Nucleotide excision repair protein ERCC1 and tumour-infiltrating lymphocytes are potential biomarkers of neoadjuvant platinum resistance in high grade serous ovarian cancer.

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Abstract:

Objective

ERCC1 is a nucleotide excision repair protein that may have a role in drug resistance in high grade serous ovarian cancer (HGSOC). We hypothesized that ERCC1 expression and tumor infiltrating lymphocytes (TILS) are induced by chemotherapy in HGSOC, which may be prognostically useful.

Methods

115 HGSOC patients were used for this study. 92 (80%) of the tissue analysed had not been exposed to platinum chemotherapy. The remaining 20% (n=23) of cases received combination or monotherapy with carboplatin before tissue was collected.

Immunohistochemistry was used to score for ERCC1 expression and morphology to score for TILs. Correlation analysis of all clinical parameters, TILs and ERCC1 and Kaplan-Meier survival analysis was performed using the ERCC1 and TILs scoring parameters (0, 1, 2 or 3).

Results

ERCC1 expression was 2-fold higher in the neoadjuvant chemotherapy group compared to the primary cytoreductive surgery group ($p<0.0001$). The mean overall survival for the neoadjuvant group with high ERCC1 was 141.6 $\pm$ 20.2 months which was significantly longer than absent ERCC1 survival of 61 + 22.6 months ($p=0.028$). ERCC1 score strongly correlated with TILs score across the whole cohort (0.349, $p = 1.3 \times 10^{-4}$) suggesting there is a relationship between ERCC1 expression and TILs, but this requires further investigation.

Conclusion: In conclusion, ERCC1 was identified as a potential biomarker of platinum response overall survival in HGSOC undergoing neoadjuvant HGSOC treatment.

Introduction:

High grade serous ovarian cancer is (HGSOC) treated by up front cytoreduction surgery followed by chemotherapy or neoadjuvant chemotherapy followed in appropriate cases by cytoreduction surgery. Optimal first line chemotherapy is a platinum compound (cisplatin or carboplatin) plus a taxane (eg. paclitaxel). Platinum, the most effective chemotherapy agent, directly damages DNA by cross-linking nucleotides which if unrepaired results in double-strand DNA breaks and eventually induces apoptosis. Recognition of the DNA cross-links is performed by a specific DNA repair process, nucleotide excision repair (NER). Once platinum cross-links are recognized, NER can decide a cell's fate by triggering the initiation of DNA repair, or if the damage is too great by directly signalling for apoptosis (1). In HGS ovarian cancer, HR deficiency as a result of BRCA mutations, results in increased DNA damage and increased apoptosis and sensitivity to DNA-damaging agents. Conversely, reduced levels of NER in the presence of DNA-damaging agents, result in the accumulation of unrepaired DNA damage with the addition of absence of apoptotic signalling, contributing to reduced or no response to platinum chemotherapy (2-4). Protein and mRNA expression levels of factors in the pathway have been reported to predict treatment response to cisplatin, and its analogues, in ovarian, testicular and non-small cell lung cancers (2, 5, 6).

ERCC1 is one key component of NER that has been extensively studied in HGSOC. Studies that have analysed HGSOC collected by debulking surgery tissue before adjuvant chemotherapy treatment have shown no prognostic role for ERCC1 expression in predicting response to adjuvant chemotherapy (7, 8) or overall survival (7, 9-11). The explanation for this maybe multifactorial and could reflect the complex and dynamic nature of DNA repair in the face of acute genotoxicity. The optimal timing of assessing ERCC1 expression could also be crucial in understanding any possible relation to platinum-based therapy response.

NER proteins require DNA damage to be present before eliciting a response, therefore we hypothesised that ERCC1 expression will be necessarily highest after chemotherapy when DNA damage is at its highest. The HGSOC cases with the lowest ERCC1 expression after chemotherapy would be expected to be the most drug resistant, due to the lack of an apoptotic response in the setting of ongoing DNA damage. In this context, high ERCC1 would be expected to result in response to treatment and thus correlate with overall survival. Comparing levels of ERCC1 expression in tumor obtained in secondary cytoreduction after neoadjuvant chemotherapy to that obtained in up front primary cytoreduction offers the chance to test this hypothesis.

It is well established that the degree of tumor infiltrating lymphocytes (TILS) correlates positively with survival (12). Furthermore, post-chemotherapy tumour tissue shows an increased number of TILS (13). If both TILS and ERCC1 expression are increased post-chemotherapy, the question that arises is whether both ERCC1 expression and high TILS are required for improved overall survival.

To explore this hypothesis, we performed the following retrospective studies: Comparison of ERCC1 expression in tumor tissue obtained after neoadjuvant chemotherapy versus primary cytoreduction surgery; comparison of survival in high and low ERCC1 expression in the cohort treated by neoadjuvant chemotherapy; and comparison of TILS and ERCC1 levels after neoadjuvant chemotherapy and their relationship to survival.

