Abstract. Aim: The enzyme Cytochrome P450 2W1 (CYP2W1) is found in fetal colon tissue and is also detected in colorectal cancer but not in non-transformed tissue. In a pilot study, we reported that the immunohistochemically-detected expression of CYP2W1 might be of prognostic value since high expression of CYP2W1 was indicative of a worse prognosis. The aim of this study was to validate the pilot study’s results using a larger, independent group of patients with colon cancer. Materials and Methods: Immunohistochemical detection of CYP2W1 in 235 malignant colon tumors of stage II and III, was carried out using a polyclonal antibody. Grading of staining was carried out by two independent readers. The highest grade that involved more than 5% of the tumor area on each slide was used for the classification of CYP2W1 expression. Results: CYP2W1 was expressed at high levels in 30% of the tumors. In the entire colon cancer group it was an independent prognostic factor in multivariate analysis (p=0.04), where high expression (grade 3) correlated with worse outcome. CYP2W1 expression was an independent prognostic factor in the subgroup of patients with colon cancer stage III (p=0.003), but not for those with stage II. In 107 cases, two slices from different areas of the same tumor were available, and no significant difference in CYP2W1 expression between the slices was observed (r=0.53, p<0.001). Conclusion: The results of the current study were in agreement with those of the previous pilot study and show that higher expression of CYP2W1 seems to be of prognostic value in colon cancer.

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HepG2 human hepatoma cell line and in some transformed tissues (7). CYP2W1 expression is high in gastrointestinal tumors, especially in colonic and rectal tumors (8). Analysis of mRNA levels in colon tumors showed that 60% of the tumors had overexpression of mRNA compared to HepG2 cells. The protein levels detected with western blotting showed that the colon tumors could be divided into two phenotypes: those with high CYP2W1 expression and those with low expression (8).

A polyclonal antibody against a CYP2W1 15-amino acid C-terminal peptide has been produced and described (7). Our group performed a pilot study including 162 patients with colorectal cancer stages II and III to detect the intratumoral CYP2W1 expression and to assess whether high expression, detected by immunohistochemistry, correlated with prognosis. That study showed that expression of the enzyme was an independent prognostic factor for overall survival in the entire group, where a high expression was associated with worse clinical outcome (9). When analyzing the colonic cancer and rectal cancer groups separately, CYP2W1 was an independent prognostic factor in colonic cancer but not in rectal cancer.

The present investigation was carried out in order to validate this preliminary finding. Since the result was most obvious in the colonic cancer group, we chose to analyze another, twice as large, independent group of patients with colorectal cancer from the same cohort with the same method as the one used in the previous study. We also addressed the question of homogeneity in CYP2W1 expression, whether or not the expression was equal in slices from two different areas of the same tumor.

Materials and Methods

Patients. The primary tumors of 235 patients with colonic cancer of stages II and III from 20 different Swedish hospitals were examined with respect to CYP2W1 expression. The surgical specimens were derived from adjuvant Nordic trials where patients up to the age of 75 years, with radically-resected colorectal cancer of stages II and III were included. Parameters of clinical outcome were obtained from the Regional Oncological Centers. The patients’ demographics and tumor characteristics are listed in Table I. All patients were randomized to surgery alone or surgery followed by adjuvant chemotherapy. The adjuvant chemotherapy regimens included 5-fluorouracil (5-FU)/levamisole for 12 months or 5-FU/leucovorin for 4-5 months according to either a modified Mayo Clinic schedule or a Nordic schedule. Some centers also randomized patients treated with 5-FU/leucovorin to receive or not levamisole (9). Adjuvant therapy was initiated within 11 weeks after surgery.

For 107 patients, we had access to two slices from different areas of the same tumor. These were compared in order to assess homogeneity regarding the expression pattern.

The study was approved by the local Ethical Committee at the Karolinska Institutet.

Immunohistochemical analysis. The examined colorectal specimens were derived from formalin-fixated, paraffin-embedded tumors in 4-μm thick sections. Immunohistochemical analysis of CYP2W1 expression was performed using the avidin-biotin-peroxidase complex technique (Vectastain® Rabbit IgG ABC-kit, Vector Labs, Burlingame, California, USA) and the CYP2W1 polyclonal antibody. The tumor slides were de-paraffinized in xylene and rehydrated in ethanol and thereafter incubated in a 3% hydrogen peroxide to inhibit the endogenous peroxidase activity. In order to reduce non-specific background staining, the slides were blocked with goat serum for 30 min followed by incubation with the CYP2W1 antibody, at 4°C overnight. The antibody was used at a dilution of 1:1000. The samples were then rinsed and incubated with biotinylated secondary antibodies and thereafter rinsed and incubated with avidin-biotin-peroxidase complexes. Visualization of immunostaining was achieved by immersion of slides in 0.05% 3,3’-diaminobenzidine tetrahydrochloride, followed by counterstaining with hematoxylin.

