SAFRA KESESİ KARSİNOMLARINDA MİKROSATELLİT İNSTABİLİTE, ÖSTROJEN RESEPTÖRÜ VE CerbB2 EKSPRESYONUNUN SAĞKALIMA ETKİLERİ NELERDİR?

WHAT ARE THE EFFECTS OF MICROSATELLITE INSTABILITY, ESTROGEN RECEPTOR AND CerbB2 EXPRESSION ON SURVIVAL IN GALLBLADDER CARCINOMA?

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Geliş Tarihi / Received: 25.11.2021
Kabul Tarihi / Accepted:24.12.2021
Araştırma Makalesi/Research Article
DOI: 10.38065/euroasiaorg.912

ÖZET

Safra kesesi karsinomlarında tedavi seçenekleri hala çok sınırlı olup prognostik faktörler tartışmalıdır. Bu çalışmanın amacı, mikrosatellit instabilite (MSI), östrojen/progesteron reseptörü, CerbB2 (HER2/Neu) durumu ve diğer prognostik parametrelerin sağkalım ile ilişkisini araştırarak patogenetik, prognostik ve hedefe yönelik terapötik bilgilere katkı sağlamaktır.

Bu retrospektif çalışmaya 2008-2020 arasında iki merkezde tanı alan safra kesesi karsinomları alınmıştır. Tümörlerin histopatolojik özellikleri yanısıra immünhistokimyasal olarak, MSI için MLH1, MSH2, MSH6, PMS2; östrojen reseptörü alfa (ER-α) / progesteron reseptörü (PR) ve CerbB2 durumları araştırılmıştır. CerbB2 pozitifliği, Silver-enhanced in situ hybridization (SISH) yöntemi ile doğrulanmıştır.

Çalışmaya 75 safra kesesi karsinomu olgusu alınmıştır. Bir olguda (%1.3) ER-α immünboyanması, 25 olguda (%33.3) SISH ile doğrulanmış CerBB2 pozitifliği ve 3 olguda (%4) MSI saptanmıştır. İstatistiksel analizde, grad, vasküler invazyon, perinöral invazyon ve sağkalım arasında anlamlı ilişki bulunmuştur ($p<0.05$). CerbB2 pozitif olgularda sağkalım (46 ay) negatif olgulardan (15 ay) anlamlı olarak daha uzundur ($p=0.021$).

Safra kesesi karsinomlarında yapılan bu çalışmada, tümör gradı, perinöral invazyon, ve vasküler invazyon sağkalımı olumsuz yönde etkilemiştir. CerbB2 pozitifliği her üç olgudan birinde saptanmış olup bu olgularda sağkalım daha uzundur. Östrojen pozitifliği çok nadiren gözlenmiştir. MSI durumunu araştırmak uygun olabilir.

Anahtar Kelimeler: Safra kesesi karsinomu, sağkalım, CerB2, östrojen reseptörü, mikrosatellit instabilite

ABSTRACT

Treatment options for gallbladder carcinomas are still very limited and prognostic factors are controversial. The aim of this study was to investigate the relationship between microsatellite instability (MSI), estrogen / progesterone receptor, CerbB2 (HER2 / Neu) status, as well as other prognostic parameters, and survival, in order to contribute to pathogenetic, prognostic and targeted therapeutic information.

In this retrospective study cases of gallbladder carcinoma diagnosed in two centers between 2008-2020 were included. Besides histopathological features of the tumors, the expression of MLH1, MSH2, MSH6, PMS2 for microsatellite instability (MSI), estrogen receptor alpha (ER-α) / progesterone receptor (PR) and CerbB2 status were immunohistochemically investigated. CerbB2 positivity was verified by Silver-enhanced in situ hybridization (SISH) method.

Seventy-five gallbladder carcinomas were included. In one case (1.3%) ER-α immunostaining, in 25 cases (33.3%) SISH- confirmed CerbB2 positivity and, in 3 cases (4%) MSI were detected. Statistically, a significant relationship was found between grade, vascular invasion, perineural invasion and survival ($p < 0.05$). Survival in CerbB2 positive cases (46 months) was significantly longer than negative ones (15 months) ($p = 0.021$).

In this study on gallbladder carcinomas, tumor grade, perineural invasion and vascular invasion had negative effect on survival. CerbB2 positivity was seen in every three case and they showed a longer survival. Estrogen positivity was very rarely observed. MSI might be worth investigating.

