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SHORT COMMUNICATION

VIA MEDICA

Decreased expression of *p73* in colorectal cancer

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Abstract

Introduction. The colorectal cancer (CRC) is one of the most frequent cancer in Poland and worldwide. This disease is characterized by distinct genetic alterations. *p73* belongs to the *p53* gene family; however, its role in the pathogenesis of CRC has not been completely understood. *p73* gene encodes several mRNA variants and protein isoforms with its longest and fully functional *p73α* (mRNA) and TAp73*α* (protein) isoform. The aim of the study was to investigate *p73* gene expression at the mRNA (*p73α*) and protein (TAp73*α*) levels in CRC.

Material and methods. Small sections of the CRC tumor tissue and macroscopically unchanged colon mucosa and submucosa from the dissection margin were collected from 23 patients diagnosed with CRC. *p73* mRNA levels were measured by Real-time PCR (QPCR) method and the expression level of TAp73*α* protein was assessed by Western blotting (WB) and immunohistochemical (IHC) staining.

Results. We found a 37% decrease in the level of p73*α* mRNA in neoplastically changed (tumor) compared with unchanged normal colon tissue from the surgical margin ($p = 0.041$). No correlations were found between mRNA levels in cancer tissue and clinical-pathological parameters.

The semi-quantification of TAp73*α* protein revealed lower and higher TAp73*α* protein contents in 11/23 and 12/23 of tumor samples, respectively, when compared with the median value of TAp73*α* protein in normal colon tissue ($p = 0.61$). The level of TAp73*α* protein level was 5 times lower in poorly differentiated cancer cells (G3) in comparison to moderately differentiated ones (G2; $p = 0.02$). No statistically significant correlations were observed between the level of the TAp73*α* protein and clinical-pathological patients' characteristics.

The IHC analysis of TAp73*α* protein presence in CRC samples showed decreased immunoreactivity when compared with matched sections of the unchanged colon wall in 4/9 patients, similar intensity of the IHC reaction in 4/9 patients and increased immunoreactivity in 1/9 patients. The TAp73*α* protein was localized mainly in the cytoplasm of the cancer cells. No statistically significant correlations between IHC results and clinical-pathological features of the patients were found.

Conclusions. The obtained results suggest that the *p73* gene may play a role as a tumor suppressor in the CRC progression. *(Folia Histochemica et Cytobiologica 2016, Vol. 54, No. 3, 166–170)*

Key words: *p73*; colorectal cancer; QPCR; Western blotting; immunohistochemistry

Colorectal cancer (CRC), a multifactorial disease caused by genetic, lifestyle and environmental factors, belongs to the most common malignancies in the world. Although nearly 90% of patients may be successfully cured by surgery at the early stage, CRC is frequently diagnosed at the late stage, *i.e.* Dukes' C and D, when the prognosis is poor [1, 2]. Similarly as in other cancer types, CRC development is associated with dysregulation of some important intracellular signaling pathways such as p53 or Wnt/*β*-catenin [3]. Tumor

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protein TP73 along with p63, belongs to the *p53* gene family and can induce cell cycle arrest as well as apoptosis *via* the activation of p53-responsive genes [4]. On the contrary, the over-expression of *p73* gene was observed in several types of neoplasms, suggesting the oncogenic role of *p73* gene in cancer development and progression [5]. The findings of either suppressor or oncogenic role of TP73 protein in carcinogenesis may be associated with different functions of several isoforms of the TP73 protein [4–6]. *p73α* mRNA variant encodes the longest protein isoform (69.6 kDa, 636aa) characterized by the occurrence of three domains: transactivation (TA), DNA-binding and proline-rich ones. Other *p73* mRNA variants (αTAp73 and ΔNp73) encode smaller or truncated proteins which lack at least one domain (*i.e.* αTAp73 does not contain TA domain) [7]. Although there are numerous studies related to the p53 status in CRC, the reports on the expression of *p73* gene and protein in this type of cancer are scarce and unequivocal [8–10]. In CRC the association between TP73 protein level and cancer progression was barely studied and the results were opposite [11]. Therefore, we decided to assess the expression of the *TP73* gene in paired tumor-surgical margin specimens of CRC patients at the mRNA and protein levels.

