BRCA1 and MAD2 are Co-expressed and are Prognostic Indicators in Tubo-ovarian High-grade Serous Carcinoma

BRCA1 and MAD2 are Co-expressed and are Prognostic Indicators in Tubo-ovarian High-grade Serous Carcinoma.

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ABSTRACT

Objectives: To investigate the relationship between BRCA1 and MAD2 protein expression, as determined by immunohistochemistry, and clinical outcomes in epithelial ovarian carcinoma (EOC).

Methods: A tissue microarray (TMA) consisting of 94 formalin-fixed paraffin-embedded EOC with fully matched clinicopathological data were immunohistochemically stained with anti-BRCA1 and anti-MAD2 antibodies. The cores were scored in a semi-quantitative manner evaluating nuclear staining intensity and extent. Co-expression of BRCA1 and MAD2 was evaluated and patient survival analyses were undertaken.

Results: Co-expression of BRCA1 and MAD2 was assessed in 94 EOC samples, and survival analysis was performed on 65 high grade serous carcinomas (HGSC). There was a significant positive correlation between BRCA1 and MAD2 expression in this patient cohort ($p < 0.0001$). Both low BRCA1 and low MAD2 are independently associated with overall survival (OS) due to HGSC. Low co-expression of BRCA1 and MAD2 was also significantly associated with overall survival and was driven by BRCA1 expression.

Conclusion: BRCA1 and MAD2 expression are strongly correlated in EOC but BRCA1 expression remains the stronger prognostic factor in HGSC.

INTRODUCTION

Ovarian cancer is the most lethal gynecologic malignancy in Western populations, resulting in the death of approximately 125,000 women globally per year [1,2]. Despite approximately 30 years of clinical trials with many different chemotherapeutic regimes and targeted therapies; cytoreductive surgery along with platinum and taxane chemotherapy remain the international standard of care for this cancer [3]. Relapse rates are high and there is an urgent need to identify prognostic and predictive biomarkers for this cancer. High grade serous carcinoma (HGSC) accounts for approximately 70% of ovarian cancer cases and the vast majority of deaths [4]. There is now compelling evidence that most cases arise from the fimbria of the fallopian tube and are more accurately categorised as tubo-ovarian HGSCs [5-7]. Germline and somatic *BRCA1* and *BRCA2* mutations in HGSC is associated with enhanced survival that is largely attributed to a better response to platinum chemotherapy [8-11]. Reduced BRCA1 mRNA and protein expression was also previously shown to be prognostic in epithelial ovarian carcinoma (EOC) [12-14] and predictive of response to both platinum and taxane chemotherapy [14]. Indeed, the evaluation of the BRCA phenotype or "BRCAness" lead to the identification of deficiencies in several homologous repair pathway genes that are also associated with enhanced survival and platinum responses [15,16]. Similarly, the study of other BRCA-related genes may further stratify chemotherapy responses in tubo-ovarian HGSC.

The mitotic arrest deficiency protein 2 (MAD2) is a transcriptional target of BRCA1 [17]. MAD2 is an essential spindle assembly checkpoint protein monitoring accurate chromosomal alignment at the metaphase plate before mitosis [18,19]. A predominance of evidence implicates the overexpression of MAD2 as a driver of chromosomal instability (CIN) in many cancer types [20]. Although MAD2 overexpression is largely associated with tumours in which the retinoblastoma protein (Rb) or p53 tumour suppressor proteins are also inactivated, MAD2 overexpression *per se* is sufficient to cause CIN *in vitro* and *in vivo* [20,21]. Conversely, deletion of one copy of MAD2 in mice also results in tumour formation arising from the development of aneuploidy [22]. While downregulation of MAD2 has been reported in a number of cancers, complete null mutations have not been identified and loss of the second copy was shown to result in lethality in several human cell types , [23-25] . It is therefore not surprising that MAD2 expression is prognostic in many cancers and low MAD2 levels have been shown to be associated with poorer survival in EOC [26]. Furthermore, the action of many chemotherapies rely on checkpoint activity in which the requirement for functional levels of MAD2 is well documented in many *in vitro* and *in vivo* studies of cancer cells [27].