Keywords: Gallbladder carcinoma, survival, CerbB2, estrogen receptor, microsatellite instability

1. INTRODUCTION

Gallbladder carcinoma ranks sixth among gastrointestinal malignancies and first among biliary carcinomas. Its overall incidence is less than 2 / 100,000. This cancer, whose distribution shows geographical differences, is reported with the highest rate in India, Far East Asia and South America. It is considered to be a disease of the elderly group, is more common over the age of 65 and, 2-3 times more common in women.[1,2] Since there is no serosal surface in the neighborhood, it easily infiltrates the liver and has a very aggressive course with five-year survival less than 5% and a mortality rate of 11.8/100,000.[1,2] The factors affecting its prognosis continue to be discussed and treatment options are still quite limited. The prognostic significance of lymph node metastasis and local spread of the primary tumor is well established. However, molecular studies are expected to determine specific markers for its prognosis and treatment protocol.[3,4]

In this study, in order to contribute to pathogenetic, prognostic and targeted therapeutic information, we aimed to investigate the prognostic significance of various morphological parameters in gallbladder carcinoma, as well as to investigate the relationship between microsatellite instability (MSI), estrogen / progesterone receptor, cerbB2 (HER2/Neu) status and survival,.

2. MATERIALS AND METHODS

In this retrospective study, cases of gallbladder carcinoma operated by open or laparoscopic cholecystectomy and diagnosed in two centers between 2008 and 2020, were included. After the age and gender of the cases were recorded, all diagnostic blocks and Hematoxylin-Eosin stained slides extracted from the pathology archives were re-evaluated. Histological type, histological grade, vascular invasion, perineural invasion, as well as tumor invasion in cases where lymph node and/or liver tissue was sent, were recorded. Tumor stages were classified according to the TNM classification. Survival data were obtained from the Death Notification System (DNS) of the Hospital Information Management System.

Immunohistochemical analysis

The expression of MLH1, MSH2, MSH6, PMS2 for microsatellite instability, estrogen receptor alpha (ERα), progesterone receptor (PR) and CerbB2 were immunohistochemically investigated in malignant cells. In all cases, a representative paraffin block that contained both meoplastic and normal tissue was chosen for immunohistochemical (IHC) analysis. Immunostaining was performed on 3 micron-deparaffinized sections using the standard avidin– biotin–peroxidase complex method with automated immunostainer (Ventana, Medical System BenchMark ULTRA / ISH Staining module). The following procedures were applied using the Ultraview Universal DAB Detection Kit; Antigen recovery in pH8 (CC1) with EDTA, antibody incubation for 50 minutes, background staining with Harris hematoxylin for 16 minutes, bluing reagent (Ventana Medical System) for 4 minutes, washing the slides with detergent water, rinsing with alcohol, drying and covering with xylene based sealer. The immunomarkers used are shown in Table 1.

For CerbB2 examination, according to American Society of Clinical Oncology (ASCO)/College of American Pathologists (CAP) Clinical Practice Guideline, complete membranous staining within >10% of cells was considered 3 positive (+) staining; and incomplete and/or weak/moderate staining within >10% was considered 2+, equivocal staining.[5] In ERα and PR examination, staining in the nuclei of ≥ 1% tumor cell was accepted as positive. Positive and negative controls for antibodies were also run simultaneously. In MLH-1, MSH-2, MSH-6 and PMS-2 evaluation, complete loss of nuclear staining was accepted as DNA mismach gene mutation. Adjacent gallblader tissue and fibrous stroma of the neoplasia were used as positive control.

In situ Hybridization Method

Silver-enhanced in situ hybridizasyon (SISH) analysis was done for cerbB2 (2+) cases. For HER-SISH, 4 micron sections were taken onto adhesive-coated slides and stained with automated instrument (Ventana Medical Systems, Tucson, AZ, USA). Deparaffinization, incubation in citrate buffer and ISH Protease were performed accordingly. The slides were denatured, hybridized and then, were subjected to SISH multimer for 16 min, and silver chromogen for 4 min. The samples were incubated with Mayer's haematoxylin and with bluing reagent to stain the background. Optimal incubation durations were selected for each step and material to protect the tissue morphology while visualising the signals. SISH positive cases were considered positive for cerbB2 expression.

Statistical analysis

In statistical analysis, Chi-square test was used for comparison of categorical values and Kaplan-Meier survival test, for survival analysis. Independent prognostic parameters were determined by multivariate Cox-regression analysis with the participation of parameters that were at the limit of significance (p <0, 100). Data with the p value of ≤0.05 were considered statistically significant.

Ethics Committe approval: 2020/58.

