
IV platin-based chemo	13 (3.9%)
IP platin-based chemo	10 (3.1%)
Unknown	9 (2.8%)

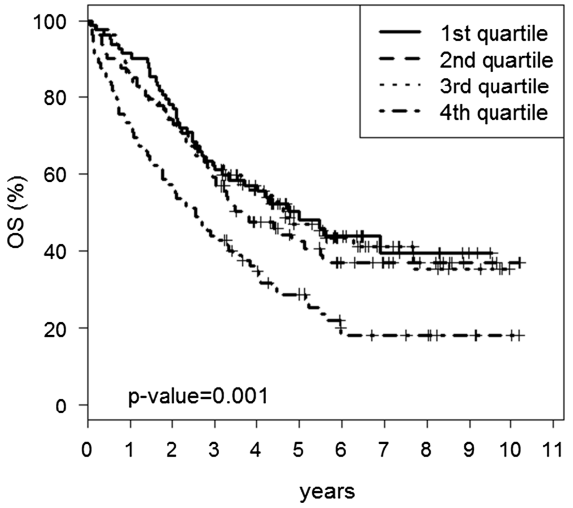
TABLE 2. Quartile grouping of AQUA NICD staining

Quartile	AQUA Staining Score	No. Patients
1	0–1377.4	82
2	1377.5–2328.5	82
3	2328.6–3676.6	82
4	3676.7–10,717.1	82
Mean	2761 (0–10,717.1)	328

NICD expression (this increment was used for its clinically relevant interpretation) (hazards ratio [HR], 1.04 [1.01–1.08]; $P = 0.023$), stage III-IV (HR, 3.79 [2.2–6.54]; $P < 0.001$), grade 3 (HR, 3.25 [1.15–9.15]; $P = 0.026$), and debulking status after surgery. Compared with microscopic debulking status, patients with residual tumors 1 cm or smaller had worse OS, HR of 1.75 (1.26–2.43), and $P < 0.001$, whereas those with residual tumors larger than 1 cm had HR of 2.58 (1.67–3.98) and $P < 0.001$ (Table 4). However, in PFS, the multivariate analysis showed that the NICD expression was not significant (HR, 1.02 [0.98–1.05]; $P = 0.31$) (Table 5) and that only advanced stage III-IV (HR, 6.03 [3.39–10.73]; $P < 0.001$) and debulking status are significant. Compared with microscopic debulking status, patients with residual tumors 1 cm or smaller had worse PFS, HR of 1.75 (1.26–2.43), and $P < 0.001$, whereas those with residual tumors larger than 1 cm had HR of 2.58 (1.67–3.98) and $P < 0.001$.

TABLE 3. Patient characteristics as related to Notch1 quartiles

	Notch1 Quartiles				
Variables	**Q1**	**Q2**	**Q3**	**Q4**	***P***
Stage					0.135
I–II	27	22	28	16	
III–IV	55	60	54	66	
Histology					0.662
Papillary serous	55	60	55	60	
Endometrioid	12	9	13	4	
Mucinous	3	1	5	2	
Clear	6	7	4	7	
Others	5	4	3	7	
Pathology grade					0.408
1	8	9	14	6	
2	7	7	4	10	
3	67	66	64	66	
Debulking status					0.88
Microscopic	46	46	55	33	
≤1 cm	23	25	17	31	
≤2 cm	2	1	3	8	
>2 cm	11	10	7	10	
Chemotherapy					0.134
No chemo	4	4	6	7	
IV	70	74	74	75	
IP	8	4	2	0	



Group (Quartiles of Notch1)	n.	Events	Median OS (months)	95% CI lower	95% CI upper
1 (≤ 1377.40)	82	43	59.89	36.07	NA
2 (≤ 2328.52)	81	48	45.67	34.89	65.94
3 (≤ 3676.65)	82	46	55.49	38.51	92.16
4 (> 3676.65)	82	63	30.77	20.07	41.33

FIGURE 1. OS based on the quartiles of NICD.

DISCUSSION

Our results confirm our hypothesis that patients with ovarian tumors that express high levels of NICD have a worse OS compared with those with lower NICD expression.

