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ERCC1 (Excision repair cross-complementing 1) expression in pT2 gallbladder cancer is a prognostic factor

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Summary. Gallbladder cancer (GBC) is the main cause of death by malignant tumour in women in Chile. There is no information regarding the role of excision repair cross-complementing group 1 (ERCC1) in GBC. Our aim is to determine the expression and significance of ERCC1 as a prognostic factor in GBC.

Tissue microarrays were prepared using 200 surgically resected GBCs and 50 non-malignant gallbladders as controls. In 190 cases, ERCC1 was determined by immunohistochemistry. The correlation between ERCC1 expression and GBC pathological characteristics and patient survival were analysed.

Ninety-five percent of the non-malignant gallbladder epithelia showed intense and diffuse ERCC1 expression. GBC cases showed ERCC1 expression in the tumour cells in 100/190 (53%) cases. The best differentiated tumours showed significantly greater expression than the less differentiated ($p<0.05$). Patients with ERCC1-positive status with subserosal carcinomas (pT2) had significantly better survival than ERCC1-negative patients at 20 and 60 months of follow-up ($p=0.005$), and the probability of dying was 6 times lower for ERCC1-positive than for ERCC1-negative patients.

Our preliminary results show that cholecystectomised patients with GBC in stage pT2 and with ERCC1 expression have significantly better survival than patients at the same stage that did not present ERCC1 expression.

Key words: Gallbladder, Gallbladder cancer, ERCC1, Immunohistochemistry, Survival, Neoadjuvance.

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Introduction

Gallbladder cancer (GBC) has been the main cause of death by malignant tumour in Chilean woman for the last twenty years (Lazcano-Ponce et al., 2001; Kapoor 2006; Andia et al., 2008) The high incidence of gallstones (almost 30% of adult Chilean women), chronic cholecystitis and the rate of cholecystectomies have been shown to play an important role in GBC pathogenesis (Chianale et al., 1990; Serra et al., 1996; Lazcano-Ponce et al., 2001).

A series of alterations at the genetic-molecular level has been described for GBC. Global genetic damage has been demonstrated amongst the most significant alterations, yet to date the exact sequence of the genetic alterations related to the progression from intraepithelial lesions (dysplasia) to invasive carcinoma is unknown (Lazcano-Ponce et al., 2001; Wistuba and Gazdar 2004).

ERCC1 (Excision Repair Cross-Complementing 1) protein plays a key role in nucleotide excision repair (NER) mechanisms. The ERCC1 gene is 15 kb in size, consisting of 10 exons, located on chromosome 19 (19q13.2-q13.3), and it encodes a subunit of the nucleotide excision repair complex. ERCC1 is one of a series of at least 20 enzymes that repairs the bulky damage to DNA that interferes with transcription and replication, inducing cell death. ERCC1 is the specific endonuclease which contains 297 amino acids and participates in recognition and excision at 5' end of damaged DNA (Boulikas, 1997; Araujo and Wood, 1999). In normal cells, the elevated expression of the ERCC1 protein is related to an increase in DNA repair activity (Yagi et al., 1997; Cheng et al., 1999; Vogel et al., 2000). An increase in ERCC1 expression with high levels of messenger RNA (mRNA) determines a phenotype that is clinically resistant to treatment with

platinum-derived drugs (Olaussen et al., 2007; Simon et al., 2007; Vilmar and Sorensen 2008) in ovarian, stomach, colon and non-small cell lung cancer (Rosell et al., 2005; Olaussen et al., 2006, 2007; Martin et al., 2008). Immunohistochemical studies have demonstrated a similar phenomenon (Handra-Luca et al., 2007; Jun et al., 2008; Lee et al., 2008).