3. RESULTS

Clinical Findings

Seventy-five gallbladder carcinoma cases (21 men, 54 women) were included in the study. Lymph node dissection was performed in 38 (50.6%) of the cholecystectomy cases, and liver tissue was present in 20 (26.6%) cases. The mean age of the patients was 64 (26-85 years). The size of the tumors was between 0.4 - 25 cm (mean 3.58 cm).

Histopatological Findings

Histopathological examination revealed 69 adenocarcinoma, NOS (92%), one mixed adenoneuroendocrine tumor, one adenosquamous carcinoma, one mucinous adenocarcinoma and

three signet ring cell carcinoma (Fig. 1). The grade, depth of invasion, vascular invasion, perineural invasion and dysplasia in the surrounding tissue of the cases are presented in Table 2. Lymph node metastasis was found in 18/38 (47.3%) cases and liver metastasis was detected in 14/20 (70%) cases.

Twenty-four (32%) patients were alive, and these patients had a follow-up period of 2 -152 months. Fifty-one patients (68%) had died within 1 day-56 months, but causes of death were not specified in DNS. The demographic and histopathological parameters of the patients and survival durations are presented in Table 2.

Immunohistochemical Findings

In immunohistochemical analysis, ERα expression was seen in only one of 75 cases (1.3%) (Fig. 2) and its characteristics are shown in Table 3. PR positivity was not detected in any case. According to the ASCO / CAP Clinical Practice Guideline, 30 cases showed (1+), 17 cases (2+) and 10 cases (3+) cerbB2 immunostaining (Fig. 3). By SISH method, 15 of 17 CerbB2 (2+) cases were found positive. With the addition of SISH positive cases, the cases were re-grouped as CerbB2 positive (n = 25, 33.3%) and negative (n = 50, 66.6%). Of the CerbB2 positive cases, 6 (8%) were Grade 1, 11 (14.6%) were Grade 2, 8 (10.6%) were Grade 3. There was no statistically significant relationship between Grade and CerbB2 positivity (p = 0.497). CerbB2 expression was not detected in T1a cases, while it was observed in 5 T1b (6.6%), 14 T2 (18.6%) and 6 T3 (8%) cases. No significant relationship was found between the depth of invasion and CerbB2 expression (p = 0.411). In univariate analysis, a statistically significant survival result was obtained with grade, vascular invasion, perineural invasion and CerbB2 (Table 2, Fig. 4). In the multivariate analysis, CerbB2 positivity (p = 0.056) and the absence of perineural invasion (p= 0.023) were determined as independent prognostic factors for survival.

When the cases were examined in terms of MSI, DNA mismatch repair (MMR) protein expression was lost in 3 cases (4%) (1 MSH2, 1 MSH2 + MSH6 and 1 PMS2) (Fig. 5 and 6). The clinical and morphological characteristics of the cases are presented in Table 4.

4. DISCUSSION

Currently, surgical resection, conventional chemotherapy and radiotherapy are the preferred treatment methods in gallbladder cancers, however, treatment success levels are still low. Therefore, there is intensive research to understand the molecular pathogenesis of gallbladder cancer and, to find targeted and personalized treatments. Identifying the pathways responsible for the molecular basis of cancer is important as it will enable more effective and safe treatment options to be used.

Gallbladder carcinoma most frequently develops through metaplasia, dysplasia, carcinoma in situ and invasive carcinoma steps, while, rarely (less than 3%), classical adenoma-carcinoma sequence is observed. Moreover, various genomic changes such as somatic mutations, microsatellite instability (MSI), heterozygous loss, gene overexpression, epigenetic changes, and those associated with miRNA have been reported.[2] Various cell signaling pathways such as the ErbB pathway, the PI3K / Akt / mTOR pathway, the MAPK / ERK pathway, and the VEGF pathway suggested to have a role in tumor development. There are probably complex interactions at the DNA, RNA level and epigenetic level in pathogenesis. The most frequently mutated genes are TP53, KRAS and ErbB pathway genes while MSI pathway is responsible in about 10% of the cases. Since gallbladder cancers are more common in women, the role of female sex hormones, estrogen and progesterone, in the pathogenesis and prognosis of gallbladder cancer is also an area of research.[2,6-9]