Materials and Methods:

Patient cohort

The Hunter Gynaecological Cancer (HGC) database was searched from 2000-2015 inclusive for first diagnoses of women with high grade serous carcinoma of the fallopian tube, ovary or peritoneum that were treated by either primary cytoreduction followed by chemotherapy or by neoadjuvant chemotherapy followed by cytoreduction.

A total of 115 HGSOC patients; 92 (80%) treated by primary cytoreduction and 23 (20%) by neoadjuvant chemotherapy followed by cytoreduction, were identified. Following primary cytoreduction, adjuvant combination platinum/paclitaxel or carboplatin monotherapy was administered. Therefore 80% of the tissue analysed had not been exposed to platinum chemotherapy at the time of collection. The remaining 20% (n=23) of cases received combination (n=18) or monotherapy (n=5) with carboplatin before tissue was collected. The outcome of cytoreduction surgery was determined as suboptimal if $\geq$1mm of residual disease was reported and optimal if $\leq$1mm or no residual disease was reported.

Full face sections of diagnostic formalin-fixed paraffin embedded (FFPE) tissue were obtained from Pathology NSW archive and FIGO staging was confirmed by a senior Anatomical Pathologist (JS). Tissue blocks with viable tissue were chosen preferentially with none or limited necrosis. Viable areas of tumour tissue were marked for scoring. Age and date of diagnosis, stage, grade, primary treatment, date of death or date of last follow-up were collated from the HGC database and confirmed from medical records. Demographic and clinical characteristics are summarised in Table 1. The Hunter New England Area Health Service Human Ethics Committee approved the study (approval number 08/08/20/5.17).

ERCC1 Immunohistochemistry

Slides were de-waxed using xylene and a serial dilution of ethanol. Antigen retrieval was performed in citric acid EDTA with an approximate pH 9.0. Slides were then treated using the SuperPicture 3 kit (Invitrogen, USA) and the anti-ERCC1 antibody 8F1 (1:300, abcam USA) and counter stained with hematoxylin. ERCC1 scoring was performed by a pathologist blinded to all clinical and pathological findings. Staining intensity was graded from 0 to 3; 0 = complete absence of ERCC1, 1 = low intensity and/or <50% cells positive, 2= medium intensity staining and >50% cells positive, 3 = high intensity staining and >50% cells positive. Examples of scoring of intensity are shown in Figure 1.

Tumour infiltrating lymphocytes (TILS)

TILs were assessed by morphology on ERCC1 IHC sections. TILS were scored as absent, mild, moderate or marked presence with a density of focal, mulitfocal or diffuse. A TIL score was calculated as described by Madore et al (14) as follows: 0 = no TILS present, 1 = mild or moderate focal / mild multifocal, 2 = marked focal / moderate or marked multifocal / mild diffuse and 3 = moderate or marked diffuse. Examples of scoring of intensity are shown in Figure 2.

Statistical analysis

Spearman's Rho was used for correlation analysis of all clinical parameters, TILs and ERCC1. Kaplan-Meier survival analysis was performed using the 4 ERCC1 and TILs scoring

parameters (0 = absent, 1 = low expression, 2 = medium expression or 3 = high expression). For 2 group ordinal analyses the groups were condensed into the following categories: low = score 0 or 1 and high = score 2 or 3. Overall survival (months) was censored for patients that were alive at last follow-up at the time of data collection.

Log Rank (Mantel-Cox) test was used to determine the Chi-squared and p-value for survival. Cox regression was used to correct the ERCC1 survival analysis for suboptimal/optimal debulking surgery, monotherapy/combination therapy and TILs score. When assessing the prognostic value of ERCC1 and TILs on survival, observation time began at the date of surgery and continued until the event or censor date. Overall survival considered death the event, and patients were otherwise censored at last follow-up.

Results:

ERCC1 expression was significantly higher in the neoadjuvant chemotherapy group (mean score = 1.61±1.16) when compared to the cytoreduction surgery group (mean score = 0.79±0.83, p<0.0001) . A small but not significant increase in TILs scores was seen in the neoadjuvant chemotherapy group (mean score = 1.57±1.24) compared to the cytoreduction surgery group (mean score = 1.30±1.02). All other demographic and clinical parameters were not significantly different between the 2 treatment groups (Table 2).

Kaplan-Meier survival analysis was used to determine that the survival distributions for the 4 ERCC1 score categories alone did not significantly differ across the whole cohort (Supplementary Table 1 and Supplementary Figure 1). While, when divided into treatment groups, ERCC1 scores in the neoadjuvant chemotherapy cohort were found to have a significantly different overall survival effect (Chi-square=6.074, df=3, p=0.014). The mean survival in the neoadjuvant group for absent ERCC1 (score = 0, n = 5) was 61.5 ± 22.6 months and for high ERCC1 (score = 3, n = 7) was 141.6 ± 20.2 months (p= 0.028) (Figure 3a). There were no significant differences in survival associated with ERCC1 scores in the primary cytoreduction group (Figure 3b) (score = 0, survival = 100.3 ± 10.8 months; score =1, survival = 89.9 ± 11.9 months; score = 2, survival = 83.7 ± 15.4 months; score = 3, survival = 98.9 ± 29.1 months).

