

# p73 Overexpression Is a Prognostic Factor in Patients with Colorectal Adenocarcinoma<sup>1</sup>

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## ABSTRACT

**Purpose:** To examine the expression of p73 in the different stages of colorectal cancer development and the association of p73 expression with patient survival.

**Experimental Design:** Expression of p73 protein was evaluated by immunohistochemistry in 221 primary colorectal cancer patients, including 58 patients with matched normal mucosa and metastases in the regional lymph nodes.

**Results:** Frequency and intensity of p73 expression were markedly increased from the normal samples (19%) to primary tumors (67%) and to metastases (95%). Overexpression of p73 predicted poor outcome in the whole group of patients ( $P = 0.014$ ) and the subgroups with left-sided ( $P = 0.002$ ) or ras-positive tumors ( $P = 0.019$ ). The prognostic significance remained in the whole group ( $P = 0.008$ ) and the subgroup with left-sided tumors ( $P = 0.019$ ) after adjustment for the patient's sex, age, tumor stage, growth pattern, and differentiation. The p73 expression was positively correlated with ras expression ( $P = 0.006$ ). The 5-year survival rates were 37, 53, 72, and 74% for the patients with p73+/ras+, p73+/ras-, p73-/ras+, and p73-/ras- tumors, respectively ( $P = 0.007$ ).

**Conclusion:** Our data indicate that overexpression of p73 independently predicted poor prognosis in the patients with colorectal cancer.

## INTRODUCTION

The p73 gene has been cloned and mapped at chromosome 1p36.2–3, a region that has highly frequent loss of heterozygosity in various human cancers (1). The p73 gene encodes a protein that contains two distinct  $\alpha$ - and  $\beta$ -polypeptides. The structure of p73 protein is highly homogeneous to p53, and p73 also shares some common functions with p53 protein, such as activating the transcription of other genes, inhibiting cell growth, and inducing apoptosis, indicating that p73 is a p53-like

tumor suppressor (2). However, the activation of a p73 allele may contribute to tumor progression, and the increased levels of p73 mRNA transcription are found in various tumors compared with the surrounding normal tissues (3–8). These factors suggest that p73 is unlikely to be a tumor suppressor gene. Moreover, several other studies show that either loss of heterozygosity or mutation of p73 is not a common genetic event in the development of human cancers (9–13), which suggests that p73 does not play an important role in tumor development. There has been no report concerning the analyses of p73 expression in different stages of colorectal cancer and its prognostic implication in patients with colorectal cancer.

In the present study, we examined the expression of p73 in primary colorectal tumor with matched normal mucosa and metastasis from 221 patients with colorectal cancer to determine whether p73 is involved in tumor development, whether p73 expression is related to prognosis, and whether the p73 expression is correlated with ras, p53, or DCC.<sup>3</sup>

## PATIENTS AND METHODS

**Clinical and Pathological Data.** Two hundred twenty-one patients with colorectal adenocarcinoma, diagnosed at the Department of Pathology, Linköping University, between 1972 and 1996 were collected. There were 58 cases with matched normal mucosa and primary and metastatic tumors. Normal samples were taken from the margin, corresponding to distant resection, and were histologically free from precancer and cancer. The patients were followed up until the end of June 1999, and 94 patients died because of cancer. The patient's sex, age, tumor site, and Dukes' stage were obtained from surgical and pathological records. Mean age was 70 years (range, 34 to 93 years). Right-sided tumors included tumors in the ascending and the transverse colon, and left-sided tumors were found in the descending and sigmoid colon and the rectum. Growth pattern and differentiation were determined by two pathologists. The numbers of unknown cases, by category, were: sex, 4; age, 11; location, 13; Dukes' stage, 24; growth pattern, 20; and differentiation, 6. Expression of ras (14), p53 (15), and DCC (16) was estimated previously by immunohistochemistry on the serial sections at our laboratory. The ras protein was detected by using monoclonal antibody ras 11, which detects both normal and mutated forms of ras p21 (Oncogene Science, Inc., New York, NY). The expression was classified as a negative reaction if <5% of tumor cells were positive and otherwise as positive. NCL-p53-CM1 rabbit polyclonal antibody (Novocastra Laboratories Ltd., United Kingdom) was used to detect both wild-type and mutated forms of p53. The expression was considered positive when tumor cells were stained, irrespective of the