Evaluation of immunohistochemistry. CYP2W1 staining intensity was defined by a visual grading scale from 0 to 3 (grade 0=no staining, grade 1=weak, grade 2=moderate, grade 3=intense staining). Each time a set of tumor samples was stained, reference slices were included as well, as one negative control slice incubated with pre-immune serum. The whole tumor slide was graded. The
grading was based on the highest intensity found in the tumor that covered at least 5% of the tumor area. Two independent investigators (K.S. and M.H.), blinded to clinical data, scored the specimens. Scoring discrepancies were resolved by consensus after re-examination.

Statistics. The Gehan-Wilcoxon univariate test was used to examine the relationships between survival and patients’ demographics and tumor characteristics. Multivariate analyses were performed using Cox regression. Adjusted hazard ratios (HR) and 95% confidence intervals (CI) were assessed by stepwise multivariate logistic regression. The Kaplan-Meier method was used to construct the survival curves. Distribution differences between groups were compared with the \( \chi^2 \) test. The Spearman correlation test was used to determine the correlation between CYP2W1 expression in two different areas of a tumor. All tests were two-tailed and considered significant at a \( p \)-value less than 0.05.

Results

The median age of the 235 patients was 66 years, with a range from 29 to 76 years. The median follow-up time for living patients was 100 months (range 54 to 120 months). Patients’ characteristics are listed in Table I.

Seven per cent of the tumors did not express CYP2W1 (grade 0). Twenty-six per cent had a weak staining (grade 1), 37% had a moderate staining (grade 2) and 30% had an intense staining (grade 3). Grades 0, 1 and 2 were considered as low-expression and grade 3 was considered as high-expression based on our previous findings (9).

Grade 3 expression of CYP2W1 was more common in patients older than the median age of 66 years compared with the younger group (\( p=0.02 \), Table I). In the elderly, stage II cancer was more frequent compared with the group of patients who were younger than 65 years. No correlation was found between CYP2W1 expression and treatment, stage of the tumor, differentiation, number of analyzed lymph nodes, sex or localization of the tumor (left/right colon).

CYP2W1 was equally expressed in the two separate slides from different areas of the same tumor (\( n=107 \), \( r=0.53 \), \( p<0.001 \)).

CYP2W1 expression and clinical outcome. In the entire group of 235 patients with stage II and III colon cancer, patients with low expression of CYP2W1 tended to have longer overall survival but without reaching statistical significance (\( p=0.1 \), Figure 1). However, when included in the multivariate analysis, CYP2W1 expression was of independent prognostic value (\( p=0.03 \)), together with stage (\( p=0.003 \)), differentiation (\( p=0.01 \)) and the number of analyzed lymph nodes (\( p=0.05 \)). The prognostic value of CYP2W1 in the multivariate analysis was independent of adjuvant treatment.

The results of the univariate analysis are shown in Table II, while those for the multivariate analysis are displayed in Table III.

In the subgroup of patients with colonic cancer of stage III (\( n=132 \)), the expression of CYP2W1 was prognostic for
Table II. Univariate analysis of prognostic factors for overall survival in the entire group of patients with stage II and III colonic cancer.

<table>
<thead>
<tr>
<th>All patients, n=235</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td>0.64</td>
</tr>
<tr>
<td>Age</td>
<td>0.78</td>
</tr>
<tr>
<td>Stage</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>No. of nodes analyzed</td>
<td>0.09</td>
</tr>
<tr>
<td>Differentiation</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Therapy</td>
<td>0.12</td>
</tr>
<tr>
<td>CYP2W1 expression</td>
<td>0.12</td>
</tr>
</tbody>
</table>

Table III. All variables from Table II were put into multivariate analysis. Only stage, number of analyzed lymph nodes, differentiation and CYP2W1 expression led to a p<0.05. In stage II, only differentiation was significant and in stage III, only CYP2W1 expression was a significant prognostic factor.

<table>
<thead>
<tr>
<th>All patients, n=235</th>
<th>p-Value</th>
<th>Hazard ratio for death</th>
<th>95% Confidence interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage III vs. II</td>
<td>0.003</td>
<td>1.81</td>
<td>1.23-2.67</td>
</tr>
<tr>
<td>No. of analyzed lymph nodes &gt;11 vs. 0-11</td>
<td>0.047</td>
<td>0.37</td>
<td>0.14-0.99</td>
</tr>
<tr>
<td>Differentiation, high/medium vs. low</td>
<td>0.008</td>
<td>0.58</td>
<td>0.39-0.87</td>
</tr>
<tr>
<td>CYP2W1 high vs. low expression</td>
<td>0.034</td>
<td>1.22</td>
<td>1.02-1.48</td>
</tr>
<tr>
<td>Adjuvant vs. surgery only</td>
<td>0.066</td>
<td>0.71</td>
<td>0.50-1.02</td>
</tr>
</tbody>
</table>

Discussion

About 30% of human colorectal cancer specimens express high amounts of the recently identified enzyme CYP2W1 (7-9). In our previously published pilot study including 162 patients with colorectal cancer of stage II and III, the results suggested that the intensity of CYP2W1 expression in colorectal cancer constitutes a prognostic marker for survival (9).