The most common clinical diagnostic pathology assessment of IHC is a 2 group classification of staining (e.g: present/absent, high/low, aberrant/wildtype). We condensed the 4 score ERCC1 catergories into low (score 0 or 1) and high (score 2 or 3) and repeated the analyses. The Kaplan-Meier survival curve displayed an increase in survival for the ERCC1 high group

compared to the ERCC1 low group (supplementary figure 3). The difference did not reach statistical significance due to the small cohort size.

The effect of all other clinical characteristics, including suboptimal/optimal debulking, monotherapy/combination therapy and TILs, on survival was also assessed to determine if there were any confounding factors influencing the survival differences due to ERCC1 expression levels. Across the whole cohort, overall survival was significantly longer for the highest TILs score (score 3: n = 24, survival =147.56+16.04 months) compared to lower or absent TILs scores (range for score 0 to 2: n = 91, = 79.17 – 108.89 months, p=0.027) and Cox proportional hazard regression analysis found TILs was the only independent risk factor for survival (p=0.008) (Supplementary tables 2 and 3).

The overall survival in the neoadjuvant chemotherapy group showed a trend towards significantly longer survival in the presence of the highest TILs score of 3. When overall survival for ERCC1 scores was corrected for TILs scores using Cox regression analysis, the increased survival in the highest expressing ERCC1 tumours remained significant (score 0: n = 6, survival = 61.5 $\pm$ 22.6 months, score 3: n = 8, survival = 141.6$\pm$20.2 months , p=0.041). ERCC1 score strongly correlated with TILs score across the whole cohort (0.349, p = 1.3 x 10^{-4}) suggesting there is a relationship between ERCC1 expression and TILs, but this requires further investigation.

Overall, the results of this study support the hypothesis that ERCC1 has potential to be a biomarker of response to platinum chemotherapy when assessed post neoadjuvant treatment. The data confirms that ERCC1 is not a predictive biomarker of response to platinum

chemotherapy when assessed in treatment naïve HGSOC tissue collected after primary cytoreductive surgery.

Discussion:

Cells have developed numerous strategies to protect themselves against DNA damaging stimuli of many kinds. When it comes to cancers, these same strategies can determine the effectiveness of chemotherapeutic agents. Platinum-based chemotherapeutic agents work by damaging tumour cells via DNA cross-links. A number of proteins are involved in repairing this type of damage (eg: XPC, DDB1, DDB2, XPA, ERCC2, ERCC3, ERCC5, ERCC6), but the most heavily studied in chemoresistance is ERCC1(2) . ERCC1 is the only member of the NER DNA repair pathway that is both highly conserved and lethal if functional mutations occur (15).

Previous studies reporting the relationship between ERCC1 and response to platinum chemotherapy in HGSOC have been conflicting, with high expression being associated with resistance in both cell lines and tumour tissue (10, 16-19) which was unable to be confirmed in follow-up studies (7). The majority of studies to date have assessed ERCC1 expression on tissue collected during primary cytoreductive surgery that occurs before chemotherapy treatment. Therefore, we reasoned that the discordant results may be due to the role ERCC1 plays in DNA repair that is only elicited after platinum-induced DNA damage occurs.

As a first step, we hypothesized that higher ERCC1 expression would be found in HGSOC treated by neoadjuvant chemotherapy compared to those treated by primary cytoreductive surgery, since the DNA damage induced by platinum would stimulate expression of ERCC1. This is the first study to compare ERCC1 in the 2 treatment groups and we confirmed the hypothesis that ERCC1 expression was higher in the neoadjuvant chemotherapy group. This result set the stage for testing of a second hypothesis: that there would be shorter survival in those patients with low/absent ERCC1 expression in the neoadjuvant cohort.

Kaplan-Meier survival analysis showed ERCC1 is a predictive biomarker of overall survival in the neoadjuvant chemotherapy group. These results require confirmation in a larger cohort but based on our results, ERCC1 has potential to be a biomarker of response to platinum chemotherapy if assessed after treatment. The data herein also confirms that ERCC1 is not a predictive biomarker of response in platinum chemotherapy when assessed in treatment naïve HGSOC tissue.

As a final step in our study, we reasoned that the predictive value of ERCC1 expression on survival in neoadjuvant patients would be of greater use clinically if it was independent of TILs. While drug resistance and TILs have largely different mechanisms of action, it is well documented that HGSOC have an inflamed phenotype with high TILs after platinum chemotherapy (13). Many studies have investigated location (stromal or intratumoral) and subtypes of TILs in relation to overall survival (20-23), including a recent extensive analysis of tumour and immune cell dynamics in HGSOC (24). Zhang et al (24) reported TILs and mutational processes, such as DNA repair deficiency, have prognostic interactions in HGSOC.