CerbB2 and its importance in gallbladder cancer

Mutations in the ErbB pathway genes, which are the major growth factor receptor genes, play a role in many types of cancer, especially breast and stomach tumors. CerbB2 expression has been reported in 5-48 % of gallbladder tumors [10,11] and, in our study, we found a similar rate (33.3%). CerbB2 expression status of a gallbladder cancer is, firstly, might have a relationship with prognosis although studies reveal conflicting data on this subject. Some have not found any significant correlation between cerbB2 and survival[12] while, in 186 surgically resected gall bladder adenocarcinoma, a higher mortality rate was detected in CerbB2 positive group compared to negative group.[11] In another study, 5-year survival was 41% in CerbB2 negative cases, and 34% in positive cases.[13] However, in a study by Kumari et al., though insignificant, CerbB2 positive cases were found to have a longer median survival than negative ones (median survival, 30 months in CerbB2 positive cases and 12 months in negative cases, P = 0.15).[14] In our study, the median survival was longer in CerbB2 positive cases compared to negative ones with a statistically significance. In gallbladder cancers, prognostic significance of lymph node metastasis, local spread of the primary tumor and presence of distant metastasis are well-accepted. When the relationship of CerbB2 positivity with those prognostic factors were investigated, in some studies, no correlation was found between the increase in CerbB2 expression and parameters such as stage, grade, tumor type or metastasis,[14] while Pujani et al. found a correlation between CerbB2 positivity and liver involvement (P = 0.05).[15] In various studies, it has been reported that most of the positive cases were elderly, female, poorly differentiated, stage IV and showed distant metastasis.[11,13] On the contrary, there are publications reporting that CerbB2 expression diminishes as the stage and grade increase.[7,14] Although CerbB2 positivity showed a positive correlation with survival in our series, causes of death could not be obtained from the registry which is a limitation of our study. Also, it was not associated with any prognostic parameter including lymph node involvement and liver metastasis. We think that, although CerbB2 is a poor prognostic marker in many tumors including breast cancer, it may not be reliable for gallbladder cancer.

The second importance of identifying CerbB2 status in cases of gallbladder cancer is related to smart drug use.[16] Treatments targeting the HER2/ EGFR pathways are preferred in various gastric and mammary malignancies and provide survival benefit.[17,18] More than 10% of the patients with advanced gallbladder cancers are expected to benefit from anti-HER treatments.[13] There are few publications about the benefit of using anti-HER therapy in gallbladder cancers and most of them are in the form of case reports. In one report, in a case of gallbladder cancer with brain metastasis, with no accompanying chemotherapy, remission was achieved with trastuzumab and lapatinib treatment after 4 months.[17] Moreover, recurrence-free 5-year survival has been reported after trastuzumab and radical salvage treatment in a stage IV gallbladder cancer.[18] Although, some authors suggest to use FDA-approved agents such as humanized monoclonal antibodies trastuzumab, pertuzumab, or dual EGFR/HER2 inhibitors lapatinib and afatinib in HER2-positive gallbladder cancers,[13] we are skeptical due to our results.

Relationship between microsatellite instability and gallbladder cancer

Microsatellites are repetitive 1-5 bases DNA units found in eukaryotic genes. The changes in the repetition of microsatellites are normally repaired by the DNA-MMR system. When MMR functions are disrupted as a result of a mutation or promoter methylation, MSI occurs.[1] Immunohistochemistry (IHC) is the most widely used modality to analyse MSI status and, it helps to identify the defective MMR protein.[5] A PCR-based technique may also be used for the detection of mutations but it requires more work and technical infrastructure. In studies conducted in various tumors, IHC is proven as sensitive as PCR in identification of MSI status.

Determining MSI status can be helpful in two aspects. One of them is to differentiate patients with Lynch syndrome. These patients are at higher risk for tumors such as gastrointestinal and endometrial cancer. In addition, the families of these patients can be genetically screened to determine the mutational status. However, in the publications so far in gallbladder tumors, a relationship between

MSI (+) gallbladder carcinoma and Lynch syndrome has not been revealed. Secondly, MMR deficient tumors are susceptible to immune checkpoint blockade and they can benefit from anti-PD-1 and anti-PDL-1 treatments.[19] Thus, differentiation of gallbladder tumors with MSI might also be important in terms of targeted treatment.[5]

In the literature, MSI has been reported between 0% and 40% in PCR-MSI and immunohistochemical MMR protein loss studies in gallbladder carcinomas.[1,12] There are also studies reporting the presence of MSI in the dysplastic epithelium of the gallbladder and chronic cholecystitis and pointing to its role in carcinogenesis.[1] Currently, there is no established relationship between MMR protein loss and tumor grade, stage, tumor morphology or survival.[1,5,12] In Roa et al.'s study where they detected MSI-H at a rate of 10%, instability was observed in 33% of the intestinal metaplasia areas and in 83% of the dysplasia areas close to the tumors, and they thought that inactivation of MMR genes started in the early stages of carcinogenesis.[13] In our series, MSI was detected in three cases (4%) by IHC analysis which were not associated with Lynch syndrome. We suggest routine examination of MSI to identify the candidates for targeted and individualized management.

