

Prognostic significance of p53-expression in colorectal carcinoma as measured by a luminometric immunoassay

Die Untersuchung der luminometrisch bestimmten p53-Expression auf deren prognostischen Wert beim kolorektalen Karzinom

Abstract

Background: Mutations of the TP53 gene induce the production of abnormal p53-protein with a prolonged half-life allowing its detection by monoclonal antibodies. In the following study we examined if elevated levels of p53 correlate with worse prognosis in colorectal cancer.

Methods: We have quantified the protein, using an immunoluminometric assay, in 144 cytosols of primary sporadic colorectal cancer tissues and in 96 specimen of normal mucosa.

Results: In 112 samples (77.8%) the p53-expression was higher than the cut-off-value of 0.15 ng p53 per mg total protein. Luminometric immunoassay did not correlate with various clinicopathological parameters. Follow-up ranged from 2.4 to 54.3 (mean 25.3) months. During this period, 61 patients developed recurrences of whom 39 died of the underlying disease. Neither univariate nor multivariate analysis showed any statistically significant differences in prognosis between high and low p53 expression.

Conclusion: Our investigation revealed that p53-overexpression as measured by a luminometric immunoassay, is not a useful predictor of prognosis in patients with colorectal adenocarcinoma. Overcoming the limit of semiquantitative immunohistochemistry for p53-protein quantitative immunoluminometry may be useful elucidating the relation between serum p53-antibodies and p53 in cytosols.

Keywords: p53-protein, colorectal cancer, prognosis, tumor markers

Zusammenfassung

Einleitung: Mutationen im TP53-Gen führen zur Expression eines abnormalen p53-Proteins mit verlängerter Halbwertszeit. Dieses kann durch monoklonale Antikörper nachgewiesen werden. In der vorliegenden Arbeit wurde unter Anwendung eines neuen Lumineszenz-Immunoassay (LIA) untersucht, ob eine erhöhte p53-Expression beim kolorektalen Karzinom mit einer schlechteren Prognose der Betroffenen einhergeht.

Methoden: Zytoplasmatisches p53 wurde in 144 Kolon- und Rektumtumoren und 96 Proben normaler Mukosa unter Verwendung eines quantitativen Lumineszenz-Immunoassay bestimmt.

Ergebnisse: Bei 112 Proben (77,8%) wurde eine p53-Konzentration nachgewiesen, die größer als der Cut-off-Wert von 0,15 ng p53 pro mg Gesamtprotein war. Das Ergebnis im Lumineszenz-Immunoassay korrelierte nicht mit verschiedenen klinisch-pathologischen Parametern. Während des medianen Beobachtungszeitraumes von 25,3 Monaten (Bereich 2,4–54,3 Monate) entwickelten 61 Patienten ein Rezidiv, was in 39 Fällen zum Tode führte.

Weder die univariate noch die multivariate Analyse der klinischen und pathologischen Variablen sowohl bezüglich der rezidivfreien Periode als auch des Gesamtüberlebens zeigten einen statistisch signifikanten Unterschied zwischen hoher und niedriger p53-Expression.

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Schlussfolgerung: Die luminometrisch bestimmte quantitative p53-Expression scheint kein Prognosefaktor für das Überleben bei Patienten mit kolorektalem Karzinom zu sein. Jedoch bietet die Methode deutliche Vorteile gegenüber der semiquantitativen Immunhistochemie und könnte insbesondere zur Klärung des Verhältnisses zwischen p53-Serumantikörpern und p53-Protein im Zytosol beisteuern.

Schlüsselwörter: p53-Protein, kolorektales Karzinom, Prognose, Tumormarker

Introduction

Colorectal cancer is the second leading cause of tumor related death in Western countries. Recurrences occur in 30–50% at which intensive follow-up could not show any significant improvement in the last decades [1]. A big problem of early diagnosis is the fact that many patients are asymptomatic for a long time [2]. There is discussion about how far follow-up has influence upon the results altogether and the individual course. Tumor growth in colorectal carcinoma is relatively slow, therefore good chances for successful therapy of recurrence is given. On the other hand, immense diagnostic effort is required to discover the relapse in a curable stage.