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<sup>3</sup> The abbreviation used is: DCC, deleted in colorectal cancer.

percentage of positive cells; otherwise, the expression was classified as negative. DCC protein expression was determined by using monoclonal antibody clone G97-449 (PharMingen, San Diego, CA). The expression was classified as positive if tumor cells were stained or negative if there were not any positive tumor cells. The numbers of available cases that matched with p73 expression were 131 for ras, 160 for p53, and 109 for DCC staining.

**p73 Antibody and Immunohistochemical Assay.** p73 is an affinity-purified goat polyclonal antibody raised against a peptide mapping at the COOH terminus of p73 $\beta$  of human origin (differs from corresponding p73 $\alpha$  sequence by two amino acids), and p73 reacts with both p73 $\alpha$  and p73 $\beta$  of human origin (Santa Cruz Biotechnology, Santa Cruz, CA). To demonstrate the specificity of the primary antibody, absorption of the primary antibody with a p73-blocking peptide (Santa Cruz Biotechnology) was performed, after which immunostaining was completely negative. Sections from paraffin-embedded tissue blocks were deparaffinized in xylene and rehydrated. Activity of endogenous peroxidase was blocked in 0.5% H<sub>2</sub>O<sub>2</sub> in methanol. To expose the masked epitopes, the sections were incubated in citrate buffer (pH 6.0) at 80°C for 10 min and then kept at room temperature. After a short rinse in PBS-Tween 20 (PBS plus 0.05% Tween 20), the sections were treated with power block (BioGenex, San Ramon, CA) for 10 min to block nonspecific background staining. After rinsing, the primary antibody in a 1:150 dilution in antibody diluent (Dako, Glostrup, Denmark) was applied at 4°C overnight. Subsequently, the sections were incubated with biotinylated donkey anti-goat IgG for 30 min, followed by goat avidin-biotin complex (Santa Cruz Biotechnology) for an additional 30 min. The peroxidase reaction was performed by an addition of 0.05% 3,3-diaminobenzidine tetrahydrochloride solution (Sigma Chemical Co., St. Louis, MO) and 0.02% H<sub>2</sub>O<sub>2</sub> in PBS for 8 min. The sections then were counterstained. The matched normal mucosa, primary tumors, and metastases in the lymph nodes were stained in the same run of the immunostaining to avoid bias on the pattern and intensity of staining. Sections known to show strong immunostaining for p73 were included in each run receiving either primary antibody or PBS, as positive and negative controls. In all of the staining procedures, the positive controls showed staining clearly, and there was no staining in the negative controls.

The sections were examined microscopically and scored independently by two pathologists in a blinded fashion without knowledge of clinical and pathological information, including survival data. To avoid artifact effect, the cells on the margins of sections and areas of poorly presented morphology were not counted. The entire histological sections were scanned at low magnification; the expression was classified as negative if there were no positive tumor cells and as equivocal if <10% of the tumor cells were positive. To assess the positive cases in which the areas of positive immunoreactivity were >10%, the cases were classified as weak, moderate, and strong based on the intensity of the staining. In fact, in the positive cases the staining for p73 covered almost all of the sections. In the 25 cases with discrepant results, a consensus score was reached after reexamination.