GBC treatment to date has been predominantly surgical (de Aretxabala et al., 1990, 1997; Taner et al., 2004). Adjuvant treatment might have an impact on survival in locally advanced GBC (Gallardo et al., 2001; Alberts et al., 2005; Misra et al., 2006); however, there is no information about abnormalities of the NER systems, particularly ERCC1, in GBC. The aim of this study was to determine the levels of ERCC1 protein expression in GBC and correlate it with clinico-pathological features, including survival.

Materials and methods

Cases

200 GBC were selected from the GBC files of the hospital in Temuco, Chile between 1990 and 2004. In all cases, the diagnosis was made from a cholecystectomy specimen. The level of tumour infiltration was established using a serial study of the entire gallbladder (mapping) (Roa et al., 1990, 1999). Thirty early pT1 carcinomas (15 mucosal and 15 muscular) and 170 advanced (135 pT2 subserosal carcinomas, and pT3 35 serosal carcinomas) were selected. None of the patients received adjuvant chemotherapy or radiotherapy. Fifty gallbladders without acute inflammation, dysplasia or cancer from the Pathology Service at the Clinica Alemana in Santiago were used as controls. The tumours were classified according to the WHO and AJCC classifications (Albores-Saavedra et al., 1992; AJCC, 2002).

Preparation of Tissue Microarrays (TMA)

Three representative areas of each tumour and the controls were selected for TMA; the semiautomatic Tissue MicroArrayer Pathology Device, Inc.™ was used with 2 mm needles.

Immunohistochemistry

The standard technique for formalin-fixed and paraffin-embedded tissue was used. The 4-micron histological sections from the TMA were deparaffinised and hydrated in decreasing alcohol concentrations. Antigen retrieval was performed in citrate buffer pH 6.0 in a microwave oven and washed in PBS pH 7.4. The anti-ERCC1 antibody (monoclonal antibody ERCC1 Ab-2 [clone 8F1] Labvision™) was used, diluted to 1/100. The primary antibody was incubated at room temperature for 60 minutes and then incubated with the complex Super Picture Polymer Detection Kit™ Zymed in a Dakoautostainer™ .

Immunohistochemical analysis

The intensity control was evaluated in endothelial cells in tonsils (assigned an intensity of 2), and graduated as: 0, negative; 1+, weak; 2+, moderate; and 3+, intense. T control (Olaussen et al., 2006).

In GBC cases, stains of epithelial cells from adjacent non-tumorous mucosa were used as a positive internal control. Positivity was measured in each case by two observers and classified based on the intensity and percentage of positivity of the tumour cells. According to the criteria used by other authors (Olaussen et al., 2006; Soria 2007), a case was considered positive where at least 10% of the tumour cells had a strong nuclear stai ning intensity of (3+), or 50% of the tumour cell nuclei had a staining intensity of (2+).

Statistical analysis

This was done using chi-square and Fisher's exact tests for the contingency tables. Multiple logistic regression analysis was performed and the ERCC1 status was adjusted for age, gender, histological differentiation degree and metastasis at the initial diagnosis. Patient follow-up cut off at 120 months and Kaplan-Meier actuarial survival curves were calculated with a significance analysis using a log-rank test (Cox-Mantel).

Results

GBC case characteristics

Of the 200 cases immunostained for ERCC1 in the TMAs, staining was evaluable in 190 cases. In the 10 remaining cases, the quantity of tumour tissue did not allow their evaluation. Patients' clinico-pathological characteristics are summarised in Table 1. In all patients

Table 1. Histopathological characteristics of the cases.

Patients	Total	Female	Male
n	190	166	24
Age	62.7 (±14.5)	61.7 (±14.4)	69.8 (±13.2)
Tumours			
Gallbladder Wall Infiltration		ERCC1(+)	%
Mucosa		9/13	69.23
Muscular		10/15	66.67
Subserosa		61/127	48.03
Serosa		20/35	57.14
Histology Differentiation		ERCC1(+)	%
Well		14/23	60.87
Moderate		70/128	54.7
Poor		16/39	41.0

the presence of gallstones was confirmed prior to cholecystectomy. In only 21% of the patients was the GBC diagnosis suspicious pre-operatively. In 41% of the patients the preoperative diagnosis was chronic cholecystitis and in 38% an acute cholecystitis was coexistent. Cystic lymph node examination was possible in only 54 cases (28.8%) of which 31 cases were negative (57%) and 23 lymph node showed metastasis.

