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Evidence for a time-dependent association between FOLR1 expression and survival from ovarian carcinoma: implications for clinical testing. An Ovarian Tumour Tissue Analysis consortium study

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Background: Folate receptor 1 (FOLR1) is expressed in the majority of ovarian carcinomas (OvCa), making it an attractive target for therapy. However, clinical trials testing anti-FOLR1 therapies in OvCa show mixed results and require better understanding of the prognostic relevance of FOLR1 expression. We conducted a large study evaluating FOLR1 expression with survival in different histological types of OvCa.

Methods: Tissue microarrays composed of tumour samples from 2801 patients in the Ovarian Tumour Tissue Analysis (OTTA) consortium were assessed for FOLR1 expression by centralised immunohistochemistry. We estimated associations for overall (OS) and progression-free (PFS) survival using adjusted Cox regression models. High-grade serous ovarian carcinomas (HGSC) from The Cancer Genome Atlas (TCGA) were evaluated independently for association between *FOLR1* mRNA upregulation and survival.

Results: FOLR1 expression ranged from 76% in HGSC to 11% in mucinous carcinomas in OTTA. For HGSC, the association between FOLR1 expression and OS changed significantly during the years following diagnosis in OTTA ($P_{interaction} = 0.01$, $N = 1422$) and TCGA ($P_{interaction} = 0.01$, $N = 485$). In OTTA, particularly for FIGO stage I/II tumours, patients with FOLR1-positive HGSC showed increased OS during the first 2 years only (hazard ratio = 0.44, 95% confidence interval = 0.20–0.96) and patients with FOLR1-positive clear cell carcinomas (CCC) showed decreased PFS independent of follow-up time (HR = 1.89, 95% CI = 1.10–3.25, $N = 259$). In TCGA, *FOLR1* mRNA upregulation in HGSC was also associated with increased OS during the first 2 years following diagnosis irrespective of tumour stage (HR: 0.48, 95% CI: 0.25–0.94).

Conclusions: FOLR1-positive HGSC tumours were associated with an increased OS in the first 2 years following diagnosis. Patients with FOLR1-negative, poor prognosis HGSC would be unlikely to benefit from anti-FOLR1 therapies. In contrast, a decreased PFS interval was observed for FOLR1-positive CCC. The clinical efficacy of FOLR1-targeted interventions should therefore be evaluated according to histology, stage and time following diagnosis.

Folate receptor 1 (FOLR1) is a member of the folate receptor family that has restricted expression in normal epithelial cells, but is reported to be highly expressed in various tumours of epithelial origin including the majority of ovarian carcinomas (Kelemen *et al*, 2005; Parker *et al*, 2005; Chen *et al*, 2012; Crane *et al*, 2012; Despierre *et al*, 2013; O'Shannessy *et al*, 2013). FOLR1 has high affinity for binding folic acid (Parker *et al*, 2005). Because folate is essential for DNA synthesis and one-carbon transfer, it has been hypothesised that FOLR1 might confer a growth advantage to the tumour by modulating folate uptake (Kane *et al*, 1988) or generating regulatory signals (Miotti *et al*, 2000). However, others showed that FOLR1 was not the major transport route for intracellular accumulation of physiological folates or anti-folates in various cell lines including the IGROV-1 ovarian cancer cells (Kamen and Smith, 2012), thereby disputing the theory of FOLR1-medicated tumour uptake of folate for proliferation.

Studies evaluating the prognostic value of FOLR1 expression in ovarian carcinoma have produced conflicting results. Chen *et al* (2012) reported that *FOLR1* mRNA upregulation was an unfavourable prognostic marker in a study of 91 serous ovarian carcinomas. In two studies that used immunohistochemistry (IHC), women with FOLR1 expressing ovarian carcinomas had no difference in survival rate: RR 0.86, 95% CI 0.57–1.31, $P = 0.49$, $N = 186$ (Kalli *et al*, 2008) and RR 0.80, 95% CI 0.5–1.1, $P = 0.2$, $N = 361$ (Crane *et al*, 2012). These two studies contained a mixture of all histological types, which may mask associations when both the marker prevalence (Kalli *et al*, 2008) and survival rates (Kobel *et al*, 2008) differ between the histological types.

The prognostic significance of FOLR1 may have therapeutic relevance. FOLR1 selectivity, affinity and restricted tissue distribution has christened it as a universal target for exploitation (van Dam *et al*, 2011) for therapy or imaging of solid tumours (Leamon and Reddy, 2004) or circulating tumour cells (He *et al*, 2008). Different treatment modalities have been proposed including folic acid-conjugated toxins that bind FOLR1 and release the toxin upon internalisation to kill tumour cells (Kelemen, 2006), and anti-tumour immune-based therapies that enhance the body's natural immunity against tumour cells (Knutson *et al*, 2006). However, clinical trials testing therapies against FOLR1 in ovarian carcinoma have produced mixed results. For example, farletuzumab (Konner *et al*, 2010; Spannuth *et al*, 2010; Armstrong *et al*, 2013) is a humanised monoclonal antibody against FOLR1, which was shown to mediate antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity and to inhibit cell growth of a Chinese hamster ovary cell line transfected with *FOLR1* when grown in low-folate medium (Ebel *et al*, 2007), although subsequent studies showed it minimally altered cell growth in cell lines naturally expressing FOLR1, including IGROV-1 ovarian cancer cells (Kamen and Smith, 2012). The results from a phase III clinical trial (NCT00849667) found that farletuzumab in combination with carboplatin and taxane did not meet the study's primary end point of progression-free survival (PFS), and analyses are ongoing in patient subsets (Walters *et al*, 2013). More recently, a phase II clinical trial reported longer median PFS among 100 selected patients with recurrent platinum-resistant ovarian carcinoma who were treated with a combination of vintafolide, a folic acid–desacetylvinblastine conjugate that binds to the folate receptor, and pegylated liposomal doxorubicin (PLD) compared with 49 patients who received PLD alone (5.0 *vs* 2.7 months PFS, respectively; $P = 0.03$) (Naumann *et al*, 2013). The drug was particularly effective in patients diffusely positive for FOLR1. No difference in overall survival (OS) was detected, although the trial was not powered to detect a statistical difference (Naumann *et al*, 2013).