MAD2 is a ubiquitously expressed protein in most tissues of the body where it is localised in the nuclear, perinuclear and cytoplasmic compartments[28-30]. It is reliably measured by real-time PCR, western blots or immunohistochemical methods. The majority of studies have predominantly evaluated MAD2 expression by immunohistochemistry. MAD2 stains the nucleus of EOCs with varying degrees of intensity and often MAD2 negative and positive cells co-exist in the same tumour sample[31,32]. MAD2 has also been examined in various other cancers including cervical[33], prostate[34], oesophageal[35], gastric[30] and breast[36]. Most cancer types were shown to exhibit varying levels of positive staining ranging from 11% to 100% positivity [26]. EOCs are associated with relatively high levels of MAD2 expression while lung, tonsillar, and oral cancers seem to be characterised by low MAD2 expression. The interpretation of these studies is limited as only one study exists for most cancer sites. Both over-expression and low MAD2 expression was shown to be associate with survival depending on the tumour type[26].

BRCA1 and MAD2 share common cellular activities in response to taxane chemotherapy in which both MAD2 and BRCA1 are described as essential mediators of paclitaxel-induced G_2M arrest [27]. Deficiencies in BRCA1 and MAD2 have also been described in the underlying mechanisms of therapy induced senescence (TIS) [37,38 39]. In addition, the aberrant expression of BRCA1 or MAD2 can contribute to CIN [20,40]. To date, no studies have evaluated the co-expression of BRCA1 and MAD2 in clinical samples. In this study we performed BRCA1 and MAD2 immunohistochemistry on a TMA consisting of mixed EOC histologies and assessed the relationship between BRCA1 and MAD2 expression. We sought to determine if the co-expression of BRCA1 and MAD2 was prognostic in the HGSC subtype.

MATERIALS AND METHODS

Tumour Samples and Patient Characteristics

A tissue microarray (TMA) (Beecher Instruments, Silver Springs, MD, USA) was constructed using 0.6 mm tissue cores of EOC cases that were retrospectively identified through the Northern Ireland Centre for Gynaecological Cancer (NICGC), in association with the Northern Ireland Biobank (NIB) between 1994 and 2007. All histopathological material used in this study was acquired ethically. NIB has approval from ORECNI (reference 16/NI/0030) to collect, store and distribute samples to researchers. Tumour blocks with at least 80% invasive carcinoma cells were available for 202 of 287 requested cases (70.4%), of which 199 were used to create the TMAs, with four cores per case.

Relevant clinical data was collated via a manual trawl of clinical record databases and pathological records by a specialist clinician. Data was anonymised and aligned to a unique sample identifier. The median age at diagnosis was 59 years. From this TMA, 94 cases, including 65 cases of HGSC, in which IHC staining data was available from all 4 cores were included in the study. Cases with missing cores were excluded from the study. Of the 94 patients, all except three received adjuvant carboplatin or cisplatin and 74 patients also received a taxane. Six patients received a combination of a platin with a taxane and either erlotinib (2 patients) or gemcitabine (4 patients). The treatment of three patients is unknown. Cytoreductive status was defined as no visible residual disease, less than 1cm, more than 1 cm. All the cases were from patients who had cytoreduction. Table 1 shows clinicopathological features of the 94 cases included in this study.

Immunohistochemistry

The TMAs were stained with MAD2 primary antibody (BD Biosciences, CAT#610678) at a 1:100 dilution using the Ventana Discovery XT immunostainer and standard immunohistochemical techniques as previously published (Furlong et al., 2012). BRCA1 staining was performed using the mouse anti-BRCA1 (AB-1) mouse monoclonal (MS110) antibody (Calbiochem, UK) at a 1:200 dilution as previously described [14,41].

