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The expression of the novel CYP2W1 enzyme is an independent prognostic factor in colorectal cancer – A pilot study

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ABSTRACT

Aim: Cytochrome P450 (CYP) enzymes are important for drug metabolism. A novel cytochrome P450 enzyme, CYP2W1, has recently been identified. This enzyme is mainly found in foetal colon tissue and in tumour tissue. In this pilot study, we have investigated the expression of CYP2W1 in 162 tumours from patients with stages II and III colorectal cancer.
Methods: The expression of CYP2W1 enzyme was immunohistochemically detected using a polyclonal antibody. Staining intensity was defined using a visual grading scale from 0 to 3. Grades 0–2 were classified as low, and grade 3 was classified as high expression of CYP2W1.
Results: About 64% of the tumours expressed a low level of CYP2W1-expression, and 36% expressed a high level. CYP2W1-expression was an independent prognostic factor for overall survival ($p = 0.007$), where a high expression was associated with a worse clinical outcome.
Conclusions: Immunohistochemically assessed expression of CYP2W1 is an independent prognostic factor in patients with stages II and III colorectal cancer.

1. Introduction

About 70–75% of all phase I-dependent metabolism of clinically used drugs is carried out by cytochrome P450 enzymes, which are mainly expressed in the endoplasmic reticulum of liver.[1] The major enzymes in this respect are CYP3A4, CYP2D6, CYP2C9 and CYP2C19. In addition, other enzymes in the families 1–3 also contribute. Besides the metabolism of drugs, these CYP enzymes are also active in the metabolism of endogenous substrates such as steroids, cholesterol derivatives and fatty acids. Many precarcinogens are bioactivated by P450s to ultimate carcinogens. With respect to cancer therapy, the P450s act as bioactivators of prodrugs such as cyclophosphamide, dacarbazine, ifosfamide, procarbazine and tegafur, as well as participate in the metabolism of active anti-cancer agents such as docetaxel, etoposide, gefitinib, idarubicin, irinotecan, paclitaxel, tamoxifen, teniposide and vinblastine.[2]

Many of the hepatic P450 enzymes are also expressed in extrahepatic tissues but at lower levels.[3] Their contribution to overall clearance of drugs by the extrahepatic P450s is minimal, but one has to take into consideration the possibility for

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local action including tissue-specific drug metabolism as potential importance for the tissue in question. One such challenging aspect is the possibility to use P450 enzymes expressed in tumour tissue for targeted anticancer therapy using the prodrug concept. Previous studies have shown that several P450s are expressed in human tumours,[4] for example, the CYP4Z1 which is overexpressed in breast carcinoma[5] as well as CYP1B1 and CYP2J2[6] which are present in many different types of tumours. In colorectal cancer, several P450s including CYP1B1, CYP2S1, CYP2U1, CYP3A5 and CYP51 have been identified using immunohistochemistry.[7]

The results from the human genome project revealed a new member of the P450 family 2, the CYP2W1. The human CYP2W1 gene is located on chromosome 7p22.3. This gene was cloned and found to be expressed mainly in tumour tissue at the mRNA level but not significantly in untransformed adult tissue.[8] Furthermore, we found that CYP2W1 is highly expressed in human hepatoma cell lines and specifically in human colon tumours.[8] CYP2W1 mRNA expression was found in half of the tumour samples analysed, including bladder, breast, liver, pancreas, stomach and thyroid, and in 78% of the colon cancer samples that were examined.[8] RT-PCR analyses of whole-mice RNA indicated that the CYP2W1 mRNA is expressed in mouse embryos but not in adult animals.[9] In accordance with this finding, an examination of CYP2W1 expression in rats revealed that a significant expression was seen in foetal colon but not in any tissues from adult rats.[8] Apparently, the gene might have an important role in foetal colon, silenced at birth but is then reactivated in colon tumours.

Today, colorectal cancer is one of the most common cancer worldwide with approximately 1 million new cases and a half million deaths annually.[10] An early detection of colorectal cancer is of importance for better overall survival. Preoperative radiotherapy and better surgical techniques have improved the clinical outcome in rectal cancer. Adjuvant treatment and the development of more efficient chemotherapeutic drugs are also reasons for an improved clinical outcome.