To address discrepant findings and to improve upon previous study designs, we evaluated tumours from 2801 patients from the Ovarian Tumour Tissue Analysis (OTTA) consortium to assess the prevalence of FOLR1 protein expression and to associate prevalence with survival for the different histological types separately. The goal of the OTTA consortium is to combine tissue microarray resources to overcome the limitations of prognostic studies associated with small sample sizes. We also utilised data from The Cancer Genome Atlas (TCGA) as a large, independent sample to evaluate these associations.

MATERIALS AND METHODS

Patients. Twelve individual studies comprising 2801 patients participating in the OTTA consortium were included for FOLR1 IHC expression analysis. Several of these studies also participated in previous OTTA IHC analyses (Kobel *et al*, 2013; Sieh *et al*, 2013) and were assessed using a biomarker-assisted review of patients' slides for histological classification (Kobel *et al*, 2013). Informed consent was obtained from patients in seven of 12 studies. For the Alberta Ovarian Tumor Types study (AOV), Calgary Serous Carcinoma study (CAL), Nottingham Ovarian Cancer study (NOT), Toronto Ovarian Cancer study (TOC) and British Columbia Cancer Agency study (VAN), the institutional research ethics boards waived the need to obtain consent and all local human research investigations committees approved each study. Key demographical and clinical data on the patients were merged into one common data set that was checked for consistency and completeness, and discrepancies were addressed with individual study investigators.

Immunohistochemistry (IHC). Centralised IHC was performed on a Leica Bond Max platform (Leica Microsystems, Wetzlar, Germany). The submitted glass slides from tissue microarrays were subjected to heat-induced antigen retrieval for 20 min using the Leica Epitope Retrieval Solution 2. Slides were incubated with the FOLR1 mouse monoclonal antibody from Novocastra (Leica Microsystems) clone BN3.2 (catalogue #NCL-F-FRalpha) for 15 min at a 1 : 50 dilution.

Scoring. Two observers (MK, JM) scored the FOLR1 staining. We combined a measure of the intensity, extent and subcellular localisation into a five-tier scoring system: absent or weak staining, strong staining of 1–50% of tumour cells irrespective of subcellular localisation, strong staining of >50% of tumour cells with membranous localisation (with no penalisation for accompanying weak cytoplasmic staining), strong staining of 50–95% of tumour cells with cytoplasmic staining (and membranous staining still present) and strong staining of >95% of tumour cells with cytoplasmic staining (Figure 1). The inter-observer reproducibility of the five-tier IHC scoring system between the two observers was evaluated in a subset of 183 patients and was high (weighted Cohen's kappa = 0.91). We also compared the five-tier IHC scores for FOLR1 obtained using the BN3.2 antibody with *FOLR1* mRNA expression data derived by RNA sequencing from a subset of 36 patients (Shah *et al*, 2009). The IHC scores correlated reasonably well with the mRNA expression values (Pearson $r = 0.77$).

The Cancer Genome Atlas (TCGA). We downloaded publicly-available data from 563 serous ovarian carcinomas from TCGA (The Cancer Genome Atlas Research Network, 2011) using the cBIOPortal for Cancer Genomics (Cerami *et al*, 2012) to assess prevalence of *FOLR1* mRNA expression in tumour relative to normal tissue (fallopian tube) and to evaluate expression with survival outcomes as described below. Gene expression in TCGA was evaluated using the Agilent 244K Custom Gene Expression G4502A_07 (Santa Clara, CA, USA) assayed at the University of North Carolina and expressed as fold change between tumour and normal tissue on the log2 scale (The Cancer Genome Atlas Research Network, 2011).