The retrospective study was approved by the local bioethics committee, and informed, written consent regarding the use of tissue was obtained before surgery from all CRC patients. The specimens were obtained from two surgical clinics from 2011 to 2014 (Department of Oncological Surgery, Warmia and Mazury Oncological Center, Olsztyn and Department of General, Endocrine, and Transplant Surgery, Medical University of Gdansk, Gdansk, Poland). Clinical and demographic data were collected at the time of enrollment.

The samples were obtained from 23 CRC patients: 17 females and 6 males; the median age was 70 years (range: 54–89 years, mean age: 69 years).The tumor location was: rectum (N = 8), sigmoid/descending colon (N = 4), transverse colon/splenic flexure (N = 4) and cecum/ascending colon (N = 7). The sizes of tumor were: < 3 cm (N = 1), 3–5 cm (N = 12), > 5 cm (N = 10). According to the UICC/AJCC classification applicable to 22 patients the following staging was obtained: stage I (N = 1), stage II (N = 7), stage III (N = 11), stage IV (N = 3). The histological G grading data was obtained for 21 patients: G2 (moderately differentiated cells; N = 18) and G3 (poorly differentiated cells, N = 3).

The collection of neoplastically changed (tumor) and normal colon tissue samples was performed as described previously [12]. For immunohistochemistry, the tissue samples were fixed in 4% buffered formalin for 3–4 days and standard histologic preparation followed [13]. For the RNA and protein isolation, part of each sample was immediately stored in RNA--later buffer (Ambion Inc., Austin, TX, USA) at 4°C overnight or immersed and kept in liquid nitrogen overnight; eventually all samples were stored at –80°C until further use. Total RNA Prep Plus kit (A & A Biotechnology, Gdynia, Poland) was used for RNA extraction according to manufacturer's protocol and the obtained RNA was stored at –80°C. 1 μg RNA was reverse-transcribed (RT) with M-MuLV RT enzyme (Thermo Fisher Scientific, Fitchburg, WI, USA) and oligodT_{18} primers (Sigma-Aldrich, Munich, Germany) were added to obtain 20 μL of cDNA. The relative levels of *p73α* mRNA were assessed by quantitative real-time PCR (QPCR) method (StepOne apparatus, Life Technologies-Applied Biosystems, Grand Island, NY, USA). cDNA was 4× diluted and qPCR reaction was prepared in 10 μL final volume: 1 μL cDNA, 200 nM each primer, 2× SensiFastSybr™ No-Rox kit (Bioline, London, UK). Primers were designed with the use of PrimerBlast online software: *p73α*: 5'-CACCACGTTTGAGCACCTCTGG; 5'-TGCTCAGCAGATTGAACTGGGC, *PGK1* (normalization gene): 5'-GTTGACCGAATCACCGACCTCTC; 5'-AGAACAGAACATCCTTGCCCAGC. The qPCR time-temperature protocol was applied as previously described [13].

The semi-quantification of TAp73α protein was performed by Western-blot (WB) technique as previously described [14] with some modifications: we used rabbit polyclonal anti-TAp73α antibodies in 1:1000 dilution in 2% TBS buffer (Sigma-Aldrich) and the overnight incubation at 4°C followed. After 3× wash in TBS with 2% Tween (TBS-T) for 15 min, the incubation for 2 h was carried out with secondary anti-rabbit monoclonal horseradish peroxidase-conjugated antibodies (Sigma-Aldrich). TAp73α protein level was normalized to the level of GAPDH protein by using rabbit anti-GAPDH antibodies at 1:5000 dilution.