**Statistical Analysis.**  $\chi^2$  and McNemar's methods (17) were used to test significance of the difference in frequency of

*Table 1* Expression of p73 in normal mucosa, and primary and metastatic tumors from patients with colorectal adenocarcinoma

| p73      | Normal mucosa (%) | Primary tumor (%) | Metastatic tumor (%) |
|----------|-------------------|-------------------|----------------------|
| Positive | 11 (19)           | 147 (67)          | 55 (95)              |
| Negative | 47 (81)           | 74 (33)           | 3 (5)                |
| Total    | 58 (100)          | 221 (100)         | 58 (100)             |

p73 between normal samples and primary tumor and metastases and the association of p73 expression with clinical and pathological factors and expression of ras, p53, and DCC. Cox's proportional hazards model (18) was used to estimate the relationship between the p73 expression and survival. The curves describing survival were computerized according to Kaplan and Meier (19). Tests were two-sided, and  $P < 0.05$  was considered statistically significant.

## RESULTS

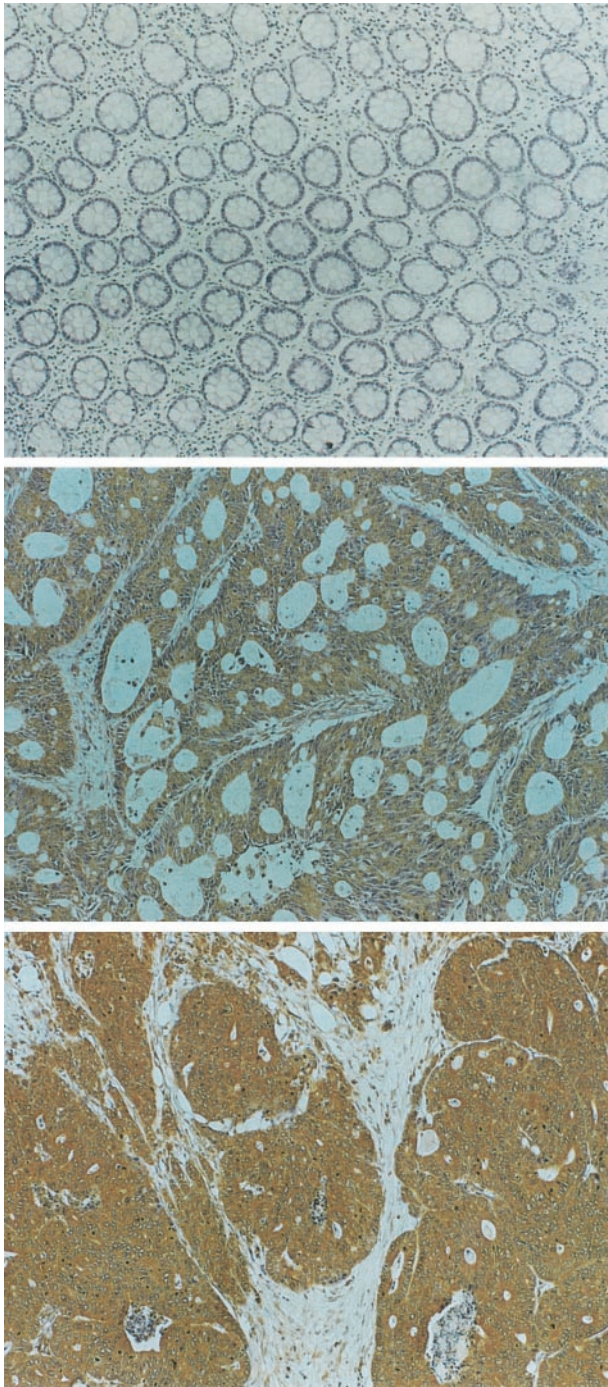
**p73 Expression in Normal Mucosa and Primary and Metastatic Tumors.** There were no significant differences with respect to clinicopathological characteristics between negative and equivocal staining, nor with weak, moderate, or strong staining. Therefore, the former two groups were classified as negative, and the latter three groups were classified as positive in our following analyses. As shown in Table 1, 19% of normal samples exhibited p73-positive characteristics, 67% in primary tumors, and 95% in metastases. In matched samples, the frequency and the intensity of p73 immunostaining were increased markedly from normal tissue to primary tumor and to metastasis ( $P < 0.05$ ; Fig. 1). In most of the negative primary tumors, the corresponding metastatic tumors were p73 positive. None of the normal samples revealed stronger p73 staining than that in the matched primary tumors.