In 1979 a new phosphoprotein named p53, which is located on the short arm of human chromosome 17, was discovered [3]. Nowadays, this alteration accounts for the most common genetic abnormality in solid human malignancies. Mutations in the p53-gene are present in 70–90% of colorectal carcinoma and they are thought to be a late event in the multistep progress of tumorigenesis [4]. Wildtype-p53-protein plays an important role in cell-cycle regulation by arresting cells with mutagenic damage in late G1 phase before entering the S-phase, thus acting as a tumor-suppressor gene [5]. Mutant-p53 usually undergoes conformational changes that prolong the wildtype's very short half-life of a few minutes to several hours, leading to intracellular accumulation [6]. Thus, the mutant form not only loses suppressor activity but can even promote tumor growth [7].

In solid neoplasms including carcinomas of the lung [8], ovaries [9], pancreas [10], stomach [11] and thyroid gland [12], immunohistochemically detected p53-expression has been correlated with an unfavourable prognosis. So far two publications have reported about a shortened disease-free and overall survival in mammary carcinoma using a modern immunoluminometric assay [13], [14]. By contrast, Daifard could find no correlation between p53-overexpression and poor prognosis in breast carcinoma [15]. Furthermore Ferrero stated that the prognostic power of p53 is no higher than the well-recognized prognostic parameters in node-negative breast cancer [16]. However, this relationship has not been consistently observed in colorectal tumor and reports of different groups are contradictory [17], [18].

In the present study, we applied a luminometric assay (LIA) to quantify p53-protein in the cytosol of 144 colorectal carcinomas and 96 reference tissues of normal colorectal mucosa. The findings were correlated to several

clinicopathological parameters. Our aim was to compare p53-concentration with patients' outcome in terms of disease-free and overall survival in order to assess the protein's value as a prognostic factor.

Methods

Patients

Representative tissue samples of 144 patients who underwent surgical treatment for primary sporadic colorectal cancer were collected. No patient had received preoperative chemotherapy or radiotherapy. The specimens were snap frozen in liquid nitrogen and stored at -80°C until examination in laboratory. Seven carcinomas were located in the cecum, 15 in the ascending colon or hepatic flexure, 6 in the transverse colon or splenic flexure, 6 in the descending colon, 29 in the sigmoid colon and 78 in the rectum. In addition, 3 synchronous tumors were found. The distribution according to UICC classification was: 35 cases Stage I (T1-2N0M0), 32 Stage II (T3-4N0M0), 41 Stage III (T1-4N1-2M0) and 36 Stage IV (T1-4N1-2M1). Histologically 3 tumors were well (G I), 119 moderately (G II) and 22 poorly (G III) differentiated. Tumor size and preoperatively detected tumor markers CEA and CA 19-9 were also recorded. Our collective contained 59 female and 85 male patients treated due to colorectal malignoma. The age ranged from a minimum of 26 to a maximum of 89 years (median 61 years). Follow-up ranged between 2.4 to 54.3 months (median 25.3 months).

Luminometric immunoassay (LIA)

Cytosolic p53-protein levels were determined applying a quantitative sandwich-type luminometric assay [13]. LIA-mat[®] p53 (Sangtec Medical, Bromma, Sweden) is a monoclonal two-site immunoassay with monoclonal antibody 1801 coated polystyrene tubes serving as solid phase. First, 100 μl of either the prepared sample or p53-standard and the same amount of a tracer solution consisting of an aminobutylethylisoluminol (ABEI)-conjugated DO1 monoclonal antibody were pipetted into the tubes and incubated for 20 hours at room temperature. The antibodies bind to different denaturation-resistant epitopes at the N-terminus of mutant and wild-type p53-protein whereby luminescence-labelled antibody DO1 is indirectly bound to the tube. Non-reacted anti-p53 was

removed by 3 washing-steps with 0.9% sodium chloride. Tracer-p53 complex bound to the tube wall was subsequently detected by a light reaction applying LIA-mat® service kit (Byk-Sangtec Diagnostica, Dietzenbach, Germany). Using LIA-mat S300 Analysator® (Stratec, Dietzenbach, Germany) oxidation of isoluminol and thereby emission of photons was started by the automatic injection of alkaline peroxide solution and catalyst solution into the test tubes. Given that this emission vanishes within short time (less than 20 seconds) a luminometer is required to catch the signals at 425 nm. The light signal measured in RLUs (relative light units) by a photomultiplier is directly proportional to the amount of existent p53. A calibration curve allows rendering the phosphoprotein's content in ng p53 per mg total protein.

Determination of the cut-off value

To differentiate between tissues with elevated levels of the phosphoprotein and those with normal p53-expression, we looked for a value in the group of healthy colorectal reference mucosa, at which 95% of data were lower and only 5% higher than this figure. By doing so we found a cut-off value of 0.15 ng per mg for p53-concentration.