Estrogen and progesterone receptor status

Estrogens play an important role in hormone-dependent cancers. In the hope of giving anti-hormone therapy in gallbladder tumors, the presence of female sex hormone receptors has been investigated, however, conflicting results have been obtained in literature.[6-8] Gupta et al.'s atudy, 28% of the cases had cytoplasmic but no nuclear ER positivity and, all were well or moderately differentiated.[7] In Hryciuk et al.'s study, not ERα, but ERβ expression was detected which was associated with shorter overall survival.[8] Shukla et al., found no ER positive cases and, determined a relationship between p53 mutation, less differentiated tumor and loss of ER expression in gallbladder cancers.[6] Studies evaluating PR expression in gallbladder tumors have also conflicting result. [6,7] Gupta et al. reported 32% cytoplasmic and 52% nuclear PR positivity.[7] Shukla et al. could not show PR expression in 98% of their cases.[6] Some researchers have observed higher PR expression than ER and recommended it to be taken into account during antihormonal therapy.[7] We, due to finding only %1 ERα immunoexpression, and no PR positivity, do not suggest a routine examination.of hormonal status.

As a conclusion, gallbladder carcinomas are usually insidious tumors and most of them are inoperable when diagnosed. In our study, tumor grade, perineural invasion and vascular invasion were found to be parameters determining poor prognosis. We found CerbB2 positivity confirmed by in situ hybridization in one of every three cases (33.3%), as well as a relation with a longer survival. Since there are promising results of the few case reports published on targeted therapy in gallbladder carcinomas, routine CerbB2 immunohistochemistry might be valuable. MSI analysis might offer an advantage to identify the candidates for targeted and individualized therapy but, ER and PR immunoanalysis seem to be irrelevant.

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FIGURE LEGENDS:

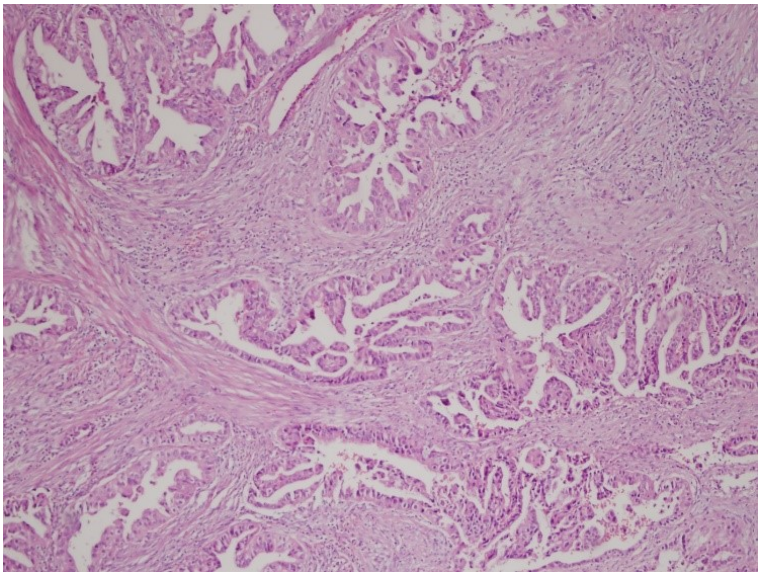

Figure 1. Adenocarcinoma, NOS (H-Ex100)