**p73 Expression in the Primary Tumors in Relation to Clinical and Pathological Variables and Expression of ras, p53, and DCC.** Association of p73 expression in the primary tumors with clinical and pathological variables is presented in Table 2. The relationships of p73 expression in the primary tumors with the expression of *ras*, *p53*, and *DCC* genes are presented in Table 3. The expression of p73 was correlated positively with the elder patients ( $P = 0.012$ ) and ras expression ( $P = 0.006$ ). We were unable to find the correlation of p73 expression with patient's sex, tumor site, Dukes' stage, growth pattern, differentiation, and expression of *p53* and *DCC* genes.

**Expression of p73 in Primary Tumors and Survival.** As shown in Fig. 2, the patients with p73-positive tumors had significantly shorter survival than those with p73-negative tumors ( $P = 0.014$ ).

Furthermore, the prognostic significance of p73 expression was evaluated in the subgroups of patients with different-sided tumors. In the patients with left-sided tumors (Fig. 3A), the positive expression of p73 implicated shorter survival ( $P = 0.002$ ). In the patients with right-sided tumors, p73 expression did not contribute prognostic significance ( $P = 0.555$ ; Fig. 3B).

In the subgroup with ras-positive tumors, the patients with p73-positive primary tumors had significantly worse outcomes than those with p73-negative tumors ( $P = 0.019$ ; Fig. 4A). We



**Fig. 1** The normal mucosa does not express p73 protein (A), the corresponding primary tumor shows p73-positive staining in the tumor cells (B), and corresponding metastasis reveals even stronger p73 staining throughout the tumor cells (C). The sections are counterstained with hematoxylin.

were unable to find the statistical significance of p73 expression in the ras-negative group ( $P = 0.163$ ; Fig. 4B). The 5-year survival rates were 37, 53, 72, and 74% for the patients with p73+/ras+, p73+/ras-, p73-/ras+, and p73-/ras- tumors, respectively ( $P = 0.007$ ).

**Table 2** Expression of p73 in relation to sex, age, tumor site, Dukes' stage, growth pattern, and differentiation in patients with colorectal adenocarcinoma

|                 | p73          |              | <i>P</i> |
|-----------------|--------------|--------------|----------|
|                 | Positive (%) | Negative (%) |          |
| Sex             |              |              | 0.126    |
| Male            | 86 (72)      | 34 (28)      |          |
| Female          | 60 (62)      | 37 (38)      |          |
| Age (yr)        |              |              | 0.012    |
| <70             | 55 (57)      | 41 (43)      |          |
| ≥70             | 84 (74)      | 30 (26)      |          |
| Site            |              |              | 0.574    |
| Right-sided     | 60 (69)      | 27 (31)      |          |
| Left-sided      | 79 (65)      | 42 (35)      |          |
| Dukes' stage    |              |              | 0.369    |
| A               | 14 (67)      | 7 (33)       |          |
| B               | 40 (57)      | 30 (43)      |          |
| C               | 52 (71)      | 21 (29)      |          |
| D               | 22 (67)      | 11 (33)      |          |
| Growth pattern  |              |              | 0.809    |
| Expanding       | 52 (67)      | 26 (33)      |          |
| Infiltrating    | 84 (68)      | 39 (32)      |          |
| Differentiation |              |              | 0.312    |
| Well/moderately | 100 (65)     | 54 (35)      |          |
| Poorly          | 44 (72)      | 17 (28)      |          |

**Table 3** Association of p73 expression with ras, p53, and DCC in patients with colorectal adenocarcinoma

|           | p73          |              | <i>P</i> |
|-----------|--------------|--------------|----------|
|           | Positive (%) | Negative (%) |          |
| Ras       |              |              | 0.006    |
| Positive  | 58 (73)      | 22 (27)      |          |
| Negative  | 25 (49)      | 26 (51)      |          |
| p53 (CM1) |              |              | 0.230    |
| Positive  | 50 (59)      | 35 (41)      |          |
| Negative  | 51 (68)      | 24 (32)      |          |
| DCC       |              |              | 0.113    |
| Positive  | 31 (70)      | 13 (30)      |          |
| Negative  | 36 (58)      | 29 (45)      |          |

In multivariate analyses, after adjustment for the clinical and pathological variables, the prognostic significance of p73 expression remained in the whole group of patients ( $P = 0.008$ ; Table 4) and those with left-sided tumors ( $P = 0.019$ , Table 5) but not in the ras-positive group ( $P = 0.269$ ).