However, there is a need for better biomarkers that help in identifying colorectal cancer patients who are at a high risk for recurrence of the disease. To date, the most important prognostic factor in colorectal cancer is still the stage of disease. Other factors of clinical importance are tumour perforation, tumour obstruction, tumour extent and involvement of adjacent organs as well as the histopathology with information about number of lymph node metastases, number of analysed lymph nodes, circumferential margin, differentiation of the tumour, blood or lymphatic vessel invasion and perineural growth. Molecular markers such as microsatellite instability,[11] thymidylate synthase expression,[12] p53[13] and K-ras expression[14,15] have been reported to be of prognostic value, but the potential clinical use in colorectal cancer remains unclear.[16] There is a need to identify new biomarkers having better prognostic information.

We had previously reported that the level of CYP2W1 mRNA and CYP2W1 protein is higher in colorectal cancer tissue than in normal colon mucosa,[17] and our hypothesis is that the immunohistochemically assessed level of CYP2W1 enzyme expression could fulfil the criteria for being a prognostic biomarker for stages II and III colorectal cancer.

2. Materials and methods

2.1. Patients

The primary tumours of 162 patients with colorectal cancer stages II and III from five different hospitals were examined with respect to CYP2W1 expression in this pilot study. The available surgical specimens were derived from adjuvant Nordic trials including patients with colorectal cancer stages II and III who were under the age of 76 years and who underwent curative surgery between 1991 and 1997.[18] The exclusion criteria in the Nordic trials were the presence of another malignancy, except squamous cell carcinoma of the skin and cervical carcinoma stage 0, previous chemotherapy or radiotherapy and severe cardiopulmonary disease.

Tumour material available in this study represented 90% of the patients included in the Nordic trials from the selected five hospitals. Parameters of clinical outcome were obtained from the centres of epidemiological oncology. The patients' demographics and tumour characteristics are listed in Table 1. All patients were randomised to surgery alone or surgery followed by adjuvant chemotherapy. The adjuvant chemotherapy regimens included 5FU/levamisole for 12 months or 5FU/leucovorin for 4–5 months according to either a modified Mayo Clinic schedule or the Nordic schedule. Some centres also randomised patients treated with 5FU/leucovorin to +/− levamisole. Adjuvant therapy was initiated within 49 days after surgery.[18] The study was approved by the local ethical committee.

2.2. CYP2W1 antibody

A polyclonal CYP2W1 antibody against a CYP2W1 15 amino acid C-terminal peptide was produced and characterised as previously described.[8] Several attempts were made to produce specific antibodies against this protein including isolation of hybridomas of mice injected with the purified recombinant enzyme and immunisation of rabbits with the full-length recombinant CYP2W1 protein, but analyses of the specificity revealed that the polyclonal C-terminal peptide antibody was the best, data not shown.

2.3. Staining of cells and immunofluorescence microscopy

Human embryonic kidney cells (HEK293) were grown and transfected with a pCMV4–CYP2W1 construct as previously mentioned.[8,19] About 6 h after transfection, the cells were trypsinised and transferred to poly-L-lysine-coated cover slips in 12-well plates.

Two days after the transfection, the cells were fixated with 2% formaldehyde (Merck Chemicals, Darmstadt, Germany), permeabilised with 0.2% Triton X-100 (Sigma–Aldrich, Stockholm, Sweden) and blocked with 10% foetal bovine serum (FBS) (Invitrogen, Rockville, MD), all diluted in PBS (Invitrogen, Rockville, MD). Thereafter, the cells were incubated with the CYP2W1 antibody diluted 1:1000 in PBS containing 3% bovine serum albumin (BSA) (Sigma–Aldrich, Stockholm, Sweden) followed by incubation with the secondary antibody, FITC conjugated anti-rabbit (Sigma–Aldrich, Stockholm, Sweden), diluted 1:500 in 3% BSA in PBS. The cover slips were mounted

Table 1 – Characteristics in 162 patients with colorectal cancer stages II and III and the CYP2W1 expression.