The GBC cases were composed of 13 mucosal (7%), 15 muscular (8%), 127 subserosal (67%) and 35 serosal carcinomas (18%). Of these, 15% were considered early carcinomas (mucosal and muscular) and 85% advanced carcinomas (subserosal and serosal). Twelve per cent (23 cases) of cases were well differentiated tumours, 67% (128 cases) moderately differentiated and 39 cases poorly differentiated tumours. None of the patients received adjuvant therapy.

Immunohistochemical results

The nuclear expression of ERCC1 in non-malignant gallbladders mucosa (controls) was positive in almost all cases with 80 to 100% positivity in the nuclei with an intensity of 2+ to 3+. The immunostaining was intense, homogeneous and diffuse in most of the epithelial cell nuclei of the non-malignant gallbladder mucosa (Fig. 1). The greatest intensity and percentage were observed in the superficial portions of the mucosa rather than in the deep areas of the glandular epithelium, and to a lesser extent in the glandular groups of the pyloric metaplastic foci.

The positive expression of ERCC1 was observed in 100/192 (52.6%) of the GBC. Most tumours presented a slight to moderate positivity and only 24% of the carcinomas had a strong positive expression over 80% of

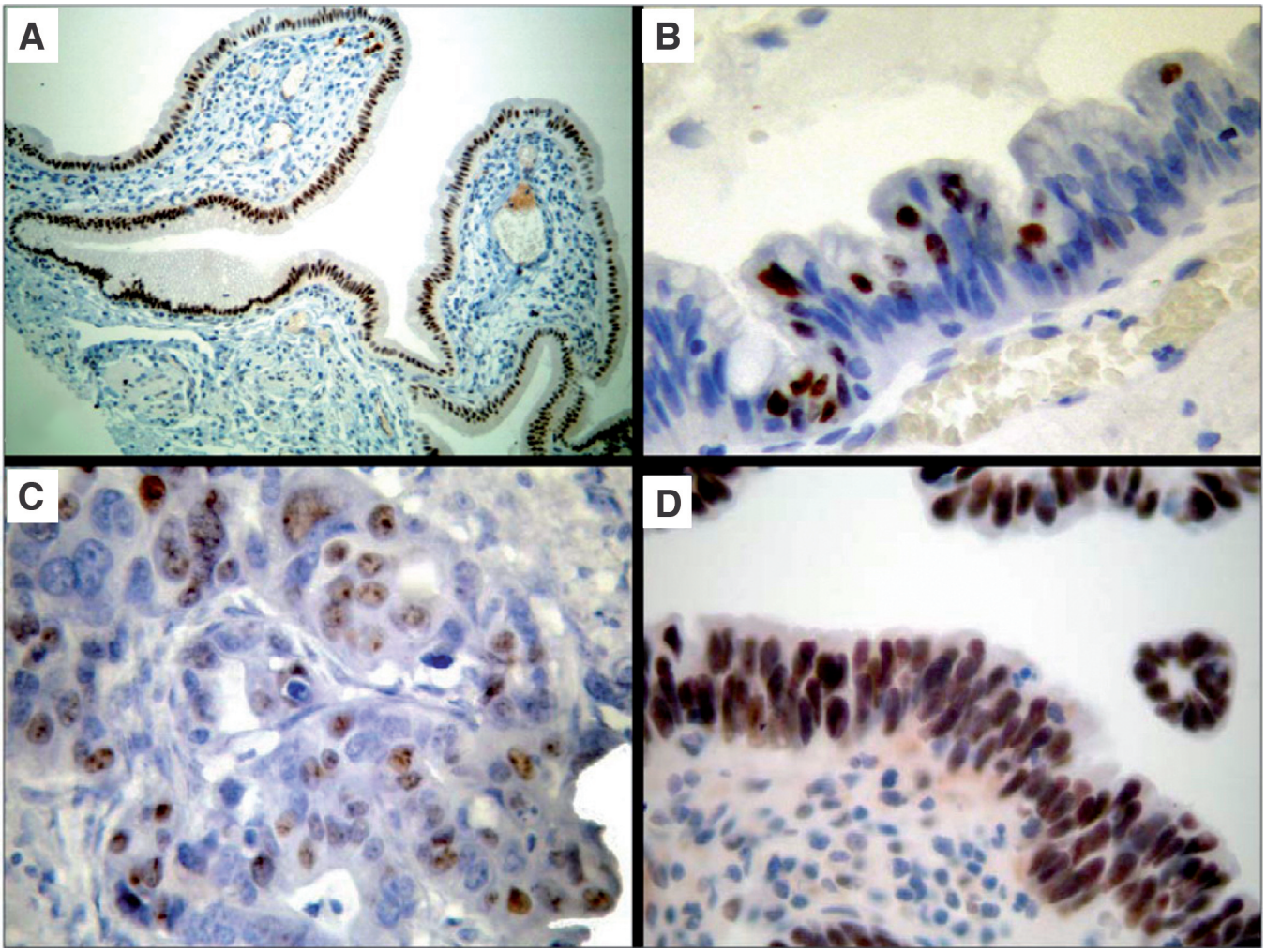


Fig. 1. ERCC1 expression in the non-malignant gallbladder mucosa. Intense, nuclear positivity can be seen in most of the epithelial cells (**A**). ERCC1 expression in gallbladder adenocarcinomas with positivity in only a few malignant cells (less than 10%) (**B**), with granular and irregular distribution of the staining (++) (**C**), and intense nuclear staining in nearly 100% of tumour cells (**D**). A, x 32; B-D, x 200

the tumour cells with an intensity of 2+ or 3+, comparable to what was observed in the controls (Fig. 1).

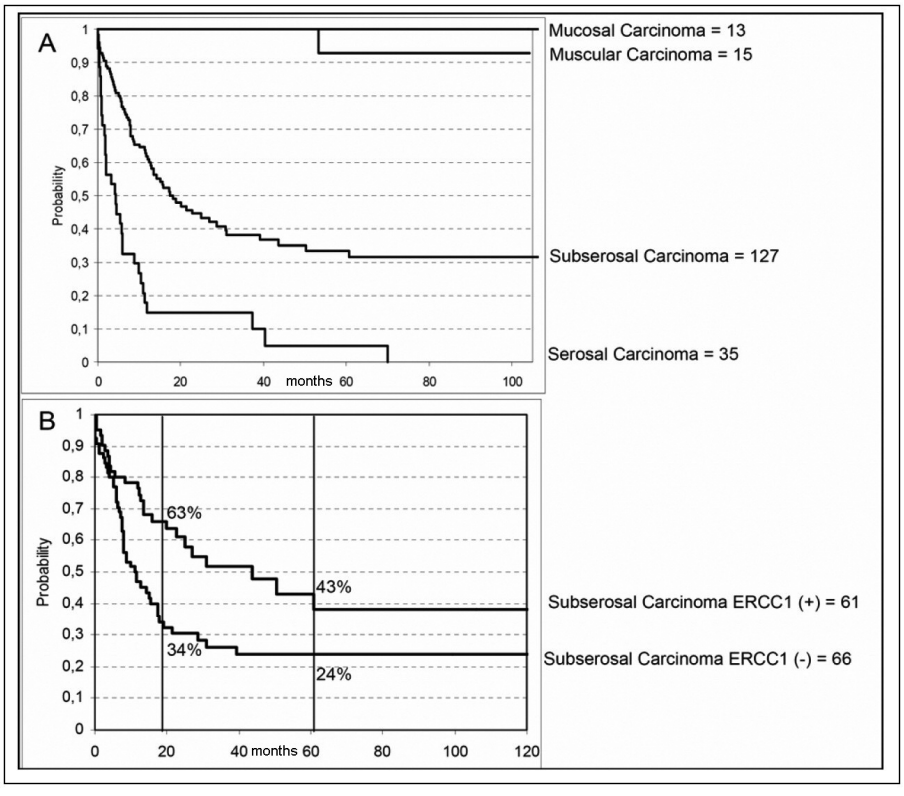
The best differentiated tumours had greater positivity than the less differentiated tumours (61% well differentiated; 55% moderately differentiated and 41% poor differentiated; p=0.05). A non-significant trend in the correlation between the level of tumour infiltration of the gallbladder wall and a lower level of ERCC1 expression was observed. In the mucosal and muscular invading carcinomas, the ERCC1 expression was detected in 69% and 67% of cases, respectively; however, in the subserosal and serosal tumours, the expression was observed in 48% and 57% of the cases, respectively.