Evaluation of BRCA1 and MAD2 Immunostaining

Three BRCA1 and MAD2 stained slides (which were derived from two immediately adjacent tissue sections of the TMA) were scored independently by 3 observers (TB, LN and WGM). Only cores containing >20% tumour were scored. Each core was assessed for maximum nuclear staining intensity and assigned a score of negative (0), weak (1), intermediate (2) and strong (3) [31,32]. A mean nuclear staining intensity was then calculated by averaging positive staining across all 4 cores and rounded up to give whole numbers; low mean nuclear intensity was regarded as scores of 0, 1 and high mean nuclear intensity as scores of 2,3. The percentage distribution of maximum nuclear staining was also recorded in the following categories; no staining was assigned a score of 0, <10% was assigned a score of 1, 10 – 25% was assigned a score of 2, 26 – 50% was assigned a score of 3, 51 – 75% was assigned a score of 4, >75% was assigned a score of 5 [41]. A quickscore was calculated by multiplying maximum nuclear intensity with corresponding distribution category [32]. This gave a maximum score of 15. Distribution groupings were assessed by median distribution or by categorising distribution as low (≤7.5) or high (>7.5).

Statistical analyses

Agreement between scorers was assessed by the intraclass correlation coefficient (ICC), obtained from a two-way random effects model for the absolute agreement using IBM SPPS 21. Values range between 0 – 1 with values above 0.7 accepted as good concordance between scorers. Kaplan-Meier estimates of overall survival were generated for disease-specific survival (DSS) calculated from time of diagnosis to time of death from ovarian cancer on 65 HGSC cases. Multivariate analysis were performed using Cox proportional hazards modelling. A Chi squared test examined the association between BRCA1 and MAD2 on 94 ovarian cancer (mixed subtypes) cases. The 94 cases were separated into a 4 X 4 contingency table which included the categories of maximum nuclear staining intensity of 0,1,2,3 for each core assessed. A Chi squared test examined the association of BRCA1 or MAD2 expression with sensitivity to chemotherapy on 65 HGSC cases. A 3 X 2 contingency table was constructed for the BRCA 1 nuclear staining intensity categories negative (0) and positive (1,2,3) staining or MAD2 nuclear staining intensity categories, low (0, 1) and high (2, 3). Chemosensitivity was calculated from date of diagnosis to date of first recurrence and in accordance with the ESMO clinical practice guidance 2017, where response is defined as disease free >12 months (1); partial response is defined as disease free for 6 – 12 months (2); and resistant is defined as disease free for < 6 months (3).

RESULTS

Statistical analysis of the association between BRCA1 and MAD2 expression in EOC.

To ensure that each tumour was well represented in the TMA, only cases in which BRCA1 and MAD2 were assessable across all 4 tissue cores were included in the assessment. The ICC for agreement between scores ranged above 0.7; this confirmed excellent agreement in the scoring. In our analysis, mean nuclear intensity was consistently more informative than the quickscore and therefore, all data presented in this study were correlated with mean nuclear intensity. Representative staining is demonstrated in Figure 1. Maximum BRCA1 and MAD2 nuclear staining intensity was assessed in 4 tissue cores representing 94 cases. This resulted in 376 observations and the frequencies of the maximum BRCA1 and MAD2 nuclear expression are recorded in supplemental Table 1. The co-expression of BRCA1 and MAD2 was assessed by a chi squared test for association and this revealed that the expression of BRCA1 was significantly associated with MAD2 expression (p

<0.0001). Low BRCA1 expression was predominantly associated with corresponding low MAD2 expression in all EOC cases.

Statistical analysis of BRCA1 and MAD2 mean nuclear staining intensity.