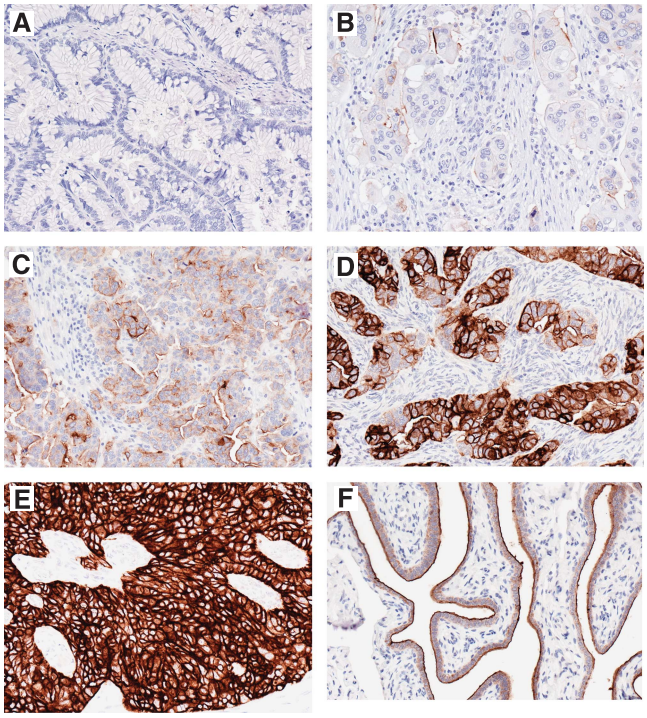

Figure 1. Immunohistochemical staining of FOLR1. (**A**) No staining in a mucinous carcinoma. (**B**) Strong staining of 1–50% of tumour cells in a high-grade serous carcinoma. (**C**) Strong staining of >50% of tumour cells with predominant membranous localisation in a high-grade serous carcinoma. (**D**) Strong staining of 50–90% of tumour cells with cytoplasmic staining (and membranous staining still present) in a high-grade serous carcinoma. (**E**) Strong staining of >90% of tumour cells with cytoplasmic staining in a high-grade serous carcinoma. (**F**) Strong staining of >50% of epithelial cells with predominant membranous localisation in normal fallopian tube.

Statistical analysis. We assessed 2801 patients from the OTTA consortium for prevalence of FOLR1 expression and included the five main histological types: high-grade serous carcinoma (HGSC), low-grade serous carcinoma (LGSC), mucinous carcinoma (MC), endometrioid carcinoma (EC) and clear cell carcinoma (CCC). Patients with absent or weak FOLR1 staining were recorded as negative, whereas all the other FOLR1 staining patterns were considered positive results. In separate analyses using the larger sample size of HGSC in OTTA, we visually inspected Kaplan–Meier survival curves and evaluated statistical associations between patients using the five-tier scoring system for FOLR1 and also with different combinations of scores (e.g., strong staining of >50% of tumour cells with cytoplasmic or membranous staining *vs* all other staining patterns) stratified by the FIGO (International Federation of Obstetricians and Gynaecologists) stage. None of these alternate combinations produced associations that were materially different from the positive/negative staining comparison (data not shown).

The Cox proportional hazard model was used to estimate hazard ratios (HRs) and 95% confidence intervals (CIs) between positive/negative categories of FOLR1 and OS or PFS stratified by histological type. In the OTTA consortium, OS was defined as death from any cause after ovarian carcinoma diagnosis and PFS was defined as survival without disease progression or recurrence determined by radiological, serological or clinical evidence or death from any cause, whichever came first. Stage at diagnosis was determined using the cancer registry and/or FIGO stage information from each site (SEER guidelines: http://seer.cancer.gov/) and categorised as FIGO stage I/II (localised and regional) and FIGO stage III/IV (distant). Because time from diagnosis to study entry was variable, we allowed for left truncation with time at risk starting on the date of diagnosis and time under observation beginning at the time of study entry. Analyses were right censored at 5 years after ovarian carcinoma diagnosis in order to reduce the number of non-ovarian carcinoma-related deaths. The proportional hazard assumption was evaluated with standard diagnostic methods, including likelihood ratio tests comparing models with and without terms that modelled covariates as a function of follow-up time on the natural logarithmic scale (Hosmer and Lameshow, 1999). Final models were fitted using Cox regression stratified by study to correct for violations of the proportional hazard assumption and adjusted for potential confounding with age at diagnosis (<70 or ⩾70 years), FIGO tumour stage (I/II, III/IV or missing) and presence of residual disease at primary surgery (macroscopic, no macroscopic or missing). An interaction term between the aforementioned covariates and follow-up time was included, where necessary, to improve model fit. In addition, we considered models separately by the FIGO tumour stage. Data for regression analyses were available for 2636 patients following the exclusion of patients with missing vital status ($N=13$), missing time from diagnosis to study entry ($N=66$), missing follow-up time ($N=43$), missing FIGO stage ($N=84$, excluded for stage-stratified analyses only) and patients with study entry >5 years from diagnosis ($N=43$).

Using TCGA data, *FOLR1* mRNA information was available for 485 patients and we defined mRNA upregulation as >1 s.d. from the mean on the log2 scale. Associations with OS and PFS were evaluated with follow-up time as described above and adjusted for the FIGO tumour stage (I/II, III/IV or missing) and presence of residual disease at primary surgery (macroscopic, no macroscopic or missing). Separate models also adjusted for platinum therapy sensitivity (resistant, sensitive, too early or missing) and treatment response (complete response, partial response, progressive disease, stable disease or missing). Age at diagnosis was not available in TCGA.

Statistical tests were two sided and were implemented with SAS (version 9.1, SAS Institute, Cary, NC, USA) and Stata/SE (version 13.1, StataCorp, College Station, TX, USA). The study followed the REMARK guidelines (McShane *et al*, 2005).

RESULTS

Study and patient characteristics. Table 1 describes the participating studies and methods of patient ascertainment and histological review. Table 2 lists the 2801 women with ovarian carcinoma that were included in the FOLR1 expression prevalence analysis, stratified by study and histological type. As expected, women with HGSC were older, were diagnosed with high tumour stage and were more likely to have macroscopic residual disease and shorter OS and PFS compared with women with EC (Table 3). Women with EC had the best prognosis.

FOLR1 expression across histological types. We observed differences in FOLR1 expression by histological type: patients with tumours of LGSC, MC, EC or CCC histology were more likely to have absent or weak FOLR1 staining compared with patients with HGSC tumours (Table 3). FOLR1-positive expression was seen in 1149 out of 1507 patients (76.2%) with HGSC, 45 out of 91 patients (49.5%) with LGSC and 141 out of 446 patients (31.6%) with CCC. Normal fallopian tube tissue was included as a control on the tissue microarrays and was FOLR1-positive in 8 out of 8 samples (100%) (Figure 1).