Whilst analysis of location of TILs was not performed as part of the current study, when overall survival for ERCC1 scores was corrected for TILs scores determined as described by Madore et al (14), in the neoadjuvant cohort, the difference in survival remained significant. Further studies to determine the relationship between ERCC1 expression and the level and location of TILs would determine if a causal or biological relationship exists and if both are required for improved response to platinum chemotherapy and overall survival.

The results reported herein indicate ERCC1 is a potential biomarker that can be further developed to assess the response to platinum chemotherapy in real-time during the course of

treatment. The short-term outcome of using ERCC1 as a prognostic marker post-neoadjuvant therapy would be to add further prognostic information to the clinical management of disease, ie: if a patient received neoadjuvant therapy and had low levels of ERCC1 detected in subsequent surgery this would be indicative of shorter overall survival, the clinician may decide to increase frequency and depth of patient monitoring/follow-up to detect relapse earlier as it is more likely to occur in the context of low ERCC1. In the longer-term as the use of ERCC1 as a prognostic marker is further assessed, it may result in changes to treatment of disease, ie: if low ERCC1 is detected no further platinum chemotherapy is used as it is unlikely to be successful. The short-term and long-term outcomes are hypothetical at this stage and would require extensive assessment in a larger cohort with enough statistical power to confirm the results and followed up in prospective cohort studies.

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Conflict of Interest statement:

The authors have no conflicts of interest to declare.

Author Contributions:

JS: Assisted in devising the project, confirmed the high-grade serous ovarian cancer diagnosis of all cases, scored the ERCC1 protein levels and contributed to preparing the final manuscript

BvZ and DG: Optimised and performed the immunohistochemistry, scored ERCC1, collated the patient cohort information and experimental data.

GO and KJ: Collated the Hunter Gynaecological Cancer (HGC) database, collected tissue and contributed to the final manuscript

JL: Contributed to the study design and the Hunter Gynaecological Cancer (HGC) database, interpretation of results and final manuscript

REV: Devised tumour-infiltrating lymphocyte scoring protocol, contributed to statistical analysis and final manuscript

NAB: Devised and designed project, oversight of collection and collation of data, scored ERCC1 and TILs, performed statistical analyses and wrote the manuscript.

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Figure legends:

Figure 1. ERCC1 immunohistochemistry scoring.

A. ERCC1 negative, score = 0; B. ERCC1 low intensity and/or <50% cells positive, score = 1; C. ERCC1 medium intensity staining and >50% cells positive, score = 2; D. ERCC1 high intensity staining and >50% cells positive, score = 3

Figure 2. Tumour-infiltrating lymphocytes (TILs) immunohistochemistry scoring.

A) Score 0 = no TILS present; B) score 1 = mild or moderate focal / mild multifocal; C) 2 = marked focal / moderate or marked multifocal / mild diffuse; and D) 3 = moderate or marked diffuse.

Figure 3. Kaplin-Meier survival analysis of ERCC1 scores associated with neoadjuvant and adjuvant chemotherapy.

A) Kaplin-Meier survival analysis determined that there was a significant difference in the distribution of survival in the ERCC1 scores in the neoadjuvant chemotherapy cases. B) No significant different in the distribution of survival between the ERCC1 scores in the adjuvant chemotherapy cases. Overall survival (months) was censored for patients that were alive at last follow-up at the time of data collection.

Supplementary Figure 1. Kaplin-Meier survival analysis of ERCC1 score.

Kaplin-Meier survival analysis determined that there was no significant different in the distribution of survival between the ERCC1 scores. Overall survival (months) was censored for patients that were alive at last follow-up at the time of data collection.

Supplementary Figure 2. Kaplin-Meier survival analysis of low/highERCC1 score.

A) Kaplin-Meier survival analysis determined that there was a large, non-significant difference in the distribution of survival in the neoadjuvant chemotherapy group between low and high groups of ERCC1 scores. B) There was no difference in survival based on the low/high groups for the cytoreductive surgery group. Overall survival (months) was censored for patients that were alive at last follow-up at the time of data collection.

4. Table

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Table 2. Clinical characteristics, ERCC1 and TILs score for neoadjuvant and primary cytoreductive surgery treated high-grade serous ovarian cancer

	Neoadjuvant Chemotherapy (n=23)		Primary cytoreductive surgery (n=91)	
	Mean	**SD**	**Mean**	**SD**
Age at Diagnosis	66.43	10.77	64.64	11.32
Stage	2.00	1.94	3.05	1.24
Grade	2.45	0.91	2.60	0.79
Overall Survival (Months)	55.18	48.00	65.46	47.07
Disease Free Survival (Months)	11.38	5.60	18.64	11.14
ERCC1 Score	1.61	1.16	0.79***	0.83
TIL score	1.57	1.24	1.30	1.02

***** p<0.0001 2-tailed t-test neoadjuvant chemotherapy compared to adjuvant chemotherapy**

Table 1. Demographic and clinical characteristics of high-grade serous ovarian cancer cohort.