Both BRCA1 and MAD2 immunohistochemical expression have been assessed previously in EOC and have been shown to be associated with survival in patients with EOC in general and in the subset of HGSC. In this study, we adopted a mean nuclear staining intensity which was superior to a quickscore in demonstrating that tumours which had a mean intensity score of zero for BRCA1 (BRCA1 negative) were significantly associated with an improved DSS in HGSC (HR, 3.305; (95% CI, 1.849 - 5.909), log rank, $p < 0.0001$) (Figure 2, supplemental Table 2). This is consistent with previous studies of BRCA1 immunohistochemistry in EOC and with the consensus that loss of BRCA1 is associated with improved chemotherapy response. A chi-squared analysis revealed that negative BRCA1 expression was significantly associated with sensitivity to chemotherapy in this patient cohort (Supplemental Table 3, $p = .005$). Conversely, while low MAD2 staining was previously demonstrated to be associated with poorer survival in EOC [26] and HGSC [31], in this study low mean MAD2 nuclear intensity was associated with improved DSS in HGSC (HR, 3.186; (95% CI, 1.789 - 5.675, log rank), $p < 0.0001$) (Figure 3, supplemental Table 2) . A chi-squared analysis also revealed that low MAD2 expression was significantly associated with sensitivity to chemotherapy in this patient cohort (Supplemental Table 3, $p = .0038$) and is consistent with the observation that BRCA1 expression is significantly associated with MAD2 expression.

Statistical analysis of BRCA1 and MAD2 co-expression

As there is a significant association between BRCA1 and MAD2 expression, the co-expression of these two proteins could be a more informative assessment of BRCA1 function in tumour cells and we aimed to determine if this could more accurately predict patient survival. Our results showed a significant association with DSS with BRCA1 and MAD2 co-expression (HR, 2.908; (95% CI, 1.592 - 5.312), log rank, $p =0.0001$) (Figure 4, supplemental Table 2), however the absence of BRCA1 expression remains the only prognostic indicator in HGSC in a multivariate analysis (HR 2.594; (95% CI, 1.358 – 4.957, $P = 0.004$) (Table 2). Therefore, the survival associated with MAD2 expression is mostly likely driven by BRCA1. While positive BRCA1 expression identifies cases with poor survival, co-expression with MAD2 failed to further stratify cases that were BRCA1 negative.

DISCUSSION

Predicting outcome in patients with EOC, particularly in the more aggressive HGSC subtype, would be extremely valuable to stratify patient management. BRCA abnormalities are widely associated with HGSC and it is indisputable that BRCA deficiency, as assessed by immunohistochemistry and mRNA levels, is prognostic in HGSC and other cancers. Loss of BRCA1 activity disrupts the homologous recombination (HR) DNA repair pathway impairing the DNA damage response in these cells: This leads to the more favourable survival responses associated with BRCA1 deficient tumours when treated with DNA damaging agents such as cisplatin. Over the last 2 decades, the HR DNA repair pathway has emerged as an important therapeutic target in oncology.

As discussed, BRCA1 deficiency is associated with improved survival in HGSC and other cancers; however, many cases with negative BRCA1 also exhibit very poor survival and therefore BRCA1 alone is insufficient as a single biomarker. Therefore, in this study we assessed if patient stratification with BRCA1 immunohistochemistry alone could be improved by the addition of MAD2. MAD2 is an attractive candidate as we and others have previously shown that high MAD2 was associated with improved survival in EOC and HGSC [26]. We therefore hypothesised that those tumours which were negative for BRCA1 and had high MAD2 expression may have more favourable responses to paclitaxel, ultimately providing a survival advantage for these patients. In our study, we demonstrate, for the first time in patient samples, a positive correlation between BRCA1 and MAD2 expression. This supports the results from *in vitro* and *in vivo* studies which suggest that MAD2 is a transcriptional target of BRCA1[17]. Secondly, we also demonstrate that MAD2 is prognostic in HGSC. However, contrary to previous studies, low MAD2 expression associated with improved DSS in this patient cohort. In a recent meta-analysis of MAD2 expression in all cancers, low MAD2 levels were more consistently associated with better survival, in keeping with the results of our study [26]. One limitation to our study is that the numbers are relatively small with few cases of negative BRCA1 and high MAD2 expression or positive BRCA1 and low MAD2 levels. Therefore, survival associated with MAD2 in this study was predominantly driven by BRCA1 expression.