Association between FOLR1 expression in HGSC tumours and survival. In OTTA, there were no statistically significant associations with OS or PFS for HGSC overall or when evaluated separately by the FIGO stage; however, the association between FOLR1 expression and OS changed when stratified by follow-up

Table 1. Description of 12 participating studies in OTTA

Study	Study abbreviation	Study location	Recruitment period	Ascertainment of patients and clinical data	Pathology data and review
Australian Ovarian Cancer Study (Merritt *et al*, 2008)	AOC	Australia	2002–2006	Treatment centers throughout Australia; cancer registries serving Queensland, South and Western Australia; longitudinal follow-up through regular review of medical records	Pathology reports and diagnostic slides reviewed by a panel of gynaecologic pathologists
Alberta Ovarian Tumor Types Study (Kelemen *et al*, 2013)	AOV	Alberta, Canada	1998–2009	Population-based Alberta Cancer Registry. Annual updates are performed for vital statistics	Pathology reports and histological slides reviewed by gynaecologic pathologist
Bavarian Ovarian Cancer Study (Hein *et al*, 2013)	BAV	Southeast Germany	2002–2006	Gynecologic Oncology Center at the Comprehensive Cancer Center Erlangen-Nuremberg	Centralised review of pathology reports and histological slides for all cases by study pathologists
Calgary Serous Carcinoma Study (Bromley *et al*, 2012)	CAL	Calgary, Canada	2003–2007	Hospital-based retrospective observational study	Histological review of all slides by study pathologist supported by centralised biomarker analysis
Novel risk factors and potential early detection markers for the Ovarian Cancer Study (Lo-Ciganic *et al*, 2012)	HOP	Western PA, Northeastern Ohio, Western NY, USA	2003–2009	Hospital registries and active surveillance of medical practices in three catchment areas	Medical chart review for all cases
Malignant Ovarian Cancer Study (Glud *et al*, 2004; Soegaard *et al*, 2007)	MAL	Denmark	1994–1999	Gynecological departments in Copenhagen, Frederiksberg and seven surrounding counties	Pathology reports reviewed for all cases and histological slides reviewed for 30% by gynaecologic pathologist
Mayo Clinic Ovarian Cancer Study (Goode *et al*, 2011)	MAY + MAC	Northcentral USA	2000–2009	Mayo Clinic medical records and State death certificates	Review by Mayo Clinic gynaecologic pathologists supported by centralised biomarker analysis
Nottingham Study (Williams *et al*, 2012)	NOT	UK	1991–2008	Hospital records and Trent cancer registry	Pathology reports reviewed by gynaecologic pathologist
Study of Epidemiology and Risk Factors in Cancer Heredity (Song *et al*, 2006)	SEA	East Anglia and West Midlands, UK	1998–2008	East Anglia and West Midlands Cancer Registry	Centralised review of pathology reports by a pathologist
Toronto Ovarian Cancer Study (Narod *et al*, 1998)	TOC	Ontario, Canada	1995–2003	Ontario Cancer Registry	Pathology reports and histological slides reviewed by a study pathologist
United Kingdom Ovarian Cancer Population Study (Balogun *et al*, 2011)	UKO	England, Wales and Northern Ireland, UK	2006–2010	10 major Gynecologic Oncology NHS centers in England, Wales and Northern Ireland; cancer registries; NHS Information Centre for Health and Social Care (England and Wales) and Central Services Agency (Northern Ireland)	Centralised review of pathology reports by a gynaecologic oncologist
Vancouver Ovarian Cancer Study (Prentice *et al*, 2007; Kobel *et al*, 2010b)	VAN	British Columbia, Canada	1984–2000	Ovarian Cancer Registry serving British Columbia, and the Cheryl Brown Outcomes unit	Histological review of all slides by University of British Columbia pathologists supported by centralised biomarker analysis

Abbreviation: OTTA = ovarian tumour tissue analysis.

period (P interaction with time = 0.01) (Table 4). FOLR1 expression was associated with a 29% non-significant increased OS ($P = 0.08$) during the first year following diagnosis and a 50% non-significant decreased OS during the fourth year. The pattern of changing survival with increasing follow-up time, however, was most evident among patients with FIGO stage I/II tumours (P interaction with time = 0.02). Among these patients, FOLR1 expression was associated with a 56% significant increased OS during the first 2 years of follow-up only (HR: 0.44, 95% CI: 0.20–0.96, $P = 0.04$). An interaction between FOLR1 expression and follow-up time was not observed with PFS.

Among 485 patients with HGSC in TCGA, *FOLR1* mRNA upregulation was present in 93 patients (19%). The associations between *FOLR1* mRNA upregulation and survival in TCGA were similar to those observed in the OTTA data for HGSC. A significant interaction with follow-up time was seen for OS (P interaction with time = 0.02) (Table 5). Following additional adjustment for platinum sensitivity and treatment response, *FOLR1* mRNA upregulation was associated with a 52% significant increased OS during the first 2 years of follow-up only (HR: 0.48, 95% CI: 0.25–0.94, $P = 0.03$) (Table 5). Although most of these patients had FIGO stage III/IV tumours, the association did not strengthen when restricted to advanced stage tumours ($P = 0.05$). A non-significant interaction between *FOLR1* mRNA upregulation and follow-up time was observed for PFS.