		Number	Percentage (%)
Cohort		115	100
Age (mean)		65 ± 11	
Stage	NS	6	5.2
	I	7	6.1
	II	3	2.6
	III	86	74.8
	IV	13	11.3
Grade	0	8	7
	1	2	1.7
	2	24	20.9
	3	81	70.4
Primary Treatment	Neoadjuvant chemotherapy	23	20
	Cytoreductive surgery	92	80
Cytoreductive Surgery	Optimal	73	63.5
	Suboptimal	27	23.5
	Unknown	15	13
Overall Survival (months)		63 ± 47	
Disease-free survival (months)		17 ± 11	
Alive at last follow-up		60	
ERCC1 scores	0	44	38.3
	1	43	37.4
	2	17	14.8
	3	11	9.6
TILS Scores	0	28	24.3
	1	42	36.5
	2	21	18.3
	3	24	20.9

5. Figure

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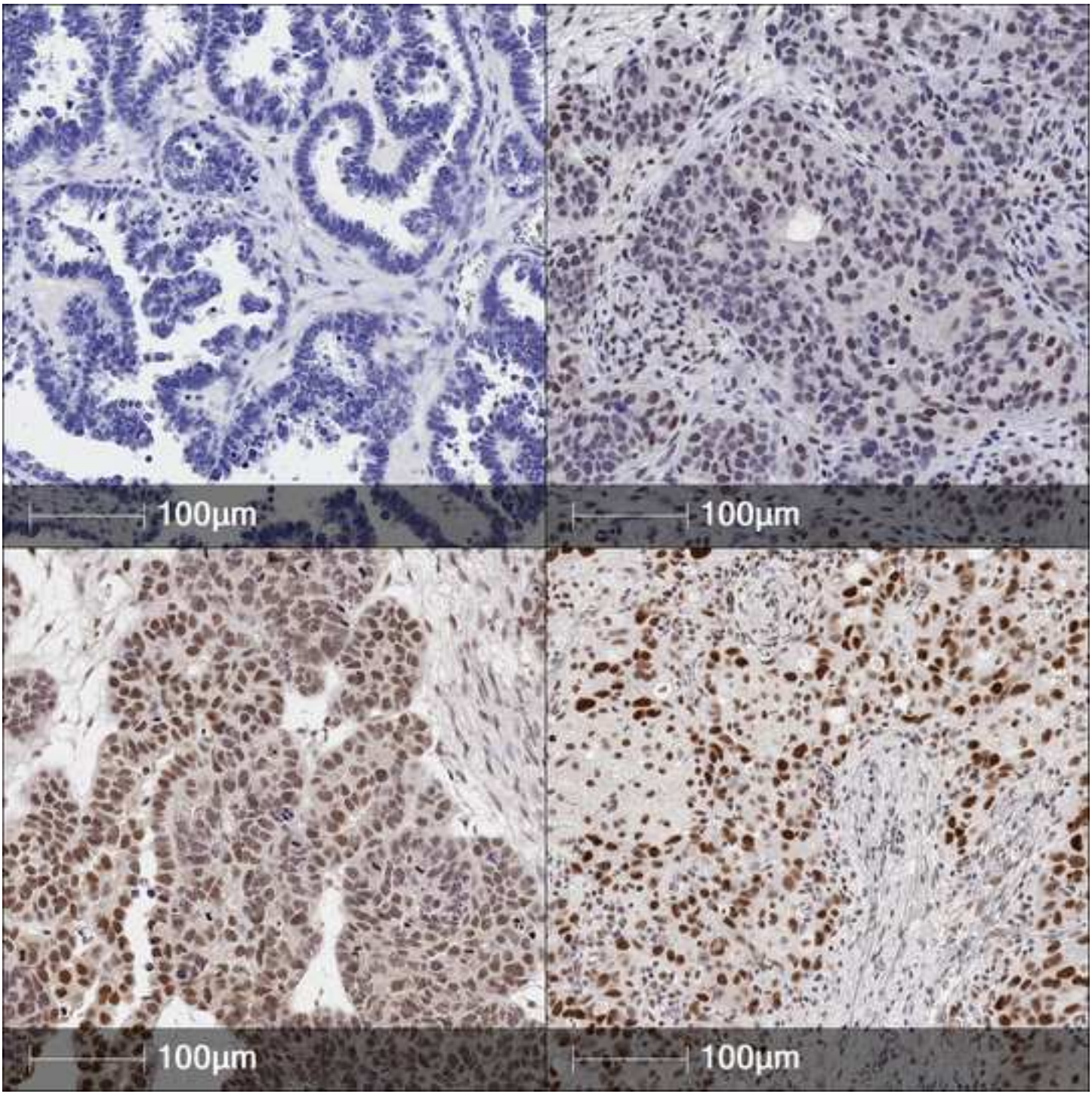