Statistical analysis

The relation of luminometric p53-expression to various clinicopathological parameters was evaluated using Wilcoxon's rank sum test for two group comparisons and the Kruskal-Wallis test for comparisons of more than two groups of categorized variables. Spearman rank correlations were performed when variables were continuous. The period from the date of resection to the date of death or last observation (if alive) was used for prognostic evaluation. The log-rank test was applied to compare Kaplan-Meier survival curves. In addition, Cox's proportional hazards model was performed with backward elimination for selection of prognostic factors using a significance level of 0.05.

Results

The assay was performed on cytosolic preparations of 144 primary colorectal adenocarcinomas with p53-protein-concentrations ranging from 0.004 ng/mg to 16.719 ng/mg (median 0.530 ng/mg). Setting the cut-off value at 0.15 ng/mg, 112 soluble fractions (77.8%) showed overexpression of the phosphoprotein. Besides, to compare these values with a reference and to determine an adequate cut-off point we analysed 96 specimens of normal colorectal mucosa (range 0.028–0.048 ng/mg, median 0.039 ng/mg) with the median value approximately 14 times lower than in malignant neoplasia. The relationship between p53 expression and several clinicopathological variables was examined and no correlation could be found with tumor stage, histological

grade, tumor diameter or localisation, age, sex or the tumor markers CEA and CA 19-9 (Table 1).

Follow-up ranged from 2.4 to 54.3 months (median 25.3 months) with survey of our collective in 15.3% less than one year, 31.9% between one and two years and 52.8% more than two years. Out of 144 patients 62 developed recurrence (43.1%), and 39 died of the underlying disease (27.1%). Five deaths were not tumor-related. Within the first postoperative year 58.1% of the recurrences occurred, increasing to 93.5% after 2 years. Recurrences were located in the liver (55.7%), lung (4.9%), abdominal wall, peritoneum, lymph nodes and in 34.4% several organs were affected.

Forty-nine out of 112 patients with p53-positive carcinomas (43.8%) developed recurrences, in comparison to the 13 out of 32 affected patients with negative protein expression (40.6%). The overall survival was 31 (29.0%) and 8 (25.0%), respectively. The 2-year relapse-free survival rate was 58.6% in the group of high p53-concentration compared to 63.6% in p53-negative lesions. The overall survival amounted 73.6% and 87.9%, respectively. After only 7.7 months a quarter of the first group had recurrences compared to 17.0 months in the latter ($p=0.43$). For overall survival the first quartiles were at 17.5 months and 32.1 months, respectively ($p=0.24$). Kaplan-Meier survival curves are shown in Figure 1 and Figure 2.

Although low p53-expression had a slightly better prognosis in terms of disease-free survival and overall survival, the log-rank test did not show statistical significance. When split into its single categories, advanced TNM-stage was associated with worse prognosis. The same was true for CA 19-9. Grading and patient's age showed significant differences in overall-survival and high levels of CEA were related to shortened relapse-free survival (Table 2). In addition, multiple Cox regression was performed to assess the influence of risk-factors on recurrence-free and overall survival (Table 3).

Only two factors were shown to have an effect on prognosis: Patients in Dukes stage IV (Dukes classification) were at higher risk for relapse by a factor of 6, and the risk for tumor-related death was seven times higher compared to Dukes stage I. Moreover, patients presenting CA 19-9 levels exceeding 37 U/l had a risk of recurrence or death that was approximately twice as high compared to tumor marker negative patients. There was no statistically significant survival advantage for patients with low p53-expression.

Table 1: Relationship between immunoluminometric p53-expression and various clinicopathological variables

p53 expression			
Variable	n =	Low (<0.15 ng/mg)	High (≥ 0.15 ng/mg)
number of patients (%)			
Tumor stage (Dukes classification)			
Stage I	35	9 (25.7)	26 (74.3)
Stage II	32	8 (25.0)	24 (75.0)
Stage III	41	11 (26.8)	30 (73.2)
Stage IV	36	4 (11.1)	32 (88.9)
Histologic grade			
Good	3	0 (100.0)	3 (100.0)
Moderate	119	26 (21.8)	93 (78.2)
Poor	22	6 (27.3)	16 (72.7)
Tumor size (cm)			
<3.0 cm	41	5 (12.2)	36 (87.8)
3.1–5.0 cm	81	24 (29.6)	57 (70.4)
>5.0 cm	22	4 (18.2)	18 (81.8)
Tumor location			
Proximal	31	8 (25.8)	23 (74.2)
Distal	113	24 (21.2)	89 (78.8)
CEA (ng/ml)			
<3.0	92	20 (21.7)	72 (78.3)
>3.0	38	12 (31.6)	26 (68.4)
CA 19-9 (U/l)			
<37	107	25 (23.4)	82 (76.6)
>37	22	6 (27.3)	16 (72.7)
Age (years)			
<60	56	11 (19.6)	45 (80.4)
60–70	55	19 (34.5)	36 (65.5)
>70	33	3 (9.1)	30 (90.9)
Sex			
Male	85	20 (23.5)	65 (76.5)
Female	59	12 (20.0)	47 (80.0)