Association between FOLR1 expression in non-HGSC tumours and survival in OTTA. No significant interactions were found between FOLR1 expression and follow-up time for the other ovarian histological types. When stratified by stage, a non-significant decreased OS interval was observed among patients with CCC and FIGO stage I/II (HR: 1.62, 95% CI: 0.97–2.71,

Table 2. Number of patients with invasive ovarian carcinoma across studies and by histological type in OTTA

Study abbreviation	Total patients $N=2801$	HGSC $N=1507$	LGSC $N=91$	MC $N=193$	EC $N=564$	CCC $N=446$
AOC	89	89	0	0	0	0
AOV	209	0	0	18	95	96
BAV	214	133	19	19	26	17
CAL	68	63	5	0	0	0
HOP	34	25	1	0	7	1
MAL	245	117	12	21	66	29
MAY	460	337	17	14	63	29
NOT	196	106	9	16	39	26
SEA	364	164	8	42	92	58
TOC	160	50	0	20	45	45
UKO	102	68	7	5	7	15
VAN	660	355	13	38	124	130

Abbreviations: CCC = clear cell carcinoma; EC = endometrioid carcinoma; HGSC = high-grade serous carcinoma; LGSC = low-grade serous carcinoma; MC = mucinous carcinoma; OTTA = ovarian tumour tissue analysis.

Table 3. Characteristics of patients with invasive ovarian carcinoma by histological type in OTTA

Characteristic	HGSC $N=1507$	LGSC $N=91$	MC $N=193$	EC $N=564$	CCC $N=446$
Age at diagnosis, mean ± s.d.	61.1 ± 11.2	55.3 ± 13.5	55.5 ± 13.9	56.1 ± 11.8	57.4 ± 11.5
Total years followed[a,b], mean ± s.d.	4.1 ± 3.2	5.8 ± 4.1	5.9 ± 4.5	6.9 ± 4.4	5.9 ± 4.6
Overall time to death[b], years, mean ± s.d.	3.2 ± 2.3	4.3 ± 3.1	2.6 ± 2.5	5.0 ± 3.9	3.5 ± 3.2
Vital status[b], *N* (%)					
Alive	421 (29.6)	41 (48.2)	114 (64.0)	371 (70.5)	239 (56.2)
Died	1001 (70.4)	44 (51.8)	64 (36.0)	155 (29.5)	186 (43.8)
Overall time to progression[a,b], years, mean ± s.d.	2.5 ± 2.7	3.6 ± 4.1	4.8 ± 4.2	5.9 ± 4.2	4.8 ± 4.5
Progression status[b], *N* (%)					
Progression-free	344 (30.0)	31 (44.9)	88 (71.5)	284 (75.3)	201 (58.9)
Progressed	804 (70.0)	38 (55.1)	35 (28.4)	93 (24.7)	140 (41.0)
Stage[b], *N* (%)					
FIGO IA, IB, IC, II (localised)	379 (26.6)	28 (32.9)	130 (73.0)	422 (80.2)	328 (77.2)
FIGO III, IV (distant)	1030 (72.4)	58 (65.9)	34 (19.1)	88 (16.7)	82 (19.3)
Unknown	13 (0.9)	1 (1.1)	14 (7.9)	16 (3.0)	15 (3.5)
Macroscopic residual disease[b], *N* (%)					
No	447 (31.4)	43 (50.6)	91 (51.1)	327 (62.2)	254 (59.8)
Yes	634 (45.6)	24 (28.2)	29 (16.3)	49 (9.3)	45 (10.6)
Unknown	341 (24.0)	18 (21.2)	58 (32.6)	150 (28.5)	126 (29.6)
FOLR1 expression, *N* (%)					
Absent/weak	358 (23.8)	46 (51.0)	171 (88.6)	398 (70.6)	305 (68.3)
Strong 1–50%	371 (24.6)	20 (22.0)	12 (6.2)	100 (17.7)	88 (19.7)
Strong membranous >50%	282 (18.7)	15 (16.5)	6 (3.1)	44 (7.8)	29 (6.5)
Strong cytoplasmic 50–95%	367 (24.4)	8 (8.8)	2 (1.0)	17 (3.0)	21 (4.7)
Strong cytoplasmic >95%	129 (8.6)	2 (2.2)	2 (1.0)	5 (0.9)	3 (0.7)

Abbreviations: CCC = clear cell carcinoma; EC = endometrioid carcinoma; FOLR1 = folate receptor 1; HGSC = high-grade serous carcinoma; LGSC = low-grade serous carcinoma; MC = mucinous carcinoma; OS = overall survival; OTTA = ovarian tumour tissue analysis; PFS = progression-free survival.
[a]Mean years follow-up among cases regardless of vital status or progression status.
[b]Smaller sample size is based on availability of OS and PFS information from patients (1422 HGSC, 85 LGSC, 178 MC, 526 EC and 425 CCC).

$P=0.07$) (Table 6), whereas a 57% significant decreased PFS interval was seen among patients with CCC overall (HR: 1.57, 95% CI: 1.06–2.34, $P=0.02$), which was more pronounced among patients with FIGO stage I/II tumours (HR: 1.89, 95% CI: 1.10–3.25, $P=0.02$) (Table 7). No other associations were found.