The immunohistochemical (IHC) detection of TAp73α protein in CRC and normal colon wall sections was performed in paired samples of 9 CRC patients by previously described technique [15] using TP73α rabbit anti-human polyclonal antibody (bs-6147R, Bioss, Woburn, MA, USA) at the dilution 1:100. After incubation for 2 h with primary antibodies at room temperature, slides were washed in PBS and incubated with an appropriate secondary antibody [ImmPRESS Anti-Rabbit Ig (peroxidase) Polymer Detection Kit, Vector Labs, Burlingame, CA, USA] for 30 min. Immunoreactive cells were visualized by

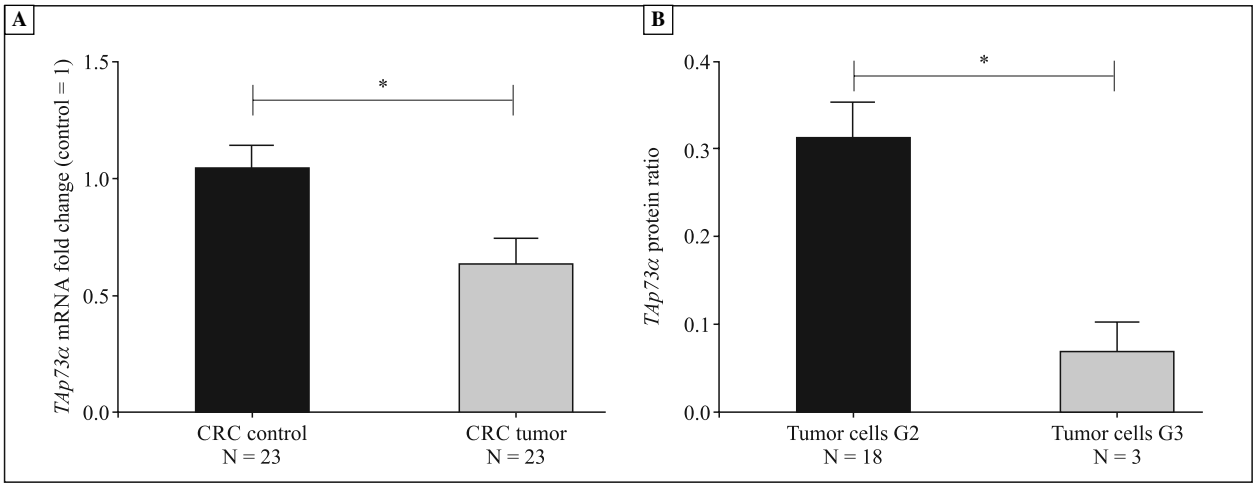

Figure 1. The expression of the *p73* gene at the mRNA (**A**) and TAp73α protein (**B**) level in colorectal cancer (CRC) and matched normal colon mucosa (CRC control). mRNA levels were determined by real-time PCR and the TAp73α content by Western blotting as described in Material and methods. The results represent the mean value and standard deviation (SD). *Statistically significant difference between groups ($p < 0.05$), Mann-Whitney U test.

addition of 3,3'-diaminobenzidine solution (DAB Peroxidase Substrate Kit, Vector Labs). The specificity of the IHC staining was determined by a negative control, in which primary antibody was replaced by 2.5% normal horse serum [ImmPRESS Anti-Rabbit Ig (peroxidase) Polymer Detection Kit] and the slides were processed under the same conditions as described.

Statistical analyses were performed using Prism ver. 6.05 program (GraphPad Software, San Diego, CA, USA) as described previously [13]. Mann-Whitney U and Kruskal-Wallis ANOVA tests were applied to check the statistical significance between two or more groups of samples/patients, respectively. Spearman's test was used to check the possible correlations. $P < 0.05$ was set as the level of statistical significance.

We found that the level of the *p73*α mRNA was by 37% lower in CRC neoplastically changed (tumor) samples than in unchanged mucosa and submucosa tissue obtained from the surgical margin ($p = 0.041$; Figure 1A). The lower *p73*α mRNA content was found in 15/23 (65%) patients. There were no differences between *p73*α mRNA level in tumor samples and age, gender, tumor location, UICC/AJCC, presence of metastasis or histological G grading; moreover, no correlations between molecular and clinical variables were found.

