Abstract. Background: To improve the prognosis of gallbladder cancer (GC) patients, a better understanding of the mechanisms of tumor development and progression is essential. The deregulation of cell cycle control is a critical step in the development of cancer. The purpose of this study was to investigate the expression of p21\(^{\text{Wafl/Cip1}}\), p57\(^{\text{Kip2}}\) and HER2/neu in an unselected GC patient population and to assess the association of these markers with p27\(^{\text{Kip1}}\) expression, p53 gene mutation status and clinical parameters of the patients. Patients and Methods: Formalin-fixed paraffin-embedded tissues from 55 operated GC patients were used to determine the expression of p21\(^{\text{Wafl/Cip1}}\), p57\(^{\text{Kip2}}\) and HER2/neu with immunohistochemistry. Results: Expression of p21\(^{\text{Wafl/Cip1}}\) was observed in 28%, of p57\(^{\text{Kip2}}\) in 19% and of HER2/neu in 13% of the patients. Absence of p57\(^{\text{Kip2}}\) expression was significantly associated with T3/T4 stage (p=0.01), positive lymph nodes (p=0.02) and advanced UICC stages (p=0.05). HER2/neu expression significantly correlated with advanced T stages (p=0.02). In the total patient population, p21\(^{\text{Wafl/Cip1}}\), p57\(^{\text{Kip2}}\) and HER2/neu had no impact on survival of the patients. Among patients with a mutated p53 gene, those without p21\(^{\text{Wafl/Cip1}}\) expression had a prolonged survival compared to patients with p21\(^{\text{Wafl/Cip1}}\) expression (p=0.004). Moreover, in p27\(^{\text{Kip1}}\)-positive patients, those without p21\(^{\text{Wafl/Cip1}}\) expression had a longer survival than those with p21\(^{\text{Wafl/Cip1}}\) expression (p=0.003). Conclusion: In the subgroup of patients with a mutated p53 gene or in p27\(^{\text{Kip1}}\)-positive patients, absence of p21\(^{\text{Wafl/Cip1}}\) expression may be associated with longer survival of GC patients. Therefore, further analyses of this protein in larger patient populations are warranted.

Gallbladder cancer (GC) still has a poor prognosis. Complete tumor resection results in the best survival outcome in these patients. However, this treatment can only be offered in 20% of patients (1) because at time of diagnosis the majority of GC patients are in advanced tumor stages and distant metastases are common. These factors mostly contribute to the poor prognosis of this malignancy, with a 5-year survival rate of less than 5% (1). To improve the prognosis of GC, a better understanding of the mechanisms of tumor development and progression is essential.

Increased attention has been focused on the deregulation of cell cycle control which is a critical step in the development of cancer. Cell cycle progression from the G1- to the S-phase of the cell cycle is regulated by the formation of cyclin/cyclin-dependent kinase (cdk) complexes (2). The activity of these complexes is inhibited by cdk inhibitory proteins which include the Cip/Kip proteins p21\(^{\text{Wafl/Cip1}}\), p27\(^{\text{Kip1}}\), p57\(^{\text{Kip2}}\) (2, 3). These proteins exert both negative and positive regulation of cdk activity at G1/S transition (4-6). Like p27\(^{\text{Kip1}}\), p53-inducible p21\(^{\text{Wafl/Cip1}}\) can induce G1 arrest (2, 7, 8) to repair damaged DNA before it is replicated and leads to cessation of cell growth. In response to severe DNA damage, apoptosis is induced by p53 (9). In GC, p27\(^{\text{Kip1}}\) expression has been reported as an independent prognostic marker and p53 gene mutations may predict response to chemotherapy in these patients (10, 11).

Another important parameter in tumor biology is the proto-oncogene HER2/neu. HER2/neu is amplified and/or overexpressed in a variety of malignancies and plays a critical role in the development and progression of cancer (12-22). Moreover, promising data were published on the use of the anti-HER2/neu antibody Herceptin\textsuperscript{®} in the treatment of some malignancies (12, 13, 23).

The purpose of the present study in GC patients was to investigate the expression of p21\(^{\text{Wafl/Cip1}}\), p57\(^{\text{Kip2}}\) and...
HER2/neu in an unselected patient population and to assess their correlation with p27Kip1 expression, p53 gene mutation status and clinical parameters of the patients.

Patients and Methods

Patients. Fifty-five unsellected patients were operated for GC between October 1991 and January 1999. Two patients were excluded from this analysis: one was suffering simultaneously from an advanced rectal carcinoma, and in the second sequencing of the p53 gene was not possible. The remaining 53 patients were included in our study. The mean age was 66.5 years (SD 10.2 years) and the median follow-up time was 64 months (range 0.3-83.6 months). The clinical characteristics of the patients are summarized in Table I. Tumor stage was classified according to the TNM system (24). Seventeen resections and 36 palliative surgeries or explorative laparotomies were performed. R2 represented macroscopic, R1 microscopic and R0 no residual tumor. Most patients with inoperable or metastatic disease received chemotherapy consisting of gemcitabine (n=10) or 5-fluorouracil/leucovorin/mitomycin C (n=11) (25).