DISCUSSION

Our investigation showed that the association between FOLR1 expression or *FOLR1* mRNA upregulation among ~1900 patients with HGSC from two independent data sets changed significantly during the years following diagnosis. In OTTA, this translated to an increase in OS by 56% among women with FOLR1-positive HGSC in the first 2 years of follow-up only and a decrease in PFS by 89% among women with FOLR1-positive CCC irrespective of follow-up time. Both findings were more pronounced among patients with FIGO stage I/II tumours.

Both our OTTA data and those we analysed from TCGA provide evidence, for the first time, that the association between FOLR1 expression and OS in HGSC changes with the duration of follow-up regardless of whether FOLR1 was measured as protein or mRNA. Previous studies reported either unfavourable prognosis

Table 4. Associations[a] between FOLR1 expression[b] and OS and PFS for 1422 high-grade serous ovarian carcinomas in OTTA

Time period	Died, *N*	Overall survival HR (95% CI)	*P*-value	Progressed, *N*	Progression-free survival HR (95% CI)	*P*-value
No stratification by follow-up	825	0.99 (0.84–1.18)	0.95	732	0.99 (0.81–1.19)	0.88
Five-period follow-up						
0–1-year follow-up	134	0.71 (0.48–1.05)	0.08		—	—
1–2-year follow-up	208	0.90 (0.65–1.26)	0.55		—	—
2–3-year follow-up	207	1.23 (0.86–1.74)	0.26		—	—
3–4-year follow-up	174	0.93 (0.64–1.34)	0.69		—	—
4–5-year follow-up	102	1.50 (0.87–2.58)	0.15		—	—
P interaction with time		0.01			0.84	
Three-period follow-up						
0–1-year follow-up	134	0.71 (0.48–1.05)	0.08		—	—
1–4-year follow-up	589	1.01 (0.82–1.24)	0.91		—	—
4–5-year follow-up	102	1.50 (0.87–2.58)	0.15		—	—
Stratified by stage						
FIGO stage I/II						
No stratification by follow-up	126	1.07 (0.72–1.58)	0.74	88	1.04 (0.66–1.65)	0.84
Three-period follow-up						
0–2-year follow-up	29	0.44 (0.20–0.96)	0.04		–	–
2–3-year follow-up	35	1.25 (0.58–2.69)	0.57		–	–
3–5-year follow-up	62	1.62 (0.88–2.96)	0.12		–	–
P interaction with time		0.02			0.70	
FIGO stage III/IV						
No stratification by follow-up	692	1.00 (0.83–1.21)	0.97	643	1.01 (0.82–1.23)	0.95
P interaction with time		0.21			0.95	

Abbreviations: CI = confidence interval; HR = hazard ratio; OS = overall survival; PFS = progression-free survival.
[a]HR and 95% CI estimated using Cox regression stratified by study and adjusted for age at diagnosis, residual disease (not macroscopic, macroscopic or missing) and FIGO stage (I/II, III/IV or missing). Models that stratified by stage did not include stage as a confounder.
[b]Positive *vs* negative staining, where negative is absent or weak staining and positive is all other stains.

Table 5. Associations between *FOLR1* mRNA upregulation and OS and PFS for 485 serous ovarian carcinomas in TCGA

Time period	Died, *N*	Overall survival[a] HR (95% CI)	*P*-value	Overall survival[b] HR (95% CI)	*P*-value	Progressed, *N*	Progression-free survival[a] HR (95% CI)	*P*-value
No stratification by follow-up	238	0.78 (0.55–1.10)	0.15	0.84 (0.60–1.19)	0.34	280	1.08 (0.81–1.45)	0.59
Five-period follow-up								
0–1-year follow-up	42	0.31 (0.10–1.01)	0.05	0.42 (0.13–1.38)	0.15		—	—
1–2-year follow-up	53	0.57 (0.26–1.27)	0.17	0.54 (0.24–1.22)	0.14		—	—
2–3-year follow-up	54	1.26 (0.68–2.36)	0.74	1.25 (0.65–2.40)	0.50		—	—
3–4-year follow-up	53	0.96 (0.48–1.91)	0.91	1.06 (0.52–2.15)	0.87		—	—
4–5-year follow-up	70	0.81 (0.45–1.46)	0.48	0.99 (0.42–2.34)	0.97		—	—
P interaction with time		0.02		0.01			0.33	
Three-period follow-up								
0–2-year follow-up	95	0.45 (0.23–0.87)	0.02	0.48 (0.25–0.94)	0.03		—	—
2–3-year follow-up	54	1.26 (0.68–2.36)	0.74	1.25 (0.65–2.40)	0.50		—	—
3–5-year follow-up	123	0.87 (0.56–1.37)	0.56	1.01 (0.59–1.73)	0.98		—	—
Stratified by stage								
FIGO stage I/II								
No stratification by follow-up	6	0.83 (0.08–8.29)	0.87	0.83 (0.08–8.29)	0.87	14	0.65 (0.17–2.48)	0.53
P interaction with time		0.12		0.12			0.07	
FIGO stage III/IV								
No stratification by follow-up	230	0.78 (0.56–1.11)	0.17	0.85 (0.60–1.21)	0.37	266	1.11 (0.83–1.50)	0.48
Three-period follow-up								
0–2-year follow-up	93	0.47 (0.24–0.91)	0.02	0.52 (0.27–1.01)	0.05		—	—
2–3-year follow-up	53	1.29 (0.69–2.42)	0.42	1.28 (0.67–2.45)	0.46		—	—
3–5-year follow-up	84	0.87 (0.50–1.50)	0.61	0.96 (0.55–1.67)	0.88		—	—
P interaction with time		0.03		0.02			0.51	

Abbreviations: CI = confidence interval; HR = hazard ratio; mRNA = messenger RNA; OS = overall survival; PFS = progression-free survival; TCGA = The Cancer Genome Atlas.
[a]HR and 95% CI estimated using Cox regression adjusted for residual disease (not macroscopic, macroscopic or missing) and FIGO stage (I/II, III/IV or missing). Models that stratified by stage did not include stage as a confounder.
[b]All models except FIGO stage I/II model additionally adjusted for platinum sensitivity (resistant, sensitive, too early or missing) and treatment response (complete response, partial response, progressive disease, stable disease or missing).