Interestingly, the semi-quantification of TAp73α protein by WB did not confirm the results of the *p73*α mRNA assessment since the lower and higher TAp73α protein contents were observed in 11/23 and 12/23 of tumor samples, respectively, when compared with the median value of TAp73α protein in normal colon tissue ($p = 0.61$; data not shown). We did not find any correlation between *p73*α mRNA and TAp73α protein levels. In reference to CRC staging, no differences in TAp73α protein content and clinical-pathological data were observed. However, in regard to histological G classification we found that TAp73α protein level was 5 times lower in poorly differentiated cancer cells (G3, $n = 3$) in comparison with moderately differentiated ones (G2, $N = 20$; $p = 0.02$; Figure 1B).

The IHC analysis of TAp73α protein presence in CRC samples showed decreased immunoreactivity when compared with matched sections of the unchanged colon wall in 4/9 patients, similar intensity of the IHC reaction in 4/9 patients and increased immunoreactivity in 1/9 patients. The TAp73α protein was localized mainly in the cytoplasm of the cancer cells. Moreover, the immunoreactive product was also present in the cytoplasm of epithelial cells and some stromal cells of the colon mucosa (Figure 2).

The analysis of the *p73* gene expression in CRC has been previously reported by other authors; however, with disparate results. Dominguez *et al.* [16] analyzed the expression of the N-terminal isoforms of *p73* in tumor tissue of 113 CRC patients both at the mRNA and protein levels using QPCR and IHC method, respectively. They found increased levels of mRNAs encoding TAp73, αTAp73 as well as αNp73 isoforms in the tumor tissue compared with the macroscopically unchanged colon mucosa. TAp73 immunoreactivity was observed in 19% of the colon cancer cases, while TAp73 protein expression correlated with mRNA quantification in 22 (88%) of the 25 colon cases. Su *et*