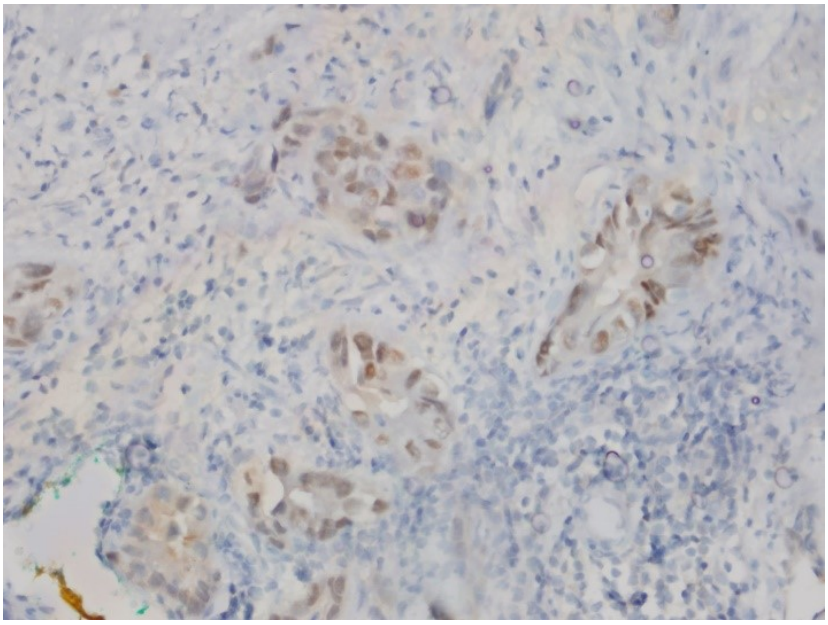

Figure 2. Positive nuclear staining with estrogen receptor in tumor cells (ERαx200)

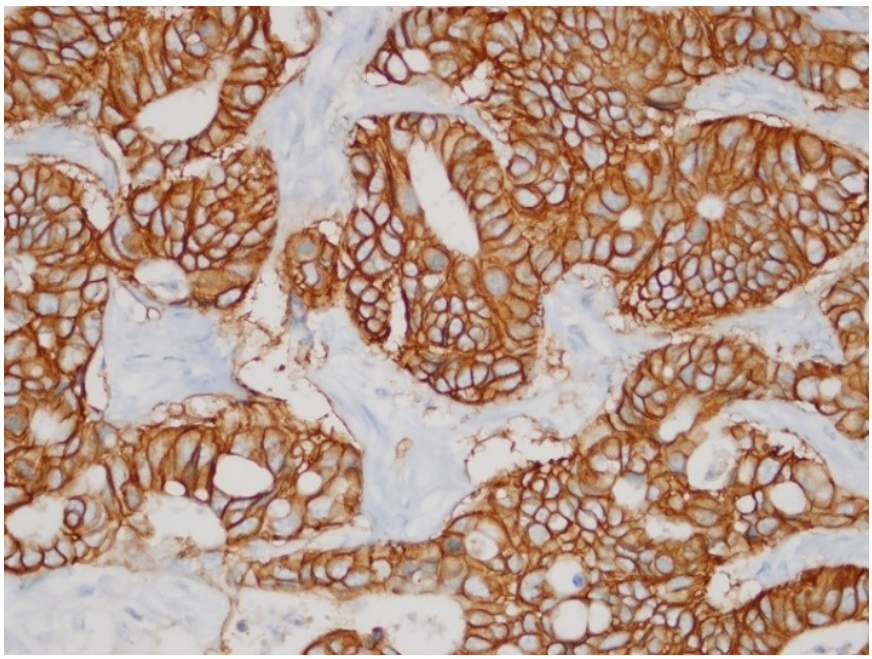

Figure 3. Complete membranous staining with CerbB2 in tumor cells (CerbB2x200)