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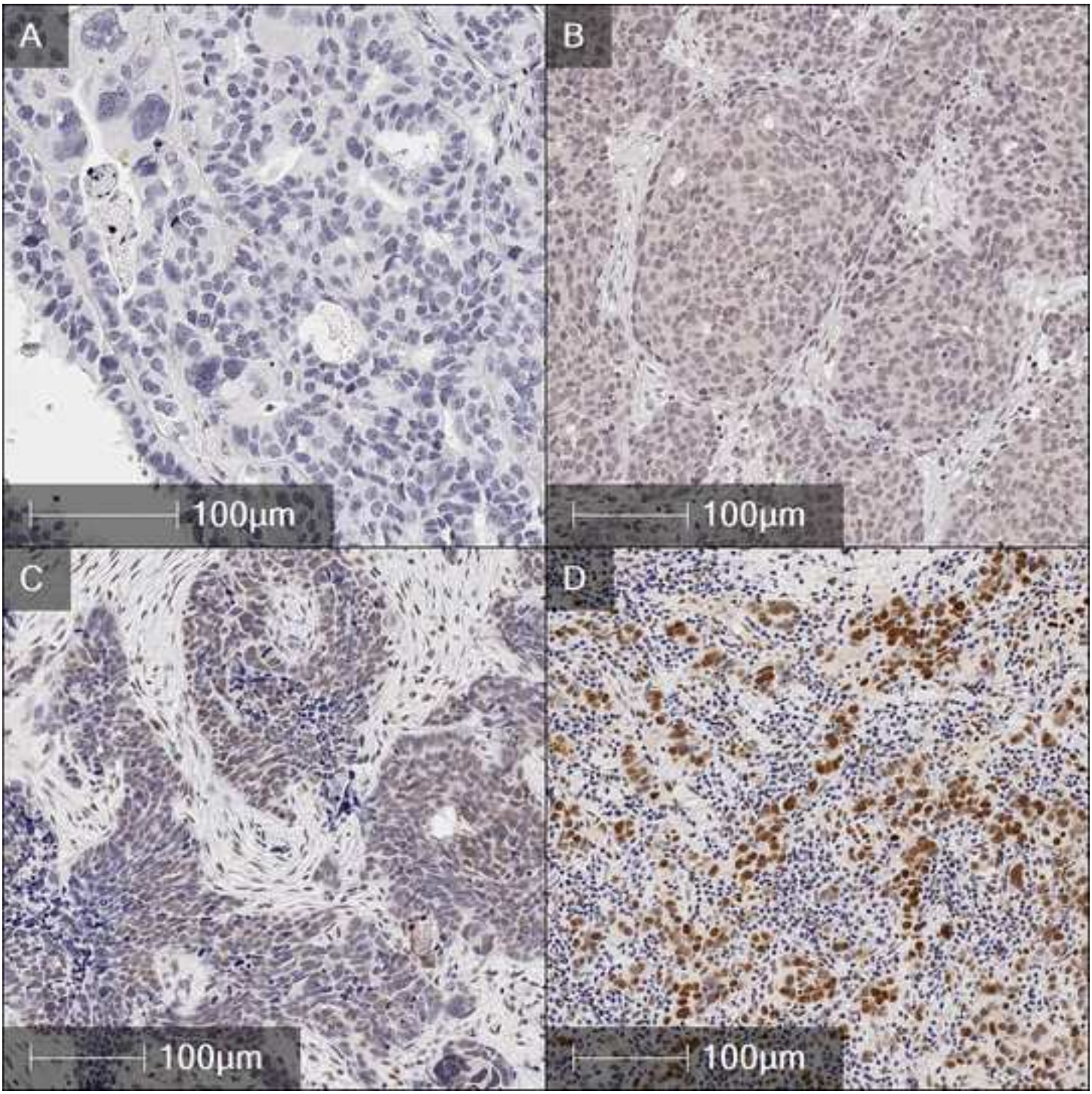