The levels of MAD2 can influence many features of tumour initiation and progression and so it is not surprising that MAD2 expression is associated with prognosis in various cancers. Contrary to all other cancers tested, low levels of MAD2 were surprisingly and uniquely shown to be a poor prognostic indicator in EOC. We now report in this study that low levels of MAD2 is associated with improved DSS in HGSC. As BRCA1 deficiency is currently the best indicator of survival in HGSC (outside of traditional factors such as tumour stage, age and cytoreductive status), it would appear more logical

that deficiencies in a BRCA1 transcriptional target would also predict better outcome. Of note, one major difference between this study and all prior studies is that the pathological review of the EOC samples in this patient cohort was recent using modern diagnostic criteria; previously there was significant interobserver variability amongst pathologists in the classification of EOC. The 5 major subtypes of EOC (HGSC, low grade serous carcinoma, endometrioid carcinoma, clear cell carcinoma, mucinous carcinoma) are pathogenetically, histologically and molecularly separate diseases and all studies of EOC should assess each subtype individually. Therefore, results from earlier studies of HGSC may have been influenced by the inclusion of other EOC subtypes. They may also have included mixed carcinomas although these are now known to be exceedingly rare, accounting for less than 1% of EOCs[42]. As we have already discussed, we were unable to assess the survival of cases that stained positive for BRCA1 with low MAD2 expression and those which were negative for BRCA1 and with high MAD2 expression, since so few cases stratified into these groupings. We would expect that low BRCA1 would have a greater influence on survival in such cases and it is conceivable that in this instance, high MAD2 would be associated with improved survival. We also employed stringent inclusion criteria for our study and only included cases with interpretable scoring in all 4 TMA cores; previous studies may have been influenced by poor tumour representation on the TMA.

This study highlights problematic aspects of using MAD2 immunohistochemistry as a biomarker in which either up- or down- regulation of the gene can result in similar genomic aberrations and contribute to poor patient survival[43,44]. Through *in vitro* and *in vivo* investigation, high MAD2 levels are known to correlate with increased cellular proliferation, cellular transformation, migration, invasiveness and cancer metastasis[45-47], while other studies have shown that low MAD2 expression has a role in chemoresistance to both DNA-damaging agents[48,49] and anti-microtubles[27,31]. Considering the heterogeneous nature of HGSC, a better understanding of the molecular subtypes could help inform the adverse role of up- or down-regulation of MAD2 on patient survival and whether it is dependent on BRCA1 levels or activity. In the absence of any major breakthrough in early detection of HGSC, patient stratification methods to predict response to therapy are extremely valuable and the role of MAD2 in chemoresistance could be informative. Future studies could focus on relating MAD2 expression to chemoresponse in which the analysis of the MAD2 conformation specific antibodies would provide an assessment of the tissue expression of the active form of MAD2.

In summary, BRCA1 and MAD2 immunohistochemistry are strongly correlated in EOC. Low expression of BRCA1 and its transcriptional target MAD2 expression is strongly correlated with improved survival in HGSC in which BRCA1 is the most important prognostic indicator.

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Table 1. Clinical Characteristics of Ovarian Cancer Cases on the NIB TMA

Table 2. Multivariate Cox proportional hazards analysis for disease specific survival (DSS).

Supplemental Table 1. Contingency table of BRCA1 and MAD2 nuclear staining intensity scores.

Supplemental Table 2. Cox regression analysis and hazard ratios (HRs) with 95% confidence intervals (CIs) for disease specific survival (DSS) adjusting for age at diagnosis, disease stage and optimal surgical debulking.

Supplemental Table 3. Contingency table for BRCA1 and MAD2 expression and sensitivity to chemotherapy with Platinum or Platinum in combination with a Taxane. A Fisher test for association of negative BRCA1 (p = 0.005) or low MAD2 (p = 0.003) with response was performed.

Figure 1: ***Path XL Core imaging of IHC staining of FFPE ovarian carcinoma tissue for BRCA1 and MAD2***. Representative images of IHC staining at 5X (inset) and 20X magnification (a -d) BRCA1 nuclear intensity score = 0, 1, 2, 3 respectively and (e – h) MAD2 nuclear intensity score – 0, 1, 2, 3 respectively.