## DISCUSSION

In the present study, we found that the patients with p73-positive tumors had a shorter survival than those with p73-negative ones in the whole group of patients. The prognostic significance of p73 expression remained, even after adjusting for the clinical and pathological variables. Tannapfel *et al.* (20) have studied hepatocellular carcinomas and found that patients with p73-positive tumors have a poorer prognosis than those with p73-negative tumors. Furthermore, in subgroups of the patients, we realized that p73 was also an independent prognostic predictor in the patients with left-sided tumors. The patients with p73-positive tumors had a remarkably shorter survival period than those with p73-negative tumors. However, we failed

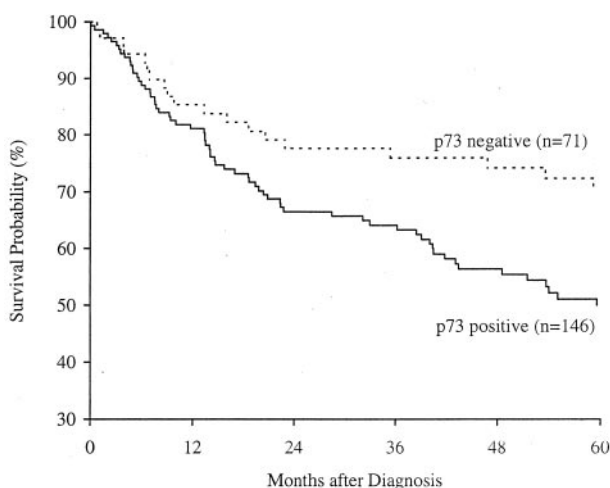


Fig. 2 Patients with p73-positive tumors had shorter survival than those with p73-negative tumors ( $P = 0.014$ ).

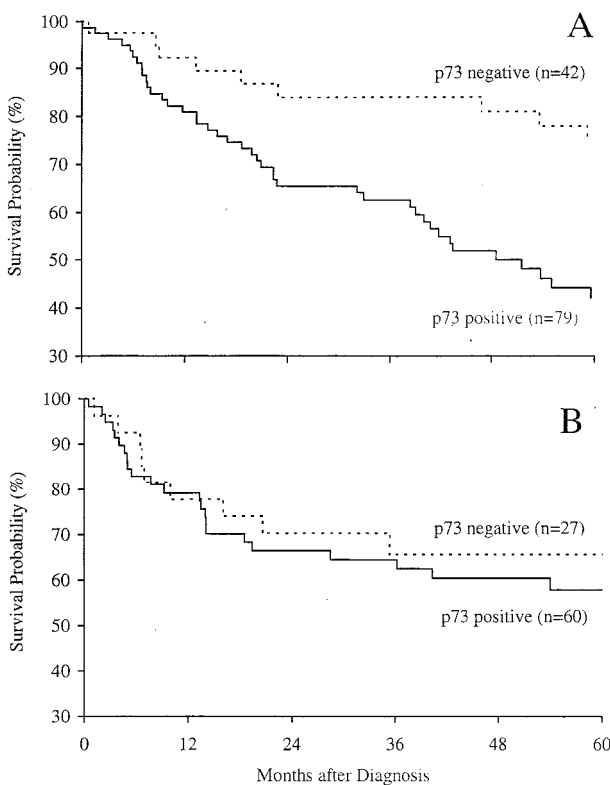


Fig. 3 In the group with left-sided tumors (A), the patients with p73-positive tumors had significantly shorter survival than those with p73-negative tumors ( $P = 0.002$ ). In the patients with right-sided tumors (B), there was no difference of survival between the patients with p73-positive and -negative tumors ( $P = 0.555$ ).