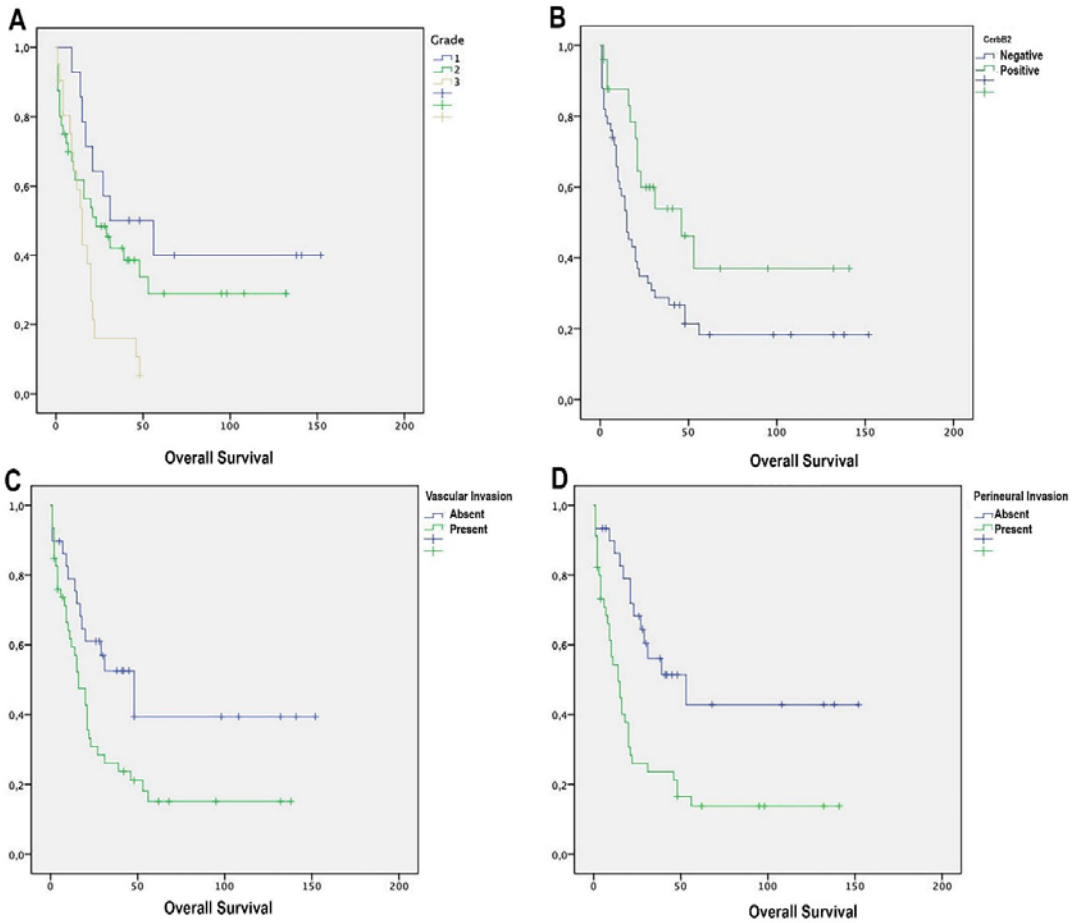

Figure 4. Kaplan-Meier overall survival curves. (**A**) Grade; (**B**) CerbB2 status; (**C**) Vascular invasion; (**D**) Perineural invasion.

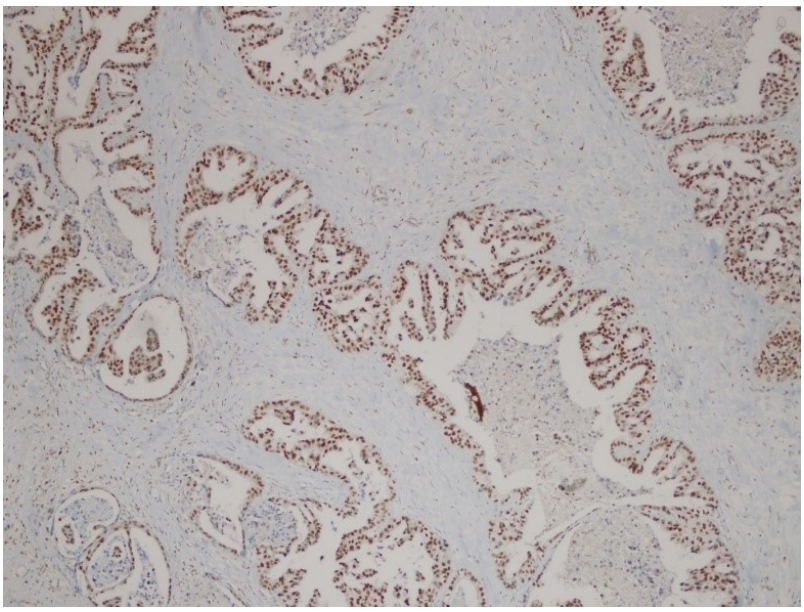

Figure 5. Gallbladder carcinoma without microsatellite instability that show PMS2 immunostaining (PMS2x100)