Figure 2: ***BRCA1 nuclear intensity significantly associates with DSS***. (a) Kaplan Mires curve of the correlation of negative BRCA1 with disease-specific survival (DSS).

Figure 3: ***MAD2 nuclear intensity significantly associates with DSS***. (a) Kaplan Mires curve of the correlation of low MAD2 with disease-specific survival (DSS).

Figure 4: ***The co-expression between low mean BRCA1 and MAD2 nuclear expression significantly associates with DSS in HGSC***. (a) Kaplan Mires curve of the correlation of neg BRCA1:low MAD2 with disease-specific survival (DSS).

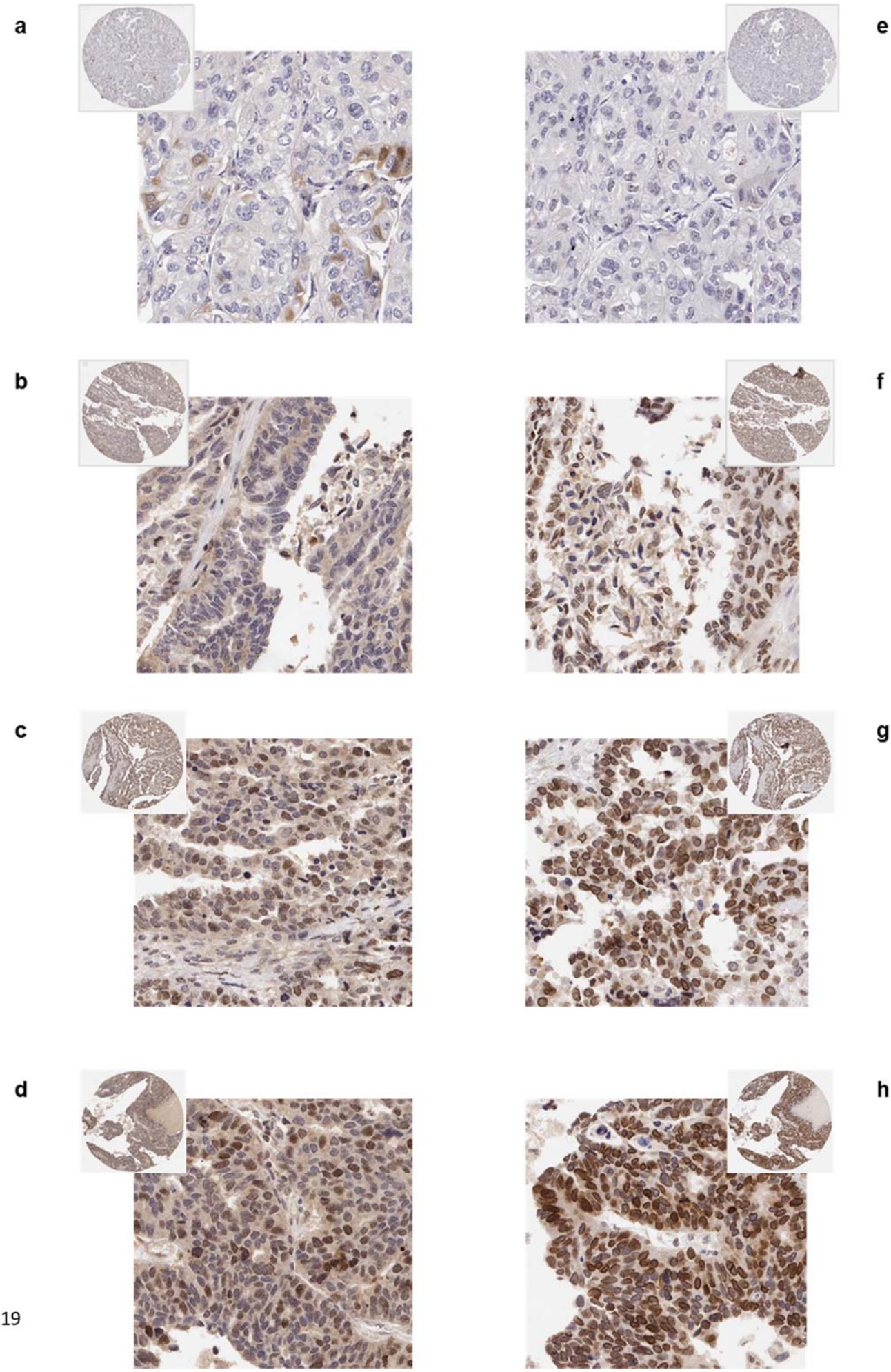
Northern Ireland Biobank (NIB) OC TMA		
Disease Stage		
I	23	24.5%
II	6	6%
III	52	55%
IV	13	14.5%
Total	**94**	**100%**
Histologic Subtype		
High Grade Serous	65	69%
Low Grade Serous	4	4%
Endometroid	8	9%
Mucinous	3	3%
Clear Cell	12	13%
Other	2	2%
Total	**94**	**100%**