Results on the clinical role of p27Kip1 expression (10) and p53 gene mutations (11) in this study population have been published.

Immunohistochemistry. Immunohistochemistry was performed on formalin-fixed, paraffin-embedded tumor specimens. Paraffin sections were mounted on poly-L-lysine-coated slides. Tissue sections (4 μm) were deparaffinized and rehydrated. Endogenous peroxidase activity were mounted on poly-L-lysine-coated slides. Tissue sections (4 μm) of formalin-fixed, paraffin-embedded tumor specimens. Paraffin sections were boiled in 10 mM citrate buffer, pH 6.0 in a pressure cooker for 10 min at room temperature and the slides were washed in phosphate-buffered saline. Sections were boiled in 10 mM citrate buffer, pH 6.0 in a pressure cooker for 10 min and antigen retrieval. After cooling for 15 min and washing in phosphate-buffered saline, the tissues were preincubated for 20 min with normal serum (normal goat serum 1:50; Dako, Glostrup, Denmark) prior to a one-hour incubation with the p21Wafl/Cip1 monoclonal antibody (EA10, dilution 1:100; Oncogene Research Products, Boston, USA) or p57Kip2 monoclonal antibody (SP706, dilution 1:100; Neomarkers, Fremont, CA, USA). Antibody binding was detected using the avidin-biotin-peroxidase method. Bound peroxidase was detected using a diaminobenzidine method. The slides were counterstained with Mayer’s Hemalum and mounted with Aquatex (Merck, Darmstadt, Germany). All washes were performed in phosphate-buffered saline.

HER2/neu expression was assessed using the HercepTest (Dako, Glostrup, Denmark) according to the instructions of the manufacturer.

Breast cancer specimens with a known expression of p21Wafl/Cip1 and HER2/neu were used as positive controls for p21Wafl/Cip1 and HER2/neu expression, respectively. With regard to p57Kip2, colon carcinoma specimens were used as positive control.

Immunostaining was examined by one observer who was blinded to the clinical outcome of the patients. Immunostaining was classified based on staining intensity and percentage of stained cells. For p21Wafl/Cip1 and p57Kip2, only nuclear staining of the tumor cells was scored as positive. For HER2/neu complete or incomplete membrane staining of tumor cells was considered as positive.

Statistical methods. Associations of p21Wafl/Cip1, p57Kip2 and HER2/neu expression with histological and clinical parameters were assessed using the Chi-square test or Fisher’s exact test. The survival of the patients was estimated from the date of surgery and determined using the Kaplan-Meier method. Differences in survival between the various groups were assessed with the log-rank test. SPSS 10.0 for Windows was used for all statistical analyses (Statistical Package for Social Science, Chicago, IL, USA).

Results

Expression of biomarkers. Staining of p21Wafl/Cip1 was nuclear and ranged from 0 to 60%. Expression of p21Wafl/Cip1 was considered as negative (≤10% p21Wafl/Cip1-positive tumor cells) in 38 patients (72%) and positive (>10% p21Wafl/Cip1-positive tumor cells) in 15 patients (28%). Expression of p57Kip2 was also nuclear and ranged from 0 to 5%. Expression of p57Kip2 was negative in 43 patients (81%) and positive (any nuclear staining of tumor cells) in 10 patients (19%). HER2/neu staining was localized at the cell membrane and ranged from 0 to 70%. HER2/neu expression was negative in 46 patients (87%) and positive (any membrane staining of tumor cells) in 7 patients (13%).

Association with clinical parameters. The clinical and pathological parameters, p27Kip1 expression and p53 mutation status of all 53 patients were associated with the expression of p21Wafl/Cip1, p57Kip2 and HER2/neu. Immunohistochemical staining of p21Wafl/Cip1 was not associated with any of the clinical and pathological parameters, p27Kip1 expression or p53 mutation. Absence of p57Kip2 expression was significantly associated with T3/T4 stage (p=0.01), positive lymph nodes (p=0.02) and advanced UICC stage (p=0.05). HER2/neu expression was significantly associated with advanced T stage (p=0.02) but was not significantly associated with the remaining parameters (Table I).

Survival. At a median follow-up of 64 months, the median overall survival of the 53 patients was 4.7 months. Univariate analysis revealed that positive lymph nodes, distant metastasis, advanced UICC stage, low p27Kip1 expression, palliative procedures and residual tumor after resection were significantly associated with shorter survival of the patients. However, expression of p21Wafl/Cip1, p57Kip2 and HER2/neu had no impact on survival in the total patient population (Table II).

We then studied the association of p21Wafl/Cip1 and p57Kip2 expression on the survival of the patients in relation to p27Kip1 expression and p53 mutation status. In these exploratory analyses, we observed that among patients with a mutated p53 gene, those without p21Wafl/Cip1 expression had a prolonged survival compared to patients with p21Wafl/Cip1 expression in their tumors (p=0.004). In contrast, in patients with a mutated p53 gene, p57Kip2 expression did not affect patient outcome (p=0.7). In the case of a wild-type p53 gene, neither p21Wafl/Cip1 nor p57Kip2 expression influenced survival. Among p27Kip1-positive patients, absence of p21Wafl/Cip1...
staining was associated with longer survival \((p=0.003)\), whereas \(p57\text{Kip}2\) expression had no impact on survival in this subgroup of patients. In \(p21\text{Waf1/Cip1}\)-negative patients, neither \(p21\text{Waf1/Cip1}\) nor \(p57\text{Kip}2\) expression affected survival of the patients. However, these analyses were based on low numbers of patients and have to be viewed with caution (Table III).