Table 6. Associations[a] between FOLR1 expression and OS by ovarian carcinoma histological type in OTTA

	LGSC	MC	EC	CCC
	HR (95% CI)	HR (95% CI)	HR (95% CI)	HR (95% CI)
Alive/died, *N*	54/31	125/53	435/91	281/143
FOLR1 expression				
Negative[b]	1.0 (ref)	1.0 (ref)	1.0 (ref)	1.0 (ref)
Positive[b]	1.31 (0.56–3.07)	0.74 (0.26–2.12)	0.93 (0.57–1.51)	1.15 (0.80–1.64)
P-value	0.53	0.58	0.77	0.45
Stratified by stage				
FIGO stage I/II				
Alive/died, *N*	26/2	107/23	379/43	254/73
Negative[b]	1.0 (ref)	1.0 (ref)	1.0 (ref)	1.0 (ref)
Positive[b]	0.58 (0.03–10.2)	0.28 (0.04–1.71)	1.32 (0.64–2.71)	1.62 (0.97–2.71)
P-value	0.71	0.17	0.45	0.07
FIGO stage III/IV				
Alive/died, *N*	27/29	5/29	41/47	17/65
Negative[b]	1.0 (ref)	1.0 (ref)	1.0 (ref)	1.0 (ref)
Positive[b]	1.16 (0.44–3.02)	0.72 (0.18–2.96)	0.66 (0.33–1.32)	0.72 (0.41–1.28)
P-value	0.77	0.65	0.24	0.21

Abbreviations: CCC = clear cell carcinoma; CI = confidence interval; EC = endometrioid carcinoma; HR = hazard ratio; FOLR1 = folate receptor 1; LGSC = low-grade serous carcinoma; MC = mucinous carcinoma; OS = overall survival; OTTA = ovarian tumour tissue analysis.
[a]HR and 95% CI estimated using Cox regression stratified by study and adjusted for age, residual disease (not macroscopic, macroscopic or missing) and FIGO stage (I/II, III/IV or missing). Models that stratified by stage did not include stage as a confounder.
[b]Negative is absent or weak staining and positive is all other stains.

Table 7. Associations[a] between FOLR1 expression and PFS by ovarian carcinoma histological type in OTTA

	LGSC	MC	EC	CCC
	HR (95% CI)	HR (95% CI)	HR (95% CI)	HR (95% CI)
Progression-free/progressed, *N*	32/38	94/28	301/77	210/123
FOLR1 expression				
Negative[b]	1.0 (ref)	1.0 (ref)	1.0 (ref)	1.0 (ref)
Positive[b]	1.14 (0.53–2.44)	1.10 (0.26–4.74)	1.02 (0.61–1.69)	1.57 (1.06–2.34)
P-value	0.74	0.89	0.94	0.02
Stratified by stage				
FIGO stage I/II				
Progression-free/progressed, *N*	14/3	86/12	271/34	191/68
Negative[b]	1.0 (ref)	1.0 (ref)	1.0 (ref)	1.0 (ref)
Positive[b]	Not estimable	2.10 (0.21–20.5)	1.81 (0.82–3.99)	1.89 (1.10–3.25)
P-value	1.00	0.52	0.14	0.02
FIGO stage III/IV				
Progression-free/progressed, *N*	18/35	8/16	28/42	15/52
Negative[b]	1.0 (ref)	1.0 (ref)	1.0 (ref)	1.0 (ref)
Positive[b]	0.97 (0.43–2.20)	0.38 (0.03–5.12)	0.78 (0.38–1.62)	1.20 (0.60–2.37)
P-value	0.94	0.47	0.51	0.60

Abbreviations: CCC = clear cell carcinoma; CI = confidence interval; EC = endometrioid carcinoma; HR = hazard ratio; FOLR1 = folate receptor 1; LGSC = low-grade serous carcinoma; MC = mucinous carcinoma; OTTA = ovarian tumour tissue analysis; PFS = progression-free survival.
[a]HR and 95% CI estimated using Cox regression stratified by study and adjusted for age, residual disease (not macroscopic, macroscopic or missing) and FIGO stage (I/II, III/IV or missing). Models that stratified by stage did not include stage as a confounder.
[b]Negative is absent or weak staining and positive is all other stains.