	Number of patients	CYP2W1 expression 0–1–2 versus 3	χ^2
CYP2W1 expression	162	104 (64%) 58 (36%)	
Gender			
Male	86	59 (69%) 27 (31%)	0.21
Female	76	45 (59%) 31 (41%)	
Age			
<68	82	49 (60%) 33 (40%)	0.23
⩾68	80	55 (69%) 25 (31%)	
Stage			
II	73	47 (64%) 26 (36%)	0.96
III	89	57 (64%) 32 (36%)	
Site			
Colon	117	70 (60%) 47 (40%)	0.06
Rectum	45	34 (76%) 11 (24%)	
Differentiation			
Low	40	25 (62.5%) 15 (37.5%)	0.42
Median	117	75 (64%) 42 (36%)	
High	3	3 (100%) 0 (0%)	
Treatment			
Surgery alone	81	56 (69%) 25 (31%)	0.19
Surgery and adjuvant treatment	81	48 (59%) 33 (41%)	
Number of analysed lymph nodes			
<12	92	59 (64%) 33 (36%)	0.6
⩾12	34	20 (59%) 14 (41%)	

on glass slides using Dako Cytomation Fluorescent Mounting medium (Dako North America, Carpinteria, CA) and were examined using a fluorescence microscope (Nikon, Tokyo, Japan).

2.4. *Immunohistochemical analysis*

The examined colorectal specimens were derived from formalin fixated, paraffin embedded tumours in 4 μm thick sections. Immunohistochemical analysis of the CYP2W1 expression was performed using the avidin-biotin-peroxidase complex technique (Vectastain Rabbit IgG ABC-kit) and the CYP2W1 polyclonal antibody in a dilution of 1:1250. The tumour slides were deparaffinised 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 in 30 min followed by CYP2W1 antibody incubation at 4° C temperature overnight. The samples were then rinsed and incubated with biotinylated secondary antibodies and thereafter rinsed and incubated with avidin-biotin-peroxidase complexes. Visualisation of immunostaining was achieved by immersion in 0.05% 3,3′-diaminobenzidine tetrahydrochloride followed by counterstaining with haematoxylin.

2.5. *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 tumour samples was stained, reference slices were included as well as one negative control slice was incubated with pre-immune serum. The whole tumour slide was thoroughly examined. The immunohistochemically detected CYP2W1 expression was classified according to the highest staining found in the tumour if the stained area exceeded 5% of the tumour area. Two independent investigators blinded to clinical data scored the specimens. Scoring discrepancies were resolved by consensus after re-examination.

2.6. *Statistics*

The Gehan–Wilcoxon univariate test was used to examine the relationships between survival and patients demographics and tumour characteristics. Multivariate analyses were performed using Cox regression. Adjusted odds ratios 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 χ^2 test. All tests were two-tailed and were considered significant at a p-value less than 0.05.

3. Results

3.1. *Patient characteristics*

The median age of the group of 162 patients included in this study was 67 years with a range from 35 to 76 years. The median followup time for patients who are still alive was 112 months (range 61–120 months). The patient characteristics are described in Table 1.

3.2. *Immunofluorescence microscopy*

Immunofluorescence analysis of transfected HEK293 cells using the CYP2W1 antibody showed that cells transfected with the pCMV4–CYP2W1 cDNA showed a significant CYP2W1 staining, whereas no staining was seen in the mock-transfected cells (Fig. 1). In addition, no staining either in CYP2W1-transfected or mock-transfected cells could be seen when using the preimmune serum.

3.3. *Immunohistochemically assessed CYP2W1 expression*

The immunohistochemically detected CYP2W1 expression was often heterogeneous in the samples, and we classified the expression according to the highest staining found in the tumour if the stained area exceeded 5% of the tumour area. Eight percentage of the tumours did not express any CYP2W1 (grade 0), whereas 18% of the tumours expressed grade 1, 38% expressed grade 2 (Fig. 2A) and 36% of the tumours expressed the highest grade of staining (grade 3) (Fig. 2B). The expression of CYP2W1 was independent of gen-

der, age, stage, site of the tumour and differentiation (Table 1). The distribution of CYP2W1 expression was the same in the two different treatment arms, surgery alone or surgery followed by adjuvant chemotherapy ($p = 0.2$).