Survival of patients with gallbladder cancer

The overall survival of the patients with GBC included in this study in relation to the tumour infiltration level in the gallbladder wall is shown in Fig. 2. The overall survival of the group studied was 50% at 20 months and 39% at 5 years. The mucosal carcinomas had 100% survival at 5 years and the muscular carcinomas 92%. By contrast, 85% of the patients with serosal compromise died before 14 months with only 4% survival at 5 years. The subserosal carcinomas (pathological T2) had 32% survival at 5 years. These results were similar to our previously published series. Thus, the tumour infiltration level of the gallbladder wall was one of the most important prognostic factors (p<0.0001) (Roa et al., 2002a,b).

ERCC1 expression and GBC prognosis

No relation was observed between survival and ERCC1 expression in the early GBC group. In the advanced (subserosal and serosal) carcinomas, survival was better in ERCC1-positive patients (p=0.01). Nevertheless, when separating the advanced carcinomas into subserosal (pT2) and serosal (pT3), this difference was observed only in subserosal tumours. In those GBCs, survival reached 63% in the ERCC1-positive versus 34% ERCC1- negative cases at 20 months after the cholecystectomy, and 43% and 24% at 5 years, respectively. The percentage difference in survival rate and ERCC1-positive status was 29% at 20 months and 19% at the 5-year follow-up (p=0.005) (Fig. 2). In the multiple logistic regression analysis, no differences were observed in the probability of dying in relation to such variables as age, gender or histological differentiation degree (Table 2). The patients with pT2 tumours with



A = p <0.001
B = p <0.005

Fig. 2. Kaplan-Meier curve showing overall survival of the 190 patients with GBC (**A**). A significant correlation is observed between tumour infiltration level in the gallbladder wall and patient survival. ERCC1 expression in subserosal (pT2) GBC (**B**). Patients with ERCC1-positive tumours show a significantly better prognosis than patients at the same stage with no expression.

Table 2. Multiple logistic regression analysis. Probability of dying in relation to age, gender or histological differentiation degree, and ERCC1 status.

Death	Odds Ratio	Std. Err	z	P>|z|	95% Conf.	Interval
Ercc1	6.16940	2.71649	4.13	0.001	2.60282	14.6231
Gender	0.948264	0.617325	-0.08	0.935	0.264723	3.39676
Age	0.684219	0.313148	-0.83	0.407	0.27901	1.67790
Metastasis	2.77397	1.48419	1.91	0.057	0.972018	7.91645
Differentiation	2.43442	2.27120	0.95	0.340	0.391077	15.1540

Number of obs: 127; LR chi2: 25.51; Prob>chi2: 0.0001; Log likelihood: -65.213216; Pseudo R2: 0.1636

metastasis at initial diagnosis had a probability of dying almost three times higher than those without (adjusted for ERCC1 status, age, gender and histological differentiation degree ($p=0.057$)). Nevertheless, the probability of dying was 6 times lower for the ERCC1-positive patients than for the ERCC1-negative patients, adjusted for age, gender, histological differentiation and metastasis ($p<0.001$).