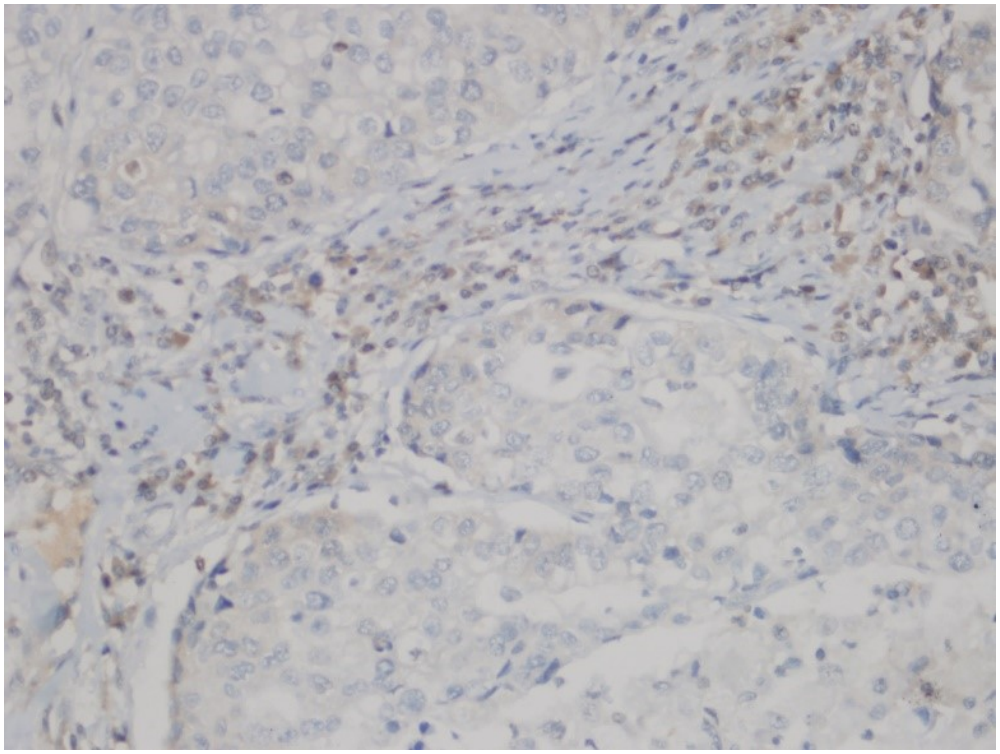

Figure 6. Gallbladder carcinoma with microsatellite instability that does not show PMS2 immunostaining (PMS2x400)

Table 1. Antibodies used and their properties

Antibody	Manufacturer/Clone	Dilution	Company information
Estrogen reseptor (ERα)	Novocastra/ 6F11	1/50	Benton Lane, Newcastle Upon Tyne, England
Progesterone reseptor (PR)	Novocastra/1A6	1/100	Benton Lane, Newcastle Upon Tyne, England
CerbB2	Thermo/ e1-4001+385	1/400	Lab vision Corporation, Fremond Blud, Fremont CA/USA
MLH-1	Dako/ E505	Ready to use	Dako North America, Via Real, CA, USA
MSH-2	Biocare/ FE11	1/100	Biocare Medical; 60 Berry Drive, Pachero, CA, USA
MSH-6	Genemed/ GM024	1/50	Genemed Biotechnologies, 458 Coriton Ct/South San Francisco, CA, USA
PMS-2	Dako/ EP51	Ready to use	Dako North America 6392 Via Real, Carpinteria, CA, USA

Table 2. Effects of various demographic and histopathological parameters on survival

	N (%) (n=75)	Median overall survival (month)	P value
Gender			0,298
Female	54 (72)	21	
Male	21 (28)	20	
Grade			0,019*
1	14 (18.6)	31	
2	40 (53.3)	23	
3	21 (28)	15	
Invasion depth			0,072
T1	13 (17.3)	29	
T2	45 (60)	21	
T3	17 (22.6)	9	
Mucin			0,857
Present	16 (21.3)	16	
Absent	59 (78.7)	21	
Vascular invasion			0,019*
Present	46 (61.3)	16	
Absent	29 (38.6)	48	
Perineural invasion			0,001*
Present	45 (60)	14	
Absent	30 (40)	53	
CerbB2			0,021*
Negative	50 (66.7)	15	
Positive	25 (33.3)	46	

* $p<0,05$

Table 3. Clinical and morphological features of the estrogen receptor alpha positive case

Features	Findings
Age and gender	52, Female
Grade and invasion depth	Grade 3, T2
Vascular invasion / perineural invasion	Present / Present
Hepatic involvement / Lymph node metastasis	Absent / Absent
Survival	4 months, alive

Table 4. Clinical and morphological features of cases with microsatellite instability

	CASE 1	CASE 2	CASE 3
Molecular analysis (loss)	MSH2	PMS2	MSH2 and MSH6
Tumor type	Adenocarcinoma, NOS	Adenocarcinoma, NOS	Adenocarcinoma, NOS
Age	73	65	71
Sex	Male	Female	Female
Invasion depth	T1a	T3	T1b
Survival	17 mo, ex	53 mo, ex	7 mo, alive
Liver involvement	No	Yes	No
Lymph node metastasis	No	Yes	No
CerbB2	Negative	Positive	Negative