

## Changes in immunoexpression of p53, Ki-67, Ets-1, APAF-1 and PTEN in serrated and conventional colon adenomas

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### Abstract

The balance between apoptosis and proliferation is tipped towards a decrease of apoptosis as the colonocyte progresses in the adenoma to carcinoma sequence of colon carcinogenesis. According to literature data, proteins like p53, Ki-67, APAF-1, Ets-1, PTEN contribute to inhibition of apoptosis and stimulation of proliferation. *Aim:* Considering the complex interference among colorectal carcinogenetic mechanisms, our aim was to study the markers Ets-1 and APAF-1 relative to p53, Ki-67 and PTEN expression in colon adenomas/polyps (A/P). *Materials and Methods:* We performed immunohistochemistry on 99 colon A/P cases from the material of the Department of Pathology, Emergency County Hospital of Tirgu Mures, Romania. Secondary EnVision Flex/HRP (Horseradish peroxidase) (20 minutes) was used for signal amplification. *Results:* The majority of A/P show increased Ki-67, p53, Ets-1 expression, decreased APAF-1 expression and preserved PTEN expression. p53, Ki-67, Ets-1 and APAF-1 demonstrated statistically significant correlations with histological type and grade of dysplasia. We also observed that expression of these proteins in the intestinal crypts has a typical distribution according to histological type and grade of dysplasia. *Conclusions:* In case of hyperplastic polyps APAF-1 expression decreases as p53 and Ki-67 expression increases, followed by a decrease in PTEN expression in serrated adenomas, and an increase of Ets-1 expression in conventional adenomas.

**Keywords:** colon polyps/adenomas, carcinogenesis, apoptosis.

### Introduction

According to recently introduced histological classifications, epithelial lesions of the colon can be conventional adenomas (tubulous, villous and tubulovillous) [1] and serrated hyperplastic polyp – HP, sessile-serrated adenoma/polyp – SSA/P, traditional serrated adenoma – TSA) [2].

Cell homeostasis depends on the balance between apoptosis and cell proliferation controlled by oncogenes and tumor suppressor genes, apoptotic and anti-apoptotic genes [3]. Development of colorectal cancer (CRC) from polyps/adenomas (A/P) requires the activation of certain carcinogenetic mechanisms that subsequently induce changes in proliferation, differentiation, cell apoptosis and angiogenesis [4, 5]. As the colonocyte progresses in the adenoma to carcinoma sequence of colon carcinogenesis, loss of the balance between apoptosis and proliferation also involves p53, Ki-67, APAF-1, Ets-1, PTEN [6].

Mutation of the p53 gene plays an important role in the adenoma–carcinoma sequence, [7] but it also is involved in the chemotherapy and radiotherapy resistance mechanism [8]. In case of DNA damage, p53 stimulates the mitochondrial apoptotic pathway through APAF-1 (apoptotic protease activating factor) [9, 10]. In human cells, in the absence of the APAF-1, chromosomal instability becomes enhanced [11]. APAF-1, along with other proteins participating in the intrinsic or mitochondrial apoptotic mechanism (Bcl-2, BAX, caspases) is studied with a great deal of interest as a potential biomarker for CRC [12]. In SW620 tumor cells resistant to apoptosis,

APAF-1 expression decreases while p53 expression is increased [6]. Loss of heterozygosity (LOH) (12q23), absent in A/P and present in CRC and hepatic metastases is the main mechanism explaining decrease of APAF-1 expression along the adenoma–carcinoma sequence, also signaled by the decrease of mRNA quantity in CRC [9].

The Ets family is characterized by the Ets domain that binds to the Ets binding site found in the promoters of the target genes [13]. These transcription factors are involved in various biological processes like cell proliferation and differentiation, hematopoiesis, apoptosis, tumor invasion, tissue remodeling, angiogenesis [13, 14]. The role of Ets-1 in the mechanism of apoptosis depends on the tissue [15]. Ets-1 may act as a pro-apoptosis factor in endothelial cells [16] of the p42-Ets-1 isoform in colon tumor cells [17]; in embryonic stem cells, it controls the post-translation activity of protein p53 [18]. The Ets-1 gene is activated *via* the Ras/Raf/MAP/MEK/ERK pathway in CRC cells [5] and in adrenal gland tumors [19] it stimulates carcinogenesis; in melanomas it contributes to the stimulation of apoptosis resistance, and it is co-activated through the AKT/PI3K pathway as well [15]. In human colorectal carcinoma cells, the FBI-1 human proto-oncogene stimulates Ets-1 transcription factor activity even through p53 and enhances Ets-1 accumulation in the nucleus [20]. It has been demonstrated that in breast cancer [21], ovary cancer, cervical cancer [20], stomach, pancreas, and thyroid cancer [22] the increased expression of Ets-1 is associated with a poor outcome [20].

Tumor suppressor phosphatase and tensin homologue deleted from chromosome 10 (PTEN) blocks the PIP3/Akt

pathway that induces cell proliferation upon activation by ErbB receptors. PTEN is able to stimulate cell apoptosis by blocking c-myc expression [23]. Inactivation of the PTEN gene occurs very frequently in human malignant lesions like glioblastomas, prostate cancer, endometrial, pulmonary, colorectal, gastric, esophageal, breast cancers and melanoma [23–25]. The presence of PTEN expression in mCRC is an adequate marker for evaluation of the efficacy of cetuximab therapy that blocks the EGFR signaling pathway [23].