to find the prognostic value of p73 expression in the patients with right-sided tumors. Our results further support the theory that initiation and progression of left- and right-sided colorectal cancers may involve different mechanisms. There may be dif-

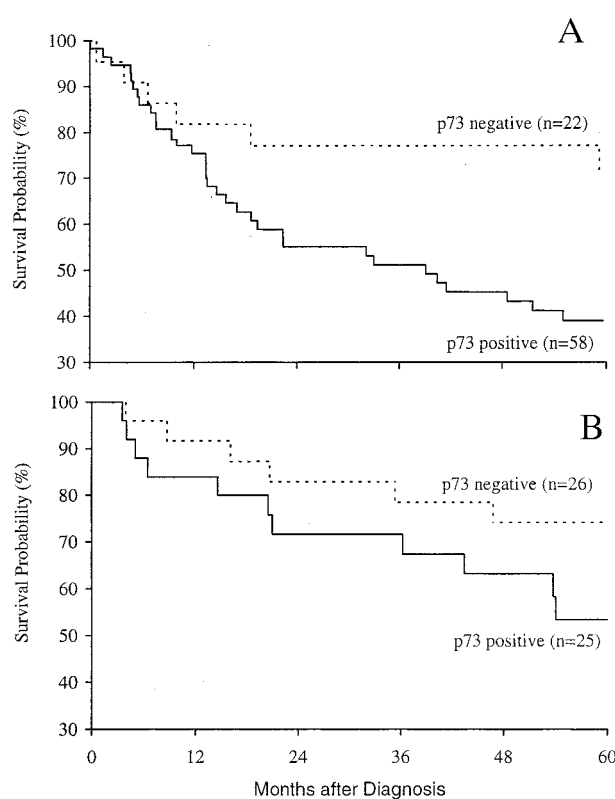


Fig. 4 In the group with ras-positive tumors (A), the patients with p73-positive tumors had significantly shorter survival than those with p73-negative tumors ( $P = 0.019$ ). In the patients with ras-negative tumors (B), the p73 expression did not contribute prognostic value ( $P = 0.163$ ).

ferent pathways by which p73 is involved to develop right- and left-sided colorectal cancers. However, the mechanism by which p73 exerts its biological action, if it does, remains to be investigated. Overall, the evidence suggested that overexpression of p73 was an indicator of prognosis in certain types of cancer patients and a subgroup of the patients.

The analyses in the combinations of p73 and ras showed that the patients with p73-/ras- tumors had the longest 5-year survival (74%), whereas the patients with p73+/ras+ tumors had the worst outcome (37%). The patients with p73-/ras+ tumors had longer survival (72%), whereas the patients with p73+/ras- tumors had shorter survival (53%). The data may suggest that p73 expression displays stronger prognostic significance than the ras protein. From the results in the present study that p73 was positively correlated with ras, it seems that p73 and ras were cofactors in affecting patient survival.

Whether p73 is functional as a tumor suppressor gene in carcinogenesis and tumor development remains unclear. Sunahara *et al.* (5) indicated that allelic loss of p73 was uncommon (17%), and there was not any mutation in 82 colorectal cancer patients. Several other groups have demonstrated that p73 mRNA transcription is increased in various tumor types, and the transcript level is higher in the tumor than that in the surrounding normal tissues (4-8). In addition, the activation of a silent p73 allele may contribute to the progression in bladder tumor