3.4. *CYP2W1 expression and clinical outcome*

In the entire group of 162 patients with colorectal cancer stages II and III, the CYP2W1 expression was found to be of prognostic value (Fig. 3, Table 2). There were no differences in overall survival between the patients whose tumours expressed a low CYP2W1 level ($n = 104$, 64%), i.e. having either a grade 0, 1 or 2 in expression. However, patients with stages II and III whose tumours expressed a high CYP2W1 (grade 3) had a worse clinical outcome, both with respect to 5-year overall survival ($p = 0.04$) and 10-year overall survival ($p = 0.01$) (Fig. 3A). The survival after 10 years was 40% for patient whose tumours expressed a high level of CYP2W1 ($n = 58$) as compared to 63% amongst patients with grades 0–2 expression in their tumours ($n = 104$). Even higher differences were noted in stage III colorectal cancer patients where survival after 10 years was only 30% in grade 3 ($n = 32$) as compared to 60% amongst patients with grades 0–2 expression of CYP2W1 ($n = 57$). This difference in CYP2W1 expression was a significant prognostic factor for overall survival, $p = 0.02$ (Fig. 3B). The CYP2W1 expression was also a prognostic factor for disease-free survival in the entire group of 162 patients, $p < 0.001$ and in the subgroup of patients with stage III colorectal cancer, $p = 0.003$. In the group of patients with stage II tumours, the CYP2W1 expression was not of prognostic value.

Tumour recurrence occurred in 33 of the 162 patients (20%). There were 12 recurrences in the group of 104 patients (12%) with low CYP2W1 expression (grades 0, 1 or 2) compared with 21 recurrences in the group of 58 patients (36%) in the group of patients whose tumours expressed the highest CYP2W1 expression ($p = 0.01$). The median time to recurrence in the group with low CYP2W1 expressors was 23 months compared with 25 months in the group with the high expressors.

Local recurrence was found in 10 of 162 (6%) patients. In the group of patients with low CYP2W1 expression, only 2 of 104 (2%) developed local recurrence compared with 8 of 58 (14%) in the group of patients with the highest CYP2W1 expression ($p = 0.003$). The same finding was seen in the smaller subgroup of 45 patients with rectal cancer. None of the 34 patients whose tumours expressed CYP2W1 grades 0–2 developed local recurrence, but in the group of 11 patients with the highest CYP2W1 expression three patients (27%) developed local recurrence ($p = 0.002$).

In the univariate analysis, the prognostic value of CYP2W1 expression, gender, age-group, site (colon or rectum), stage, differentiation, treatment (surgery alone or surgery and adjuvant therapy) and the number of analysed lymph nodes (<12 versus $\geqslant$12) was tested. Only CYP2W1 expression and stage were of prognostic value (Table 2). Age showed a trend towards prognostic value when age was calculated as a continuous variable, $p = 0.08$.

The parameters mentioned above were analysed in multivariate analysis with respect to overall and disease-free survival. In the entire patient cohort, only the stage (RH = 1.97 [95% CI, 1.19–3.27], $p = 0.009$) and the CYP2W1 expression (RH = 1.40 [95% CI, 1.10–1.78], $p = 0.007$) were independent prognostic factors for overall survival (Table 2). In stage III colorectal cancer, CYP2W1 expression was the only prognos-