Considering the complex interference among these carcinogenetic mechanisms, we studied the markers Ets-1 and APAF-1 relative to p53, Ki-67 and PTEN expression in colon A/P.

## Materials and Methods

In this study, we performed immunohistochemistry on 99 colon adenomas/polyps (A/P) (22 hyperplastic polyps – HP, seven sessile-serrate adenomas/polyps – SSA/P, three traditional serrated adenomas – TSA, 26 tubular adenomas – TA, 41 tubulovillous adenomas – TVA) from the material of the Department of Pathology, Emergency County Hospital of Tîrgu Mureş, Romania. Most of the tumors were seen in males (56.5%), in the left colon (67.7%). The mean age of the patients included in the study was 62.4±2 years (range: 34–86 years). In HP, SSA/P and TSA the signs of conventional dysplasia were absent (WD), but these were seen in all TA and TVA cases; the most frequent was low grade dysplasia (LGD) (65.3% of TA and 58.5% of TVA), and the rest were high-grade dysplasia (HGD).

The 3-µm thick sections obtained from the formalin fixed and paraffin embedded resection tissue specimens were routinely dewaxed and rehydrated followed by endogenous peroxidase blocking. Antigen retrieval was performed by pressurized steam cooking [citrate solution, pH 6 for p53, Ki-67; Tris/EDTA (ethylenediaminetetraacetic acid) buffer, pH 9 for APAF-1, PTEN, Ets-1]. We used the following mouse monoclonal antibodies for p53 (clone DO-7, DakoCytomation, Denmark) in 1/100, Ets-1 (clone IG11, Leica Biosystems Newcastle Ltd., UK) in 1/200, APAF-1 (clone NCL-APAF1, Leica Biosystems Newcastle Ltd.) in 1/600, PTEN (clone PTN-18, MOB 449, Diagnostic BioSystem, Pleasanton, USA, against the C-terminal segment of the protein) in 1/400 (overnight, 4°C), and rabbit monoclonal antibody Ki-67 (clone SP6, LabVision Fremont, CA, USA) in 1/100. Secondary EnVision Flex/HRP (Horseradish peroxidase) (Dako, 20 minutes) was used for signal amplification. 3,3'-Diaminobenzidine (DAB, Dako) development was used for detecting primary antibodies. The slides were counterstained with Hematoxylin, dehydrated and mounted. Negative controls were performed by omitting the primary antibody.

Immunohistochemical reactions were read by two independent reviewers. The percent of positive tumor cells was established in order to determine immunohistochemical grading. In case of p53, Ki-67, Ets-1 and PTEN, we had nuclear expression, while in case of APAF-1 the expression was cytoplasmic. We interpreted the results according to the following grading system: G0: 0–10%, G1: 11–50%, G2: 51–100%. We considered a positive reaction whenever over 10% of cells were labeled by p53,

Ki-67 and Ets-1, while expression was considered to be negative (decreased or absent) if less than 50% of cells showed positive labeling for PTEN and APAF-1.

Results were analyzed using the Graph Pad In Stat 3, ver. 3.06 statistic calculation software (GraphPad Software Inc., San Diego, USA). We considered the association significant when  $p < 0.05$ , with 95% confidence interval.

## Results

In the normal colon epithelium, nuclear expression of Ki-67 and p53 was seen in less than 10% of the cells located in the lower third of the crypts, and Ets-1 expression was absent. Cytoplasmic expression of APAF-1 and nuclear expression of PTEN was distributed throughout the whole thickness of the normal epithelium (Table 1).

Table 1 – Immunoeexpression of Ki-67, p53, Ets-1

	Ki-67			P53			Ets-1		
	G0	G1	G2	G0	G1	G2	G0	G1	G2
<i>Histological type</i>									
HP	13	7	2	8	11	3	21	1	0
SSA/P	2	3	2	0	6	1	5	2	0
TSA	1	2	0	0	2	1	1	1	1
TA	0	24	2	0	10	16	5	20	1
TVA	3	37	1	0	31	10	11	23	7
	$P < 0.05$			$P < 0.05$			$P < 0.05$		
<i>Grade of dysplasia</i>									
WD	16	12	4	8	19	5	27	4	1
LGD	2	39	0	0	30	11	9	28	4
HGD	1	22	3	0	11	5	7	15	4
	$P < 0.05$			$P < 0.05$			$P < 0.05$		

HP: Hyperplastic polyps; SSA/P: Sessile serrated adenomas/polyps; TSA: Traditional serrated adenomas; TA: Tubular adenomas; TVA: Tubulovillous adenomas; WD: Without dysplasia; LGD: Low-grade dysplasia; HGD: High-grade dysplasia.

### Ki-67 immunoeexpression

In most of A/P, Ki-67 expression was increased (G1 in 73.7%, and G2 in 7%, respectively), and absent in a small percent of the cases only (G0 in 7%). Ki-67 expression correlated