### Discussion

The aim of our study was to determine the clinical relevance of \(p21\text{Waf1/Cip1}\), \(p57\text{Kip}2\) and HER2/neu expression in unselected GC patients and their association with \(p27\text{Kip}1\) expression and p53 mutation status.
In our present study, which included completely resected and palliative operated GC patients, p21\textsubscript{Waf1/Cip1} expression was detected in 28% of the patients, which is comparable to a previous report (26). An increased expression of p21\textsubscript{Waf1/Cip1} was reported in a variety of malignant diseases including laryngeal carcinoma (27), gastric cancer (28), esophageal squamous cell carcinoma (29), non-small cell lung cancer (30), rectal carcinoma (31) and ovarian cancer (32). We observed no significant associations of p21\textsubscript{Waf1/Cip1} with clinical parameters or survival of the patients, which is also consistent to the results of Jarnagin et al. (26) in GC and comparable to the results in other malignancies (28-30). In some types of cancer, however, expression of p21\textsubscript{Waf1/Cip1} was associated with increased survival (29, 30). We observed no significant association of p21\textsubscript{Waf1/Cip1} expression with a functional p53 gene, which is in line with the observation that p21\textsubscript{Waf1/Cip1} is activated through p53-dependent (7, 8) but also p53-independent (33, 34) mechanisms. Interestingly, among patients with a mutated p53 gene, those without p21\textsubscript{Waf1/Cip1} expression might have a longer survival, which is supported by similar results of a recent publication in ovarian carcinoma patients (32) and supports the conclusion that a positive p21\textsubscript{Waf1/Cip1} expression might be a poor survival parameter in GC if p53 is mutated. Due to the small number of patients included in our study, further conclusions about the relationship between p53 mutations and p21\textsubscript{Waf1/Cip1} expression can only be drawn after analysis of larger cohorts of patients. There was also no association between absence of p27\textsubscript{Kip1} and p21\textsubscript{Waf1/Cip1} expression, which is also consistent with the literature (29). Generally, a high p27\textsubscript{Kip1} protein expression is associated with an improved survival in cancer patients (10, 35, 36). In this prognostic favorable subgroup, patients without p21\textsubscript{Waf1/Cip1} expression had an improved survival. This finding is different to Kapranos et al. (37), who
reported that patients suffering from head and neck cancer who stained positive for p27\(^{kip1}\) and negative for p21\(^{waal/cip1}\) had the poorest survival. This contradiction shows that some clinical associations of the Cip/Kip family are still poorly understood and have to be further investigated.

Previous reports suggested that loss of p57\(^{kip2}\) expression is involved in development (38) and progression of various malignancies (39). We observed an association between p57\(^{kip2}\) expression and early T stages, negative lymph nodes and early UICC tumor stages in GC, suggesting that p57\(^{kip2}\) expression might be associated with early tumor stages. In other hepatobiliary carcinomas, low p57\(^{kip2}\) expression correlated with a high biological aggressiveness (39, 40) and poor prognosis of the patients (40). Expression of p57\(^{kip2}\) had no impact on survival of the patients in our analysis. In hepatocellular carcinoma, Ito et al reported a shorter disease-free survival for patients with low p57\(^{kip2}\) expression in the univariate but not in the multivariate analysis (39). In addition, p57\(^{kip2}\) expression had no impact on survival in esophageal carcinoma patients (41).

It has been shown that a constitutive expression of HER2/neu in gallbladder epithelium results in the development of adenocarcinomas of the gallbladder (42). HER2/neu expression in GC was reported in the broad range between 9\% up to 65\% (43-47). In our analysis, 13\% of the cases showed expression of HER2/neu. HER2/neu staining was associated with advanced local tumor stages but it did not have an impact on survival of the patients. Suzuki et al. reported that even HER2/neu gene amplification is not useful as prognostic parameter (45). The therapeutic options of HER2/neu in GC will rarely ever be used because of the overexpression in just a minority of patients. Our results suggest that HER2/neu-targeted therapies may not improve treatment of GC.

Clinical parameters such as positive lymph nodes or distant metastasis, advanced UICC stages, palliative operative procedures or residual tumor stage are the strongest prognostic factors in GC patients. To date, early detection, including low UICC stages and resectability, are still the most important factors for improved survival in GC patients. Among the proteins of the Cip/Kip family, p27\(^{kip1}\) was an independent prognostic parameter, as shown previously (10), and p21\(^{waal/cip1}\) may also be of clinical relevance in patients with a mutated p53 gene. Further analyses of the Cip/Kip family in larger patient populations will improve our understanding of the molecular mechanisms of GC and are the prerequisite for new treatment strategies based on modulation of Cip/Kip family members in the future.

References


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