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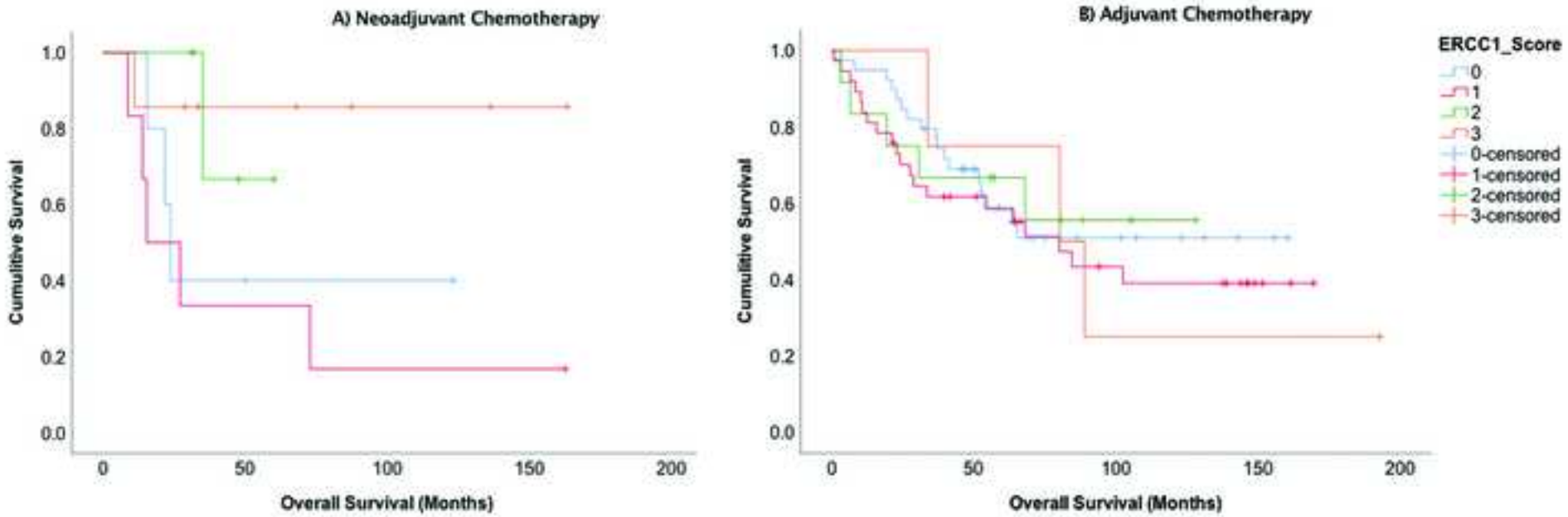

Supplementary Table 1. Mean survival time for ERCC1 and TILs expression levels in whole HGSOC cohort

ERCC1 Score	Mean Survival Estimate	Std. Error	95% Confidence Interval		χ2	df	P-value
			Lower Bound	Upper Bound			
0	97.238	10.331	76.990	117.486	3.009	3	0.39
1	84.096	10.923	62.687	105.504			
2	88.029	13.009	62.532	113.526			
3	126.110	24.746	77.608	174.612			
TILs Score	**Mean Survival Estimate**	**Std. Error**	**95% Confidence Interval**		**χ2**	**df**	**P-value**
			Lower Bound	**Upper Bound**			
0	79.168	11.383	56.858	101.478	9.172	3	0.027
1	74.376	10.870	53.070	95.682			
2	108.893	14.618	80.242	137.543			
3	147.561	16.035	116.133	178.990			

6. Supplementary Material

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Supplementary Table 2. Cox proportional hazard regression analysis for independent risk factors affecting overall survival

	B	SE	Wald	df	Sig.	Exp(B)
Age	.033	.021	2.503	1	.114	1.033
Stage	.242	.184	1.733	1	.188	1.273
Grade	-.479	.342	1.956	1	.162	.620
ERCC1 Score	.232	.360	.415	1	.520	1.261
TILs Score	-.961	.364	6.966	1	.008	.383