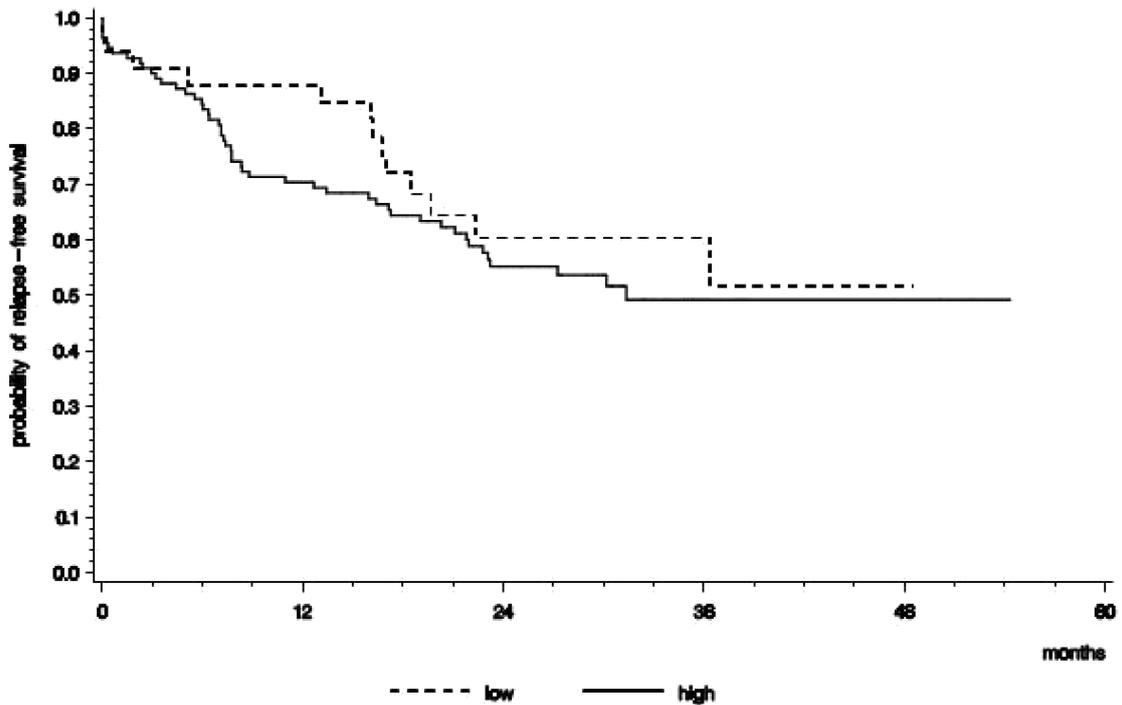


Figure 1: Relapse-free survival of patients whose tumors expressed p53-protein in a concentration higher than 0.15 ng/mg (solid curve) compared to low p53-expression (hatched curve)