	Adjusted hazard ratio	95% Lower confidence interval	9% upper confidence interval	*P*-value
Age	1.025	0.993	1.059	0.127
Stage	1.916	1.220	3.009	0.005
Cytoreduction/ Cytoreductive surgery	0.576	0.330	1.004	0.052
Neg BRCA1 V pos BRCA1	2.594	1.358	4.957	0.004
Low MAD2 V High MAD2	1.420	0.719	2.804	0.312

		MAD2 nuclear staining intensity				
BRCA1 nuclear Staining Intensity		**0**	**1**	**2**	**3**	Total
	0	96	5	4	0	105
	1	66	19	8	1	94
	2	49	21	21	8	99
	3	19	13	33	13	78
Total		230	58	66	22	376

	BRCA1 (neg V pos)		MAD2 (low V high)		BRCA1:MAD2 (co-stain)	
	HR (95% CIs)	*P*-value	HR (95% CIs)	*P*-value	HR (95% CIs)	*P*-value
unadjusted	3.305 (1.849 - 5.909)	<0.0001	3.186 (1.789 - 5.675)	<0.0001	2.908 (1.592 - 5.312)	<0.0001
Age/Stage/ Cytoreduction/ Cytoreductive surgery	2.787 (1.501 - 5.174)	0.001	2.033 (1.059 - 3.905)	0.03	1.934 (0.994 - 3.761)	0.052

		Chemotherapy Sensitivity		
		1 (sensitive)	2 (partial)	3 (resistant)
BRCA1	Negative	25	6	1
	Positive	13	11	8
MAD2	Low	25	4	2
	High	13	13	7