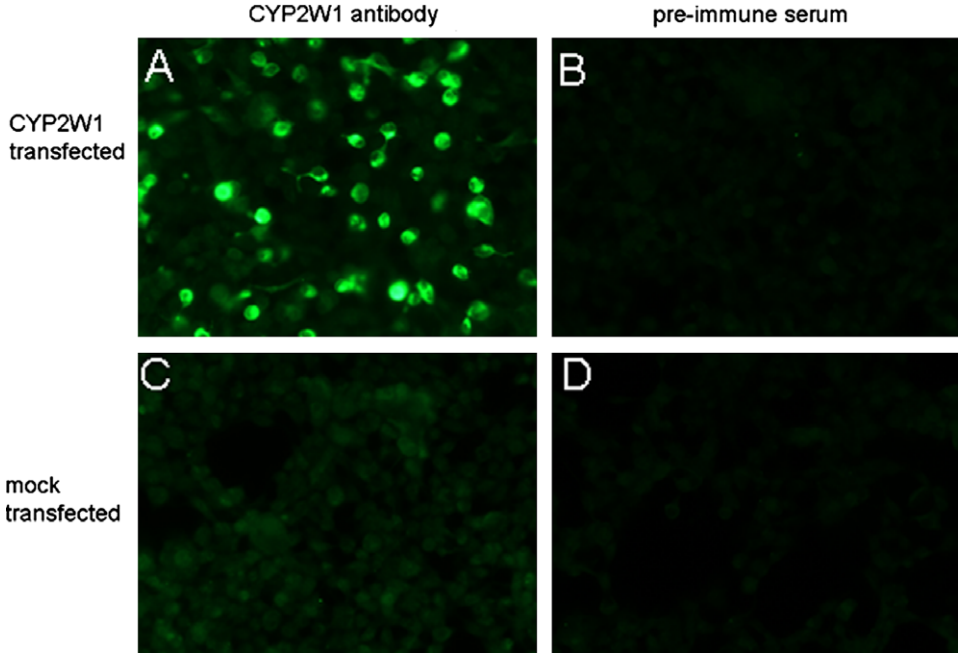

Fig. 1 – Immunofluorescence staining of the CYP2W1 protein in transfected HEK293 cells. (A) CYP2W1 transfected cells stained with the CYP2W1 antibody. (B) CYP2W1 transfected cells stained with the pre-immune serum. (C) Mock-transfected cells stained with the CYP2W1 antibody. (D) Mock-transfected cells stained with the pre-immune serum. All sera were used at a dilution of 1:1000, and as a secondary antibody a FITC-conjugated anti-rabbit was used at a dilution of 1:500.

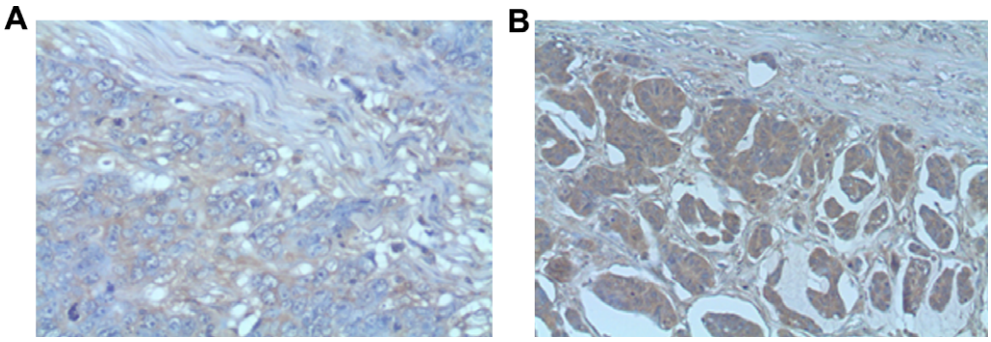

Fig. 2 – Immunohistochemical expression of CYP2W1. (A) Low expression, grade 2, (B) high expression, grade 3.

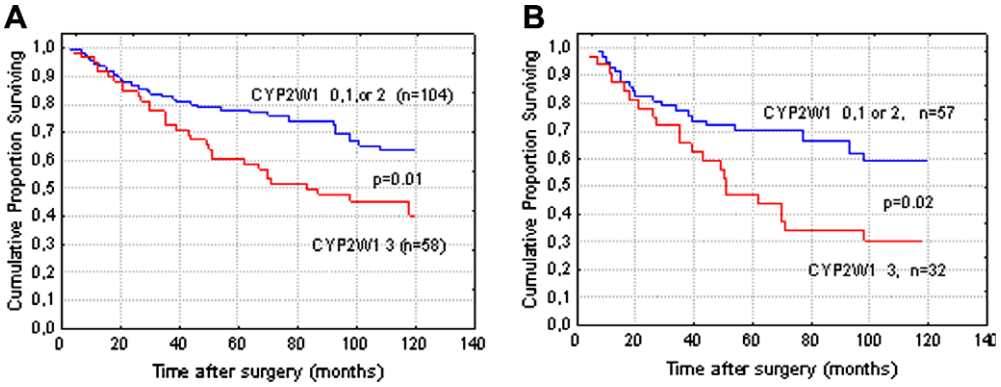

Fig. 3 – Survival of patients with stages II and III colorectal cancer in relation to the extent of CYP2W1 expression in the tumours. The expression of CYP2W1 is defined as intensities 0, 1, 2 and 3 as explained in the text. (A) Overall survival in the group of 162 patients with stages II and III colorectal cancer, (B) Overall survival in the subgroup of 89 patients with stage III colorectal cancer.