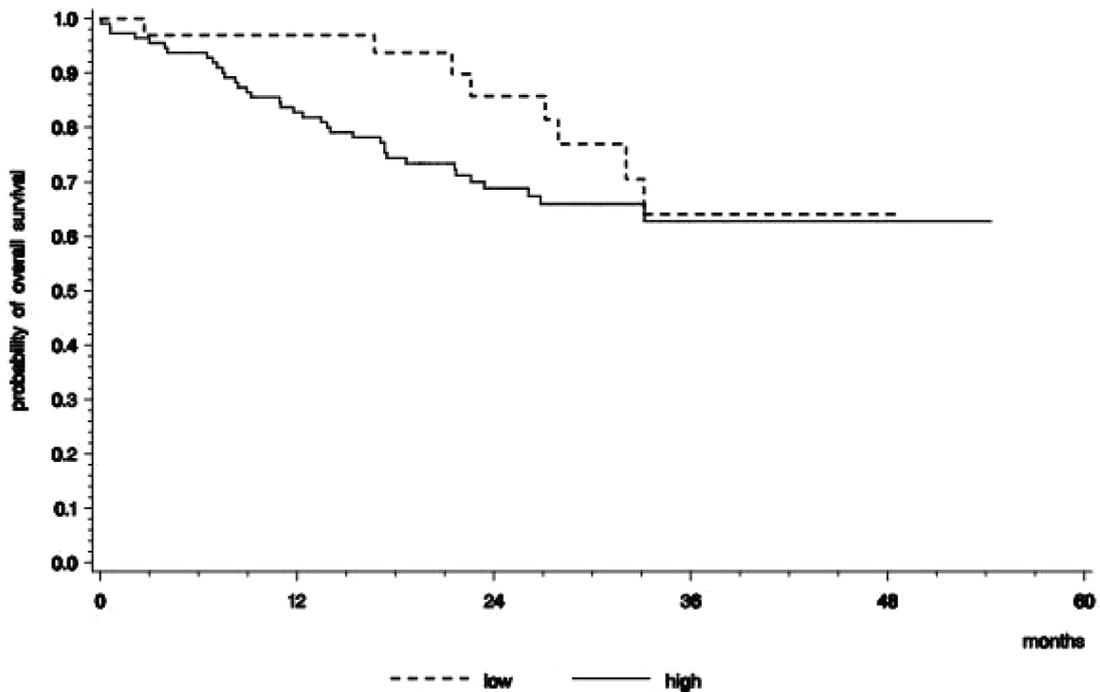


Figure 2: Overall survival of patients whose tumors expressed p53-protein in a concentration higher than 0.15 ng/mg (solid curve) compared to low p53-expression (hatched curve)

Table 2: Recurrence-free and overall survival in months related to different clinicopathological parameters (25%-rf = a quarter of the patients were free of recurrences after the observation period, 25%-os = a quarter of the patients were still alive after the observation period, n.r. = value not reached)

	Groups	Case number	Recurrence	25%-rf	p-value	Death	25%-os	p-value
Grading	G1/G2	122	51	12.6	0.59	30	27.1	0.04
	G3	22	11	8.8				
Tumor size	<4 cm	56	20	8.3	0.16	14	26.8	0.33
	≥4 cm	88	42	10.9				
Tumor location	Colon	66	29	7.1	0.58	18	21.5	0.67
	Rectum	78	33	16.7				
Age	<65 years	85	38	7.7	0.29	27	17.4	0.04
	≥65 years	59	24	17.1				
Sex	Male	85	36	13.0	0.67	19	26.1	0.31
	Female	59	26	7.8				
CEA	<3.0 U/l	92	30	16.7	0.002	19	32.1	0.12
	≥3.0 U/l	38	24	7.7				
CA 19-9	<37 U/l	107	36	17.1	<0.001	22	32.1	0.005
	≥37 U/l	22	17	5.1				
Invasion	T1/T2	43	11	22.3	0.01	7	n.r.	0.03
	T3/T4	101	51	7.7				
Lymph nodes	N0	70	19	23.1	<0.001	9	n.r.	<0.001
	N1-3	74	43	7.0				
Distant metastasis	M0	109	34	20.3	<0.001	15	n.r.	<0.001
	M1	35	28	1.5				
Luminometric immunoassay	Low	32	13	17.0	0.43	8	32.1	0.24
	High	112	49	7.7				
Immunohistochemistry	Negative	25	11	56.0	0.84	8	14.0	0.72
	Positive	51	24	52.9				
Tumor stage (Dukes classification)	Stage I	35	8	22.3	<0.001	5	n.r.	<0.001
	Stage II	32	10	23.2				
	Stage III	41	16	17.1				
	Stage IV	36	28	1.5				

Table 3: Influence of risk-factors upon relapse-free and overall survival in multivariate analysis by multiple Cox regression analysis

Prognostic factor	Groups	Case number	Recurrence		Tumor-related death	
			Hazard ratio	p-value	Hazard ratio	p-value
CA 19-9	<37 U/l	107	1.0 (reference)	0.01	1.0 (reference)	0.10
	≥37 U/l	22	2.21		1.82	
Tumor stage (Dukes classification)	Stage I	35	1.0 (reference)	<0.001	1.0 (reference)	<0.001
	Stage II	32	1.11		0.60	
	Stage III	41	1.46		1.20	
	Stage IV	36	6.06		6.98	

Discussion

In the present study, p53-expression in 144 colorectal adenocarcinomas was measured by a quantitative luminometric immunoassay and related to various clinicopathological parameters and prognosis, with the emphasis on examining the phosphoprotein's potential role as an independent prognostic factor. We used monoclonal antibodies D01 and PAb 1801, both of which are able to recognize not only the mutant forms, but also wildtype-p53 protein at denaturation resistant epitopes at the N-terminus of the molecule [19].