Discussion

Immunohistochemical expression of ERCC1 in the non-malignant gallbladder epithelium was observed in most of the mucosal epithelial nuclei in over 90% of the cases. In the chronically inflamed gallbladder mucosa, this fact could suggest the indemnity and/or overexpression of the DNA repair mechanisms related to the NER system due to chronic mucosal damage (Cheng et al., 1999; Latimer et al., 2003). The up-regulation of DNA repair genes has been associated with inflammatory activity in the liver. Increased DNA repair activity may reflect increased DNA damage as a result of chronic liver injury (Zindy et al., 2005).

In the neoplastic gallbladder epithelium, ERCC1 expression was considered positive in 53% of the cases with significantly less intensity than in the non-malignant epithelium. In only 11 cases (6%) did the tumour cells show positivity comparable to that observed in the non-tumorous gallbladder epithelia. Of these cases, 7 were mucosal carcinomas and 4 were muscular, all patients with 100% survival at 5 years. This may suggest a relation between the loss of protein expression and the most advanced stages of the disease, probably associated with a decrease in histological differentiation, as has been demonstrated in other neoplasias (Liang et al., 1995; Reed et al., 2003; Takenaka et al., 2007). The early tumours showed a greater expression of ERCC1 than the advanced tumours. These differences might be a result of the progressive loss of DNA repair mechanisms during tumour cell progression (Cheng et al., 2000; Andrew et al., 2003). Some studies have proven a link between the degree of cell differentiation and the mRNA levels of this gene (Rubin, 1988; Handra-Luca et al., 2007). It has been suggested that an intact DNA repair mechanism may reduce the accumulation of genetic aberrations and the intratumoral ERCC1 that is involved in tumour DNA repair contributes to a decrease in malignant potential, and therefore in the risk of relapse after definitive treatment (Simon et al., 2005).

In the group with subserosal carcinomas (pT2), the patients with ERCC1-positive tumours had a significantly better survival at 20 and 60 months of follow-up. The multiple logistic regression analysis did not show any differences in the probability of dying in relation to age, gender or histological degree differentiation at this tumour stage. The patients with pT2 tumours with metastasis had a probability of dying almost three times higher than those without metastasis at the time of diagnosis. The probability of dying in the ERCC1-positive patients was 6 times lower than the ERCC1-negative patients, adjusted for age, gender, histological differentiation degree, and presence of metastasis ($p<0.001$). Thus, new elements should be considered in the evaluation of pT2 patients, such as: quantification of subserosal invasion (Roa et al., 1990), morphometry of tumour size, location and volume (Elpek et al., 1999), Rokitansky-Aschoff sinus evaluation (Yamaguchi et al., 1992), or markers like ERCC1, which may contribute to distinguishing groups of patients with different prognoses.

Our results show that ERCC1 status in patients with pT2 GBC could be considered a prognostic factor in those patients who do not undergo adjuvant therapy. These findings will need to be validated using molecular techniques such as ERCC1 mRNA RT-PCR. The relation between the novel biomarkers, such as ribonucleotide reductase M1 (RRM1) and ERCC1 expression (Zheng et al., 2007), opens up a range of possibilities in the clinical use of the levels of these proteins as predictive factors in the GBC response to platinum- and gemcitabine-based chemotherapy (Bepler et al., 2006; Rosell et al., 2006). In the future, ERCC1 status may be a factor to consider in the selection of adjuvant therapy when treating GBC.

In our opinion, evidence for the clinical utility as a prognostic factor of ERCC1 status in pT2 gallbladder cancer is still insufficient, and new studies are needed. Nevertheless, these findings should be taken into account, especially in gallbladder cancer, where the existing information is very scarce.

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