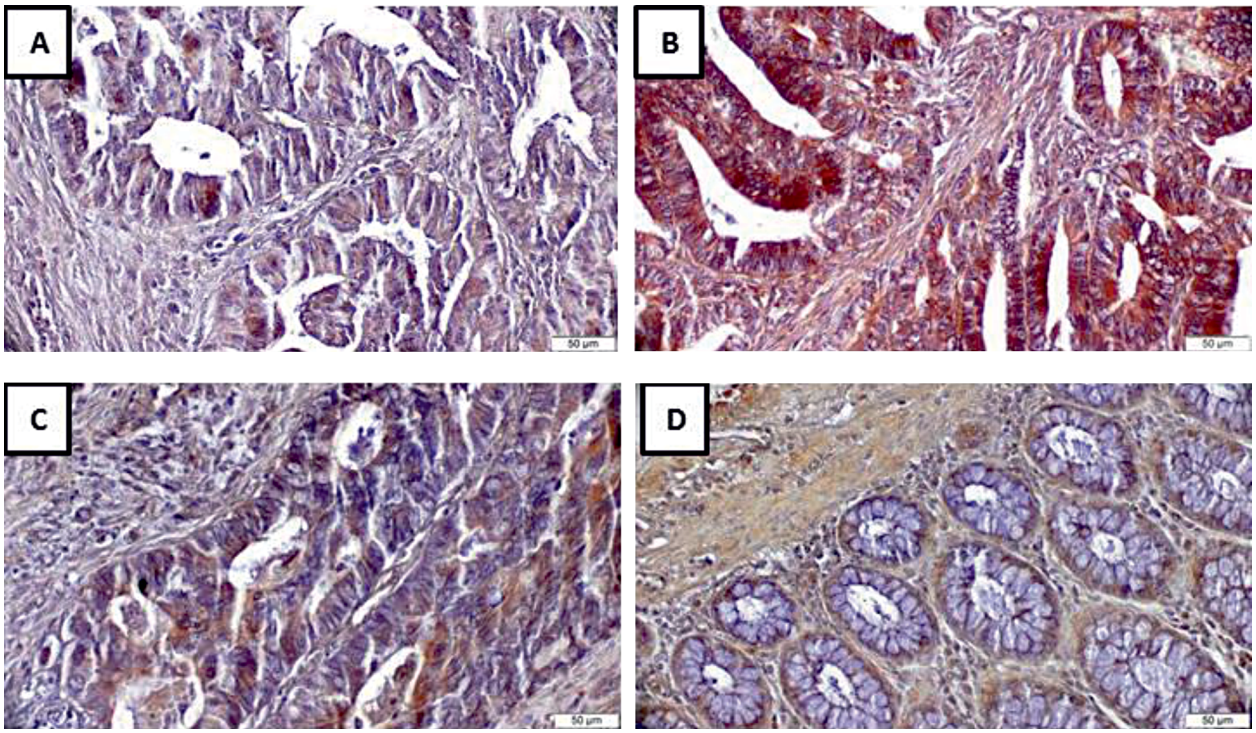

Figure 2. Representative examples of p73 protein immunoreactivity in colorectal cancer and normal colon tissue. Weak immunoreactivity of the TAp73α protein in CRC tumor tissue (**A**) and strong expression in the corresponding normal mucosa of the same patient (**B**). Comparable intensity of the TAP73α protein immunoexpression in tumor tissue (**C**) and normal mucosa (**D**) of the same patient. The immunohistochemical staining was performed as described in the body text. Magnification: ×400.

al. analyzed *TP73* gene expression at the mRNA and protein level in CRC by *in situ* hybridization and IHC technique [11] and found higher *p73* mRNA and TP73 protein levels in all samples of CRC neoplastically changed tissue than in paracancerous tissue. Guan *et al.* [17] investigated the expression of C-terminal TP73α protein isoforms by WB and both p73α and p73α by immunohistochemistry in tumor tissue and macroscopically unchanged colon tissue of 56 CRC patients. The p73 protein staining was confined to the nuclear area. Positive staining frequently showed a heterogeneous distribution and the intensity of staining also varied, whereas in our observations the staining intensities were similar (Figure 2). In normal colon mucosa 70% (39/56) cases were negative while CRC tissue demonstrated a high level of expression: 73% (41/56) exhibited p73 characteristics (17). WB results showed that 82% (46/56) samples had high level of p73 protein expression, 14% (8/56) did not show a significant difference in p73 protein expression and 4% (2/56) of cases even expressed lower than the normal level of the control. The authors found positive correlation between the results of WB and IHC analyses [17].

The discrepancies between results of our study and those of other authors might have been caused by the differences in the applied methods, low number of the analyzed patients and diversity of the study group in terms of clinical and pathological features. Moreover, some disadvantage of our study may be connected with the different number of cases assessed by QPCR, WB and IHC methods. In fact, our study focused only on TAp73α isoform, which has pro-apoptotic function due to the presence of the TA domain. As a result, the TAp73α isoforms act intracellularly in the similar way as p53 protein [4]. Since other authors studied the whole group of TP73 isoforms [17] or the whole *p73* mRNA without the isoform differentiation [11], our results may be only compared with the above mentioned data of Domininquez *et al.* [16].

Although other authors revealed opposite to our data of *p73* gene expression between tumor and unchanged tissues of CRC patients, they also did not observe any associations between molecular and clinical and pathological parameters (such as sex, age or TNM status) [11, 16, 17]. On the contrary to our observations of decreased TAp73α protein in G3 samples, Su *et al.* [11] demonstrated overexpression of the *p73*

gene in CRC assessed as G2 and G3 compared with G1, both at the mRNA and protein levels. Again, such observation cannot be compared with our study, since none of our analyzed CRC sample was classified to G1 (well differentiated cells) grade.

In conclusion, our preliminary study confirms the observed contribution of the *p73* gene to the progression of CRC; however, our results should be confirmed by larger number of cases with wider clinical-pathological spectrum.

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