a
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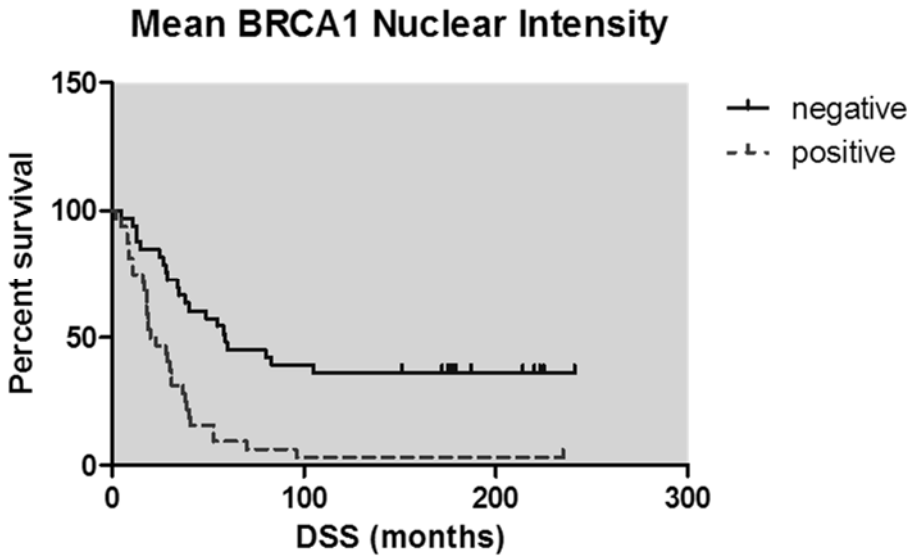
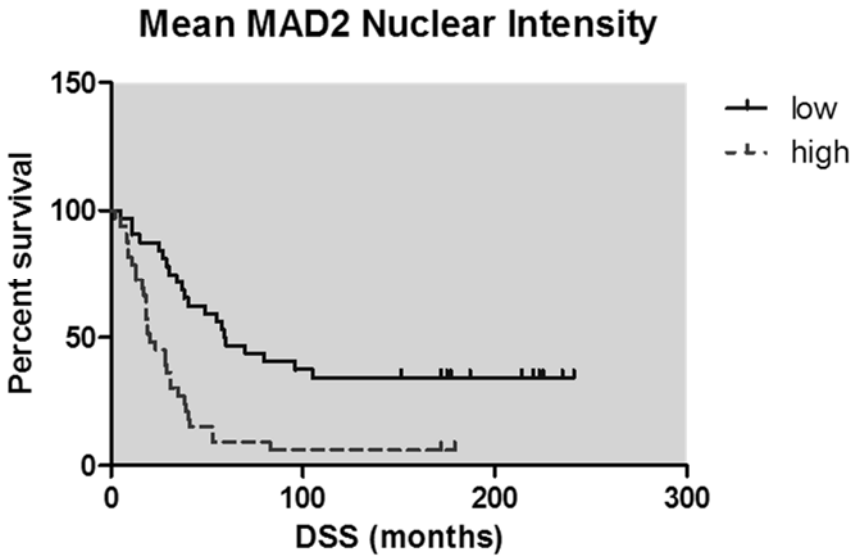
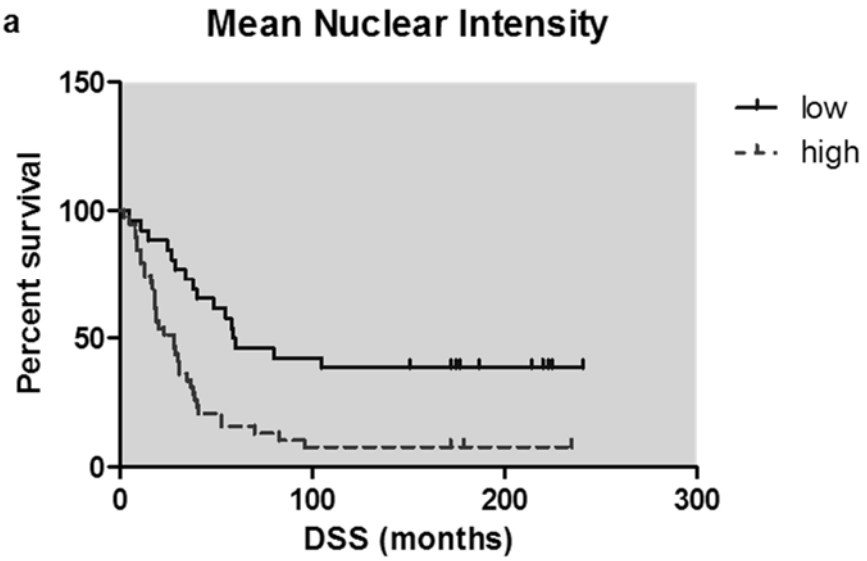

Mean BRCA1 Nuclear Intensity
Percent survival
150
100
50
0
0
100
200
300
DSS (months)
negative
positive

a


Mean MAD2 Nuclear Intensity
Percent survival
150
100
50
0
0
100
200
300
DSS (months)
low
high

a


Mean Nuclear Intensity
Percent survival
150
100
50
0
0
100
200
300
DSS (months)
low
high