TABLE 4. Cox proportional hazard model for OS

Parameter	HR	95% CI	*P*
Univariate analysis			
Notch1	1.06 (per 500 units of Notch increase)	1.02–1.09	0.002
Multivariate analysis			
Notch1	1.04 (per 500 units of Notch increase)	1.01–1.08	0.023
Age, y	1.02	1.0–1.03	0.016
Stage (III–IV vs I–II)	3.79	2.2–6.54	<0.001
Grade			
2 vs 1	2.39	0.76–7.51	0.135
3 vs 1	3.25	1.15–9.15	0.026
Debulking status			
≤1 cm vs microscopic	1.75	1.26–2.43	<0.001
>1 cm vs microscopic	2.58	1.67–3.98	<0.001

TABLE 5. Cox proportional hazard model for PFS

Parameter	HR	95% CI	*P*
Univariate analysis			
Notch1	1.04 (per 500 units of Notch increase)	1.01–1.08	0.25
Multivariate analysis			
Notch1	1.02 (per 500 units of Notch increase)	0.98–1.05	0.31
Age, y	1.01	1.0–1.02	0.186
Stage (III–IV vs I–II)	6.03	3.39–10.73	<0.001
Grade			
2 vs 1	1.46	0.57–3.74	0.434
3 vs 1	2.16	0.97–4.81	0.061
Debulking status			
≤1 cm vs microscopic	1.86	1.35–2.57	<0.001
>1 cm vs microscopic	2.52	1.62–3.92	<0.001

Although Notch1 has been studied in a variety of cancers, its potential role in ovarian cancer has only recently been investigated. Hopfer and colleagues[15] were the first to report on Notch1 in ovarian cancer. While investigating a small number of tumor samples, they did not find differences in the expression of extracellular Notch1, but interestingly, they did find that overexpression of the active NICD conferred a growth advantage to ovarian carcinoma cells. Piqued by this finding, Rose et al[11] investigated the expression of NICD in a small subset of ovarian tumor samples and found that NICD expression was found in 16 (76%) of 21 primary ovarian tumors. More recently, Oktem and colleagues[16] found that increased immunoexpression of Notch1 was associated with metastasis in serous ovarian carcinoma. Our finding that NICD expression is associated with poor OS for patients with epithelial ovarian cancer is consistent with these previous results. These findings represent the first clinical correlation of NICD expression with poor prognosis and establish NICD as an independent poor prognostic factor in epithelial ovarian cancer.

Given the association with worsened OS, the Notch1 pathway is of potential interest for development of targeted therapies. Our group has previously shown that depletion of NICD with siRNA leads to growth reduction in cell lines that express NICD.[11] In addition, we have shown that novel compounds can reduce NICD expression and inhibit ovarian cancer cell growth. Xanthohumol, an abundant chalcone found in the hop plant, was found to be a potent inhibitor of ovarian cancer cell growth while down-regulating Notch1 transcription.[17] A recent study by Kim et al[18] showed that the use of xanthohumol inhibited the invasion of triple negative breast cancer cells. Recently, Groeneweg et al[19] showed that inhibition of notch signaling in combination with paclitaxel reduced platinum-resistant ovarian tumor growth. Compounds such as xanthohumol, paclitaxel, and others that target the Notch1 pathway should be further investigated as a potential treatment in epithelial ovarian cancer.

Our study has many strengths, including the large sample size, the central treatment facility with consecutive cases collected, the long period of follow-up with comprehensive data collection, and a dedicated research facility for construction and testing of TMA. Representative images of ovarian carcinomas for each quartile of Notch1 expression as quantitated by AQUA is seen in Figure 3 (Supplemental Digital Content, http://links.lww.com/IGC/A248).

However, there are limitations that should be noted. First, this is a retrospective study that includes patients over a time frame in which chemotherapy treatments, especially the second, third, and fourth line, may have changed. Although we did not see any association in our quartiles to chemotherapy type, there were very few patients who received IP chemotherapy, and analysis of chemotherapy for recurrence was not completed. However, the majority of patients received IV chemotherapy in a timeframe when carboplatin (area under the curve, 6) and paclitaxel (175 mg/m^2) every 21 days were the standard of care, making this a relatively homogeneous group. Second, heterogeneity in the expression of NICD across tumor volumes may have skewed results, as it is possible that we may not have adequately sampled the tumor in our cellblocks. However, we tried to prevent this by having a gynecologic oncologist and a pathologist review the tumor slides and ensure that the area of highest tumor concentration correlated with the area of the paraffin block that was sampled. Furthermore, previous studies in lymphoma suggest that duplicate cores do not provide significant advantages of single-core TMAs.[14] In addition, by combining TMA and quantitative immunofluorescence technologies, we have effectively minimized the contribution of any nonspecific NICD staining, which can skew immunohistochemical studies.

Due to the unique characteristics of each histologic type of ovarian cancer, subsequent analysis according to histology and debulking status should be considered to extend the current analysis.

In conclusion, our results confirm ovarian tumor NICD expression as an independent poor prognostic factor in patients with epithelial ovarian cancer. Further studies targeting inhibition of the Notch1 signaling pathway should be pursued as an added adjuvant therapy with the current standard of care.

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