**Table 4** Multivariate analysis of p73 expression, sex, age, tumor site, Dukes' stage, growth pattern, and differentiation in relation to survival in patients with colorectal adenocarcinoma

|                 | No. of patients | Cancer death rate ratio (95% confidence interval) | P       |
|-----------------|-----------------|---|---------|
| p73 expression  |                 |   | 0.008   |
| Negative        | 63              | 1.0   |         |
| Positive        | 115             | 2.136 (1.191–3.831)                               |         |
| Sex             |                 |   | 0.510   |
| Male            | 98              | 1.0   |         |
| Female          | 80              | 1.182 (0.719–1.943)                               |         |
| Age (yr)        |                 |   | 0.028   |
| <70             | 92              | 1.0   |         |
| ≥70             | 86              | 1.796 (1.057–3.052)                               |         |
| Site            |                 |   | 0.459   |
| Left-sided      | 107             | 1.0   |         |
| Right-sided     | 71              | 1.206 (0.732–1.972)                               |         |
| Dukes' stage    |                 |   | <0.0001 |
| A               | 17              | 1.0   |         |
| B               | 62              | 1.916 (0.432–8.516)                               |         |
| C               | 70              | 5.457 (1.295–22.98)                               |         |
| D               | 29              | 26.905 (6.143–117.84)                             |         |
| Growth pattern  |                 |   | 0.017   |
| Expanding       | 69              | 1.0   |         |
| Infiltrating    | 109             | 1.863 (1.1–3.155)                                 |         |
| Differentiation |                 |   | 0.984   |
| Well/moderately | 125             | 1.0   |         |
| Poorly          | 53              | 1.005 (0.611–1.654)                               |         |

**Table 5** Multivariate analysis of p73 expression, sex, age, Dukes' stage, growth pattern, and differentiation in relation to survival in patients with left-sided colorectal adenocarcinoma

|                 | No. of patient | Cancer death rate ratio (95% confidence interval) | P       |
|-----------------|----------------|---|---------|
| p73 expression  |                |   | 0.019   |
| Negative        | 36             | 1.0   |         |
| Positive        | 68             | 2.975 (1.048–7.714)                               |         |
| Sex             |                |   | 0.497   |
| Male            | 57             | 1.0   |         |
| Female          | 47             | 1.29 (0.625–2.68)                                 |         |
| Age (yr)        |                |   | 0.005   |
| <70             | 53             | 1.0   |         |
| ≥70             | 51             | 2.841 (1.339–6.025)                               |         |
| Dukes' stage    |                |   | <0.0001 |
| A               | 14             | 1.0   |         |
| B               | 34             | 4.876 (0.605–39.29)                               |         |
| C               | 37             | 13.74 (1.784–106.17)                              |         |
| D               | 19             | 75.39 (9.337–609.11)                              |         |
| Growth pattern  |                |   | 0.234   |
| Expanding       | 37             | 1.0   |         |
| Infiltrating    | 67             | 1.494 (0.756–2.948)                               |         |
| Differentiation |                |   | 0.145   |
| Well/moderately | 77             | 1.0   |         |
| Poorly          | 27             | 1.629 (0.858–3.096)                               |         |

(3). The studies on esophageal cancers (10), breast cancer (11), melanoma (13), and neuroblastoma (21) have shown that allelic loss, mRNA transcription, and mutations of *p73* gene are not main genetic events in carcinogenesis and tumor development. To understand further the essential roles of *p73* and provide an insight into the development of colorectal cancer, it is of interest to investigate the expression of *p73* in the different stages of tumors. Therefore, in the present study, the expression of *p73*

protein was examined in the primary and metastatic tumors, together with the corresponding normal colorectal mucosa from patients with colorectal cancer. The results showed that both frequency and intensity of *p73* expression were markedly increased from normal tissue to primary tumor and to metastasis, which indicated that *p73* may be involved in the development of colorectal cancer, including metastasis. Up to now the data do not provide genetic evidence that the inactivation of *p73* is required for tumorigenesis. The activation of a silent allele or overexpression of *p73*, rather than a tumor suppressor function, may contribute to tumor development.

In summary, the data suggest that *p73* was up-regulated during tumor development from the normal mucosa to the primary and metastatic tumors. The overexpression of *p73* was a valuable prognostic marker to predict poor outcome for the patients with colorectal cancer.

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