(Chen *et al*, 2012) or no adverse association (Kalli *et al*, 2008; Crane *et al*, 2012) between FOLR1 protein or mRNA and OS. Time following diagnosis in those studies ranged from ~6.5 years (Chen *et al*, 2012) to 15 years (Kalli *et al*, 2008) and an average of the HR would obscure the modifying effect of follow-up time, as observed in the current investigation when we did not stratify by follow-up time. The change in association between FOLR1 expression and OS was more evident among FIGO stage I/II tumours in OTTA, whereas TCGA was composed mainly of FIGO stage III/IV tumours. For HGSC that did not express FOLR1, patients were observed to have an increased rate of death within 2 years after diagnosis, which attenuated with follow-up time. We speculated whether the absence of FOLR1 expression in the primary HGSC tumour was an indicator of tumours that responded poorly to standard platinum chemotherapy. Inclusion of treatment response indicators in statistical models that were available in the TCGA data analysis, however, did not weaken the associations with OS and did not support this hypothesis. Although it is possible that FOLR1 expression itself may have changed with follow-up time to explain our observations, this is not supported by studies that showed no significant change in FOLR1 expression following chemotherapy in patients with HGSC-matched tumour from primary surgery and either interval debulking surgery or surgery for recurrent disease (Crane *et al*, 2012; Despierre *et al*, 2013). It is possible, therefore, that differences between patients with FOLR1-negative poor-prognosis HGSC and FOLR1-positive tumours, which showed a survival advantage, may be from differences in intrinsic tumour biology.

The association between FOLR1 protein expression and decrease in PFS interval in patients with CCC and, specifically FIGO stage I/II tumours, is potentially interesting and will require independent confirmation. FIGO stage I/II tumours comprise the majority of CCC (almost 80% of CCC and 12% of all patients in our study) and their optimal management represents a clinical dilemma, because ~20% of patients relapse and are resistant to platinum-based chemotherapy (Kobel *et al*, 2010a; Anglesio *et al*, 2011). Existing clinical trials that are testing therapies to FOLR1 expression in ovarian carcinoma are comprised mostly of HGSC and one reported a favourable outcome among patients with platinum-resistant recurrent ovarian carcinoma (Naumann *et al*, 2013), which is a clinical profile similar to CCC. Interestingly, that study (Naumann *et al*, 2013) included eight patients with CCC (seven in the vintafolide arm). It remains to be evaluated whether targeting therapies specifically to FOLR1 in patients with CCC and FIGO stage I/II tumours improves their survival.

We observed that FOLR1 protein was expressed in 76% of HGSC, which agrees with a recent estimate of prevalence by Crane *et al* (2012) of 82% among 210 patients with HGSC. Further, only ~30% of CCC and EC, and 11% of MC were positive for FOLR1. These estimates are lower than those reported previously and may be from the much smaller sample sizes evaluated in studies of HGSC ($N = 36$–73) (Kelemen *et al*, 2005; Despierre *et al*, 2013; O'Shannessy *et al*, 2013) and of other histological types (Kalli *et al*, 2008; Crane *et al*, 2012). We also observed FOLR1 expression in normal fallopian tube tissue samples, which is increasingly accepted as the cell of origin for most HGSC (Piek *et al*, 2001; Crum *et al*, 2007). Others also reported strong FOLR1 expression in normal fallopian tube (Veggian *et al*, 1989; O'Shannessy *et al*, 2013), postulating that FOLR1 tumour expression is maintained from cell type-of-origin (O'Shannessy *et al*, 2013). However, at least two previous studies found strong FOLR1 expression in normal ovarian surface epithelium (Wu *et al*, 1999; Kelemen *et al*, 2005), including one that prospectively collected ovarian surface epithelial samples from healthy postmenopausal women undergoing oophorectomy (Kelemen *et al*, 2005). Given the difficulty to obtain viable normal ovarian surface epithelium, we question whether earlier studies (Veggian *et al*, 1989; Ross *et al*, 1994; Parker *et al*, 2005) found low expression in ovarian stroma rather than epithelium.

There are several strengths to this investigation. We showed the novel result that the association between FOLR1 expression and OS changes with the duration of follow-up and with histological cell type. Our evaluation is the largest assembly of an unselected sample of HGSC and other ovarian carcinoma types. We performed a centralised IHC assay with excellent inter-observer reproducibility that correlated reasonably well with *FOLR1* mRNA expression level among a subset of patients in our sample. Harmonisation of clinical variables contributed to robust statistical analysis and control for potential confounding. We also leveraged TCGA data to evaluate the association with HGSC in an independent data set and, although the direction and magnitude of the associations were similar between OTTA and TCGA samples, TCGA had too few tumours with FIGO stage I/II to evaluate.

In summary, we found that FOLR1 protein was widely expressed in patients with HGSC in OTTA and that expression was associated with increased OS in the first 2 years following diagnosis. The association with clinical outcome in CCC was opposite to that seen for HGSC with a decreased progression-free interval observed for FOLR1-positive CCC. Both findings were more pronounced among patients with FIGO stage I/II tumours. Our study highlights the need to evaluate FOLR1-targeted therapy outcomes by histological type, stage and time following diagnosis if FOLR1 is to be used as a target for therapeutic intervention.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

MK and LEK designed the overall study and oversaw statistical analysis. SJR, DGH, MK and LEK obtained financial support. SJR and MPI coordinated the OTTA studies database. MK and JM reviewed histological slides. AD, BG and SEJ harmonised the clinical variables. MK, BAC, PDPP, SD, DDB, KO, UM, CM, SL, WB, LS, MWB, AH, FCT, AH, DLW, MSA, EH, AJ, CH, KRK, BLF, GLK, ZCF, RAV, SL, SC, GN, PG, AG-M, SAG, EB, MW, BR, MB, HM, AO, PS, MJ-L, KED, JA, MM, JMK, HS, CE, AD, GC-T, SF, BG, SEJ, JG, LG, ELG, SKK, DGH, PAF, KBM, JDB and LEK coordinated contributing studies. MK, JDB and LEK drafted the manuscript, and all authors contributed to the final draft.

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