tic factor for overall survival (RH = 1.47 [95% CI, 1.09–1.98], $p = 0.01$). With respect to disease-free survival, CYP2W1 expression was an independent prognostic factor in the entire group of 162 patients (RH = 1.52 [95% CI, 1.20–1.92], $p < 0.001$) together with stage (RH = 1.91 [95% CI, 1.17–3.13], $p = 0.01$). The CYP2W1 expression was the only prognostic factor with respect to disease-free survival in the group of patients with colorectal cancer stage III (RH = 1.58 [95% CI, 1.18–2.12], $p = 0.002$).

4. Discussion

The role of CYP2W1 in the development of colorectal cancer is not known, but increased levels of CYP2W1 are found in colorectal cancer compared with normal colon mucosa.

In the present pilot study, immunohistochemically detected intratumoural CYP2W1 expression in a well-defined group of 162 colorectal cancer patients with a median follow-up time of 112 months had a significant prognostic value. A high expression of CYP2W1 was associated with a worse clinical outcome. Different hypothesis are needed to describe the potential role of CYP2W1 expression in colorectal cancer. The CYP2W1 enzyme might catalyse reactions which influence the properties of the tumour. Furthermore, the CYP2W1 expression can be a phenotypic marker for the extent of tumour transformation and malignancy. The finding of CYP2W1 expression in foetal colon is interesting in this respect. Many embryonally active genes, for example the carcinoembryonic antigen (CEA), which is used as a biomarker in colorectal cancer,[20] are activated in the corresponding cancer tissue in adult life.

The CYP2W1-specific antibody we used had previously been shown to recognise both endogenously and heterologously expressed CYP2W1 in HepG2 and in HEK293 cells, respectively.[8] In Western blot analyses using pre-immune serum or serum blocked with the peptide used for immunisation, the binding to CYP2W1 was abolished.[8] In the current study in which we examined the properties of four additional monoclonal and polyclonal CYP2W1 antibodies, the peptide antibody was found to have the highest specificity. Thus, no significant staining was obtained in mock transfected HEK293 cells or in cells treated with the preimmune serum (Fig. 1).

In a recent study, CYP2W1 mRNA and protein expression was determined in HepG2 and colon carcinoma cell line Caco-2TC7 cells, and in normal colon and colon tumour tissue samples. An overexpression of CYP2W1 was found in the tumour tissue compared with normal colon tissue.[17] Using Western blotting, it was possible to make a relative quantification of CYP2W1 in 14 paired samples obtained from normal tissue and tumour tissue from the same patients. The tumours could be divided in two phenotypes, those with elevated CYP2W1 expression and those with low expression. The low grade of CYP2W1 expression was similar to or only slightly

Table 2 – Characteristics in 162 patients with colorectal cancer stages II and III and overall survival.

	Number of patients	Number of deaths (%)	Uni variate analysis overall survival, *p*-value	Multi variate analysis overall survival, *p*-value
CYP2W1 expression				
0, 1 or 2	104	35 (34%)	0.01	0.007
3	58	32 (55%)		
Gender				
Male	86	40 (47%)	0.4	n.s
Female	76	27 (36%)		
Age				
<68	82	30 (37%)	0.2	n.s
⩾68	80	37 (46%)		
Stage				
II	73	23 (32%)	0.003	0.009
III	89	44 (49%)		
Site				
Colon	117	44 (38%)	0.5	n.s.
Rectum	45	23 (51%)		
Differentiation				
Low	40	17 (43%)	0.2	n.s.
Median (117)	120	48 (40%)		
High (3)				
Treatment				
Surgery alone	81	31 (38%)	0.7	n.s.
Surgery and adjuvant treatment	81	36 (44%)		
Number of analysed lymph nodes				
<12	92	40 (43%)	0.4	n.s.
⩾12	34	13 (38)%		

higher than the CYP2W1 expression in normal tissue. These two colon cancer phenotypes were also found when the level of CYP2W1 mRNA was considered.[17] An overexpression of the CYP2W1 mRNA has also been detected recently in gastric cancer specimens, as compared to tissue from normal gastric mucosa.[21] These findings indicate that a high CYP2W1 expression might be found in different types of gastrointestinal cancers.