The current study, including 235 patients with colonic cancer of stage II and III, verified that CYP2W1 is expressed to a various extent in colorectal tumors and that 30% exhibited the highest expression of CYP2W1. Only 7% of the tumors did not exhibit any CYP2W1 expression. CYP2W1 expression was not homogenous in the tumors as there were areas with stronger staining, as well as areas with weaker staining, within the same tumor slice. Scoring with immunohistochemistry is a semi-quantitative method, which represents a weakness of such studies. We tried to standardize the analysis by grading the slide using the most strongly-stained area exceeding 5% of the tumor area on each slide. We used this definition since it was used in our previous study.

A weakness of both our studies is the use of a relatively old material, where patients were treated between 1991 and 1997. At that time, the impact of examining a high number of lymph nodes was still not clear. In the group of patients reported in this latter study, the median number of analyzed lymph nodes was only six. The low number of analyzed lymph nodes may cause an underestimation of the number of stage III tumors. The TNM system was not routinely used in grading colonic tumors in the 1990s, which may also have led to underestimation of the number of more advanced tumors. The obvious strength of using an old patient material is the long follow-up time.

In our subgroup of patients from the adjuvant Nordic trial, the survival benefit of chemotherapy was insignificant. In the original Nordic trial, the treatment arm did not reach statistical significance, although there was a tendency towards better survival in the adjuvant group with stage III disease (p=0.07) (10).

The reason why CYP2W1 expression is associated with poor prognosis in stage III disease is unclear but might reside in the changed cellular phenotype during transformation whereby the CYP2W1 gene, normally expressed in fetal life, is activated. When comparing the adjuvant group with the surgical group of stage III patients, we found that CYP2W1 expression was significantly associated with worse outcome only in the group that received adjuvant treatment (data not shown), however, the sample size is too small in order to allow for any conclusions to be drawn. As mentioned above, both in the entire patient group and in the stage III group, CYP2W1 was prognostic independently of the adjuvant treatment. We do not know whether chemotherapy used for cancer treatment is metabolized to any extent by CYP2W1, although one could speculate about the potential of this enzyme metabolizing drugs that could in some way interfere with the chemotherapy given. Previous studies have shown expression of CYP3A4, a major anticancer drug-metabolizing enzyme, in colorectal cancer (11).

In a recently published study, we describe the N-linked glycosylation of CYP2W1 in vitro upon its overexpression in HEK-293 cells, and also in vivo, in normal colon tissue and in colorectal cancer specimens (6). This provides the first case, to our knowledge, of glycosylation of a human drug-metabolizing P450 enzyme. CYP2W1 has an inverted ER membrane topology, becoming therefore available to
glycosyltransferases in the ER lumen, but not available for functional interactions with cytosol-oriented P450 reductase. A fraction of both glycosylated and non-modified CYP2W1 is located on the cellular surface. In intact CYP2W1-containing HEK-293 cells, it was found that CYP2W1 was catalytically active in the transformation of aflatoxin B1 to cytotoxic products, indicating functional intracellular electron transfer and the ability to metabolically activate compounds into cytotoxic end-products. The cell surface localization of the enzyme and the ability of CYP2W1 to activate chemicals indicate that CYP2W1 might be used as a target in the therapy of colorectal cancer using either antibodies or pro-drugs. Thus, the colonic cancer-specific expression of CYP2W1 makes it a potentially interesting target for the development of novel anticancer agents.

The reason why tumor cells express an enzyme not normally expressed in adult cells is not fully understood. The tumor cell is characterized by a higher degree of genetic instability which in itself may cause genetic and epigenetic changes that alter the expression pattern of various genes, e.g. CYP2W1. Carcinoembryonic antigen (CEA) is expressed in fetal life and thereafter expressed rather specifically in colonic tumors and CYP2W1 has the same pattern of expression. We previously showed that the increased expression of CYP2W1 in tumors is associated with demethylation of the CpG island in the exon 1-intron 1 junction (8). The control for the activation of CYP2W1 by such epigenetic modulation is unknown but may be a result of the phenotypic changes during transformation of the colon cells. The role of CYP2W1 and its activation in colorectal cancer cells may be further elucidated by studies of enzyme expression in primary tumors, compared to that in nodal and distant metastases, as in pre-malignant stages such as colorectal adenomas with and without dysplasia.

**Conclusion**

In colonic cancer, immunohistochemically-assessed expression of CYP2W1 appears to be of independent prognostic value. Approximately 30% of colonic tumors exhibit high expression of CYP2W1. CYP2W1 expression is thus a promising prognostic marker for colonic cancer, although additional studies are necessary before a conclusive statement can be made.

**Declaration of Interest Statement**

The Authors report no conflicts of interest. The Authors alone are responsible for the content and writing of the paper.

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**Figure 2.** CYP2W1 expression in the group of 132 patients with colon cancer stage III.
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