6. Supplementary Material

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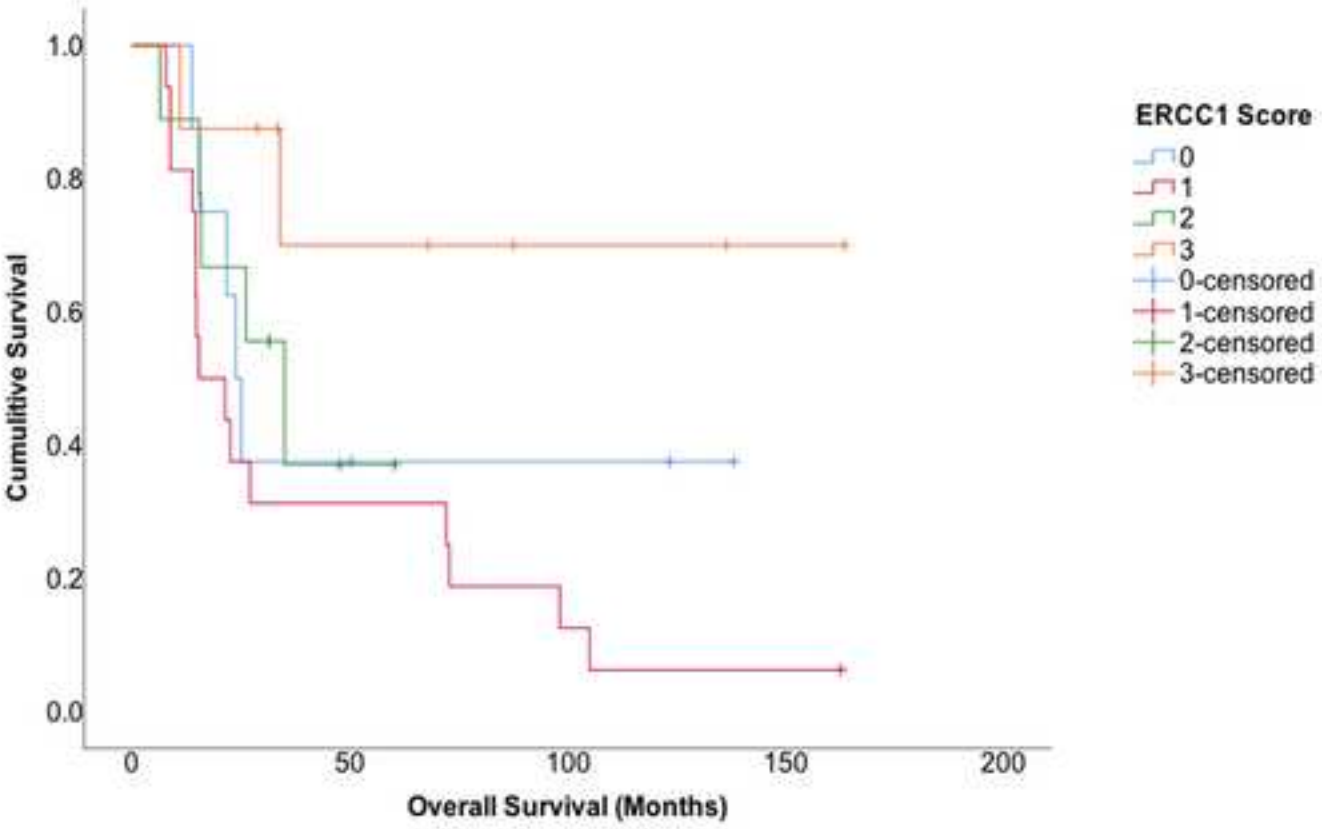
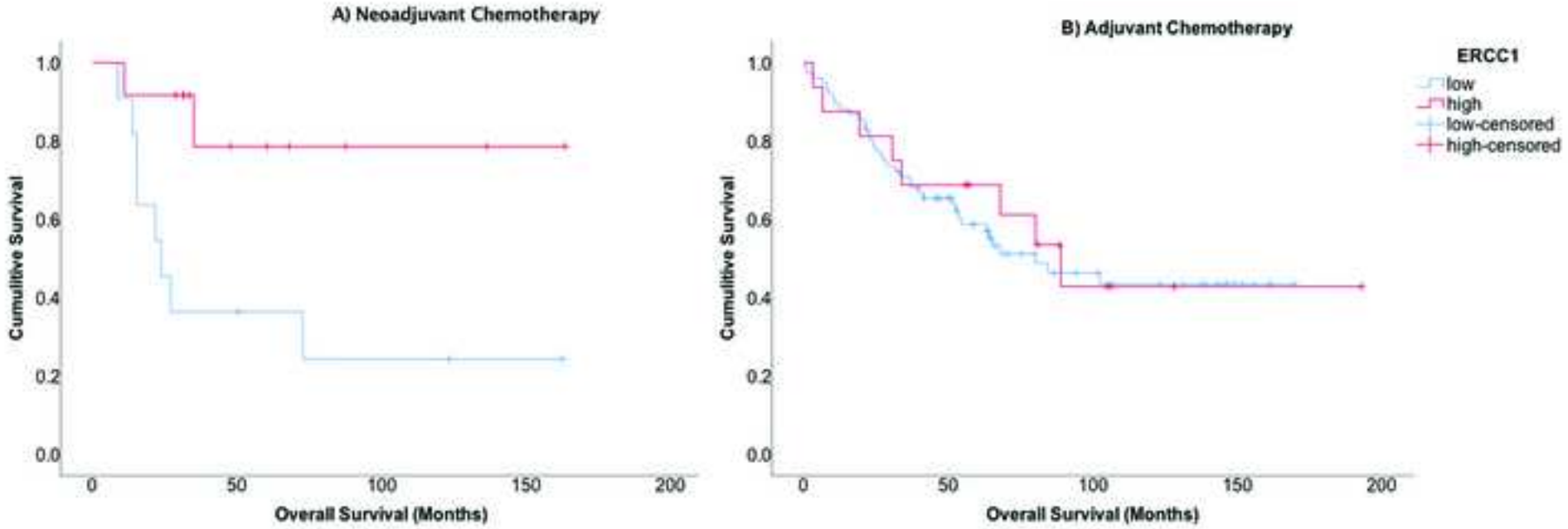


6. Supplementary Material

Click here to download high resolution image



***7. Highlights (for review)**

Highlights:

- ERCC1 is expressed 2-fold higher after platinum chemotherapy
- Significantly longer survival of patients with high ERCC1 after platinum chemotherapy
- Tumour-infiltrating lymphocytes associated with high ERCC1
- Potential for ERCC1 to be a prognostic marker of response to platinum chemotherapy