In this study, we have used immunohistochemical technique to detect the CYP2W1 protein. The evaluation with immunohistochemistry will always be semi-quantitative and is therefore hard to standardise. Even if the staining was carried out throughout for several days, the same tumour sample was used as a reference every day. We assume that the highest level of CYP2W1 is of importance for the characteristics of the tumour and if the extent of the highest staining exceeds 5% of the tumour area, the tumour is graded according to the highest staining. Further studies comparing results from immunohistochemistry, PCR-technique and protein detection using Western blot will give us more information to decide the best way to grade the expression of the CYP2W1 enzyme using immunohistochemistry.

Hypo- and hyper-methylation of genes are common events that occur in cancer cells.[22,23] For example, hypomethylation activates some protooncogenes[24,25] and promotes chromosomal and microsatellite instability. Studies have also shown that gene methylation is involved in the transcriptional regulation of some CYP enzymes. CYP1B1 may activate several procarcinogens, and is furthermore active in prodrug activation. Hypomethylation of the CYP1B1 promoter is associated with an overexpression of CYP1B1 in prostate cancer.[26] We recently found that CYP2W1 gene expression is also dependent on methylation. Accordingly, demethylation of the CpG island in exon 1-intron 1 junction of the gene was found to be a necessary but not a sufficient cellular factor for the expression of CYP2W1 in both cell lines and in the tumours. In non-transformed tissue, the methylation appeared to silence the gene.[17]

Patients in the present study underwent surgery between 1991 and 1997 when the impact of examining a high number of lymph nodes was still not clear. Only 27% of the patients had 12 or more lymph nodes analysed with a higher rate in colon cancer, 33%, compared with rectal cancer, 15% ($p = 0.04$). According to the American Joint Committee on Cancer and the International Union Against Cancer,[27,28] at least 12 lymph nodes should be examined to have an adequate staging. In larger patient material, the number of analysed lymph nodes is reported to be associated with survival.[29,30]

During the 1990s, the total mesorectal excision (TME) technique was introduced in Sweden which has had an impact on local recurrence and survival.[31] We do not know the percentage of patients with rectal cancer included in our study who were operated on using the TME technique. However, in this study the rate of local recurrence in patients with rectal cancer was 7% which is comparable with the results from patients operated on using the TME technique.[32] The rate of

local recurrence in patients with colon cancer in our study group was 6%.

There was a trend towards an association between the CYP2W1 expression and site of the tumour with more tumours having CYP2W1 grade 3 expression in the rectal cancer group compared with the colon cancer group ($p = 0.06$). The CYP2W1 expression in rectal cancer was not dependent on whether preoperative radiation was given or not. However, the total number of rectal cancers was only 45, and further studies are needed to evaluate whether CYP2W1 expression is dependent on the site of the tumour.

In the group of 162 patients in the present study, we could not find any significant benefit of adjuvant chemotherapy. In the analysis of the entire patient population of 2224 patients included in the Nordic trials, no significant difference in overall survival could be found. However, in the subgroup of patients with stage III colon cancer, an absolute difference of 7% favouring chemotherapy was seen.[18]

In this study, we have besides CYP2W1 analysed factors such as gender, age, differentiation, stage and number of analysed lymph nodes which in large patient material have been shown to be of prognostic value. We have not analysed the possible impact of tumoural perforation, obstruction, involvement of adjacent organ and prognostic factors as thymidylate synthase expression, microsatellite instability, loss of heterozygosity and DCC. This is a pilot study to examine the expression of CYP2W1 in colorectal tumours from 162 patients. Based on these numbers of patients, we have 80% power to detect the relative risk of 70% higher mortality rate for high CYP2W1 expressors compared with low expressors with the significance of 5%, two-sided test. The finding of CYP2W1 expression as a possible prognostic factor has to be confirmed in further studies.

5. Conclusion

Our results show that the immunohistochemically detected CYP2W1 expression in radically resected stages II and III colorectal cancer is associated with overall survival where a high expression of the enzyme indicates a worse clinical outcome. This is the first report on the prognostic value of CYP2W1 enzyme in colorectal cancer, and the results indeed warrant further large prospective studies.

Conflict of interest statement

None declared.

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