Using a cut-off value of 0.15 ng p53 per mg total protein, determined by the analysis of 96 normal tissue specimens, immunoluminometrically detected p53-overexpression was observed in 77.8%, which is comparable to results in immunohistochemistry [20], [21]. Our reference samples showed a median value of 0.039 ng/mg, which is almost 14 times lower than the correspondent value of the tumor group (median 0.530 ng/mg). Borg et al. [13] and de Witte et al. [14], who applied a similar immunoluminometric assay to breast carcinoma, found high levels of TP53 protein in 30% and 28% of their specimen, respectively, using cut-off points at the same level as we did in the first study and 2.5 ng/mg in the latter. The correspondence of our value with the one in the study of Borg et al. [13] seems to be accidental, as the later was determined by finding the best discrimination between good and bad prognosis while we calculated the cut-off value.

We did not find high levels of p53 to be an independent predictor of prognosis. Previous research undertaken to assess the prognostic value of p53-overexpression in colorectal carcinomas has revealed contradictory results [20], [22]. Direct comparison between these results is difficult due to the use of different monoclonal and polyclonal antibodies, various methods in preparing samples, a general lack of consensus in the interpretation of staining results and other investigator-related factors [23]. Interestingly, Bosari et al. Flamini et al. and Sun et al. described a strong correlation between immunohistochemically detected p53-overexpression in the cytoplasm of malignant cells and shorter overall-survival, with nuclear detection not being related to worse prognosis [24],

[25], [26]. Some studies have shown that wild-type p53 is restricted to the nucleus, whereas the mutant form interacts with the cytoplasmatic heat-shock-protein family and can even be found in both cellular compartments [6]. This may be of interest in measuring p53 by immunoluminometric assay, as it detects cytoplasmatic and not nuclear phosphoprotein.

Our investigation revealed that p53-overexpression, as measured by a luminometric immunoassay, is not a useful predictor of prognosis in patients with colorectal adenocarcinomas. At present, molecular techniques are still not suitable for routine clinical application. However, detection of p53-protein has been proposed as an indirect method of screening tumors for mutations within the p53-gene. One of the major advantages of this procedure, in contrast to genetic analysis, is that a statement on the protein's functional status can be made [27].

The limit of immunohistochemistry in p53-protein detection is its semiquantitative approach which is surpassed by quantitative immunoluminometry. The latter is crucial in comparison with the analytical determination of serum p53-antibodies. Suppiah et al. found that anti-p53 autoantibody sero-positivity did not influence the overall or disease-specific survival and was not related to clinical parameters of colorectal cancer, matching with our results. According to our findings, UICC Stage III/IV is the only independent prognostic factor in overall and disease-free survival [28], [29]. To our knowledge, no study has examined the correlation between serum p53-antibodies and quantitative immunoluminometric p53 in cytosols. Still TNM classification seems to be the best predictor of outcome, although no distinction between good and unfavourable prognosis within the groups can be made. To date, the numerous and complex ways by which the phosphoprotein regulates cell growth are not exhaustively understood. Furthermore, research on p53 is necessary to elucidate its mechanisms in order to allow a better interpretation of clinical studies with long-term follow-up about the protein's possible role as a prognostic factor. So far, insufficient evidence exists to recommend routine use of p53 in colorectal adenocarcinoma [30].

Conclusion

Immunohistochemical detection of p53 depends on several investigator-related factors which can be eliminated by the objective quantitative measurement of immunoluminometry. No study has examined the quantitative p53-expression in the cytosol of colorectal carcinoma before. In our study no correlation could be found between cytosolic p53-expression and various clinico-pathological parameters. Low p53-expression, however, has a slightly better prognosis in terms of disease-free and overall survival but this is not statistically significant. Expression of p53 remains an unclear prognostic parameter in colorectal carcinoma. As soon as the exact mechanisms of p53 in tumorigenesis are known, interpretation of clinical studies with long-term follow-up for p53 as a potential prognostic factor are then possible. The relationship between serum p53-antibodies and quantitative immunoluminometric p53 in cytosols of tumors could help to understand these mechanisms.

Notes

Conflicts of interest

We certify that there is no actual or potential conflict of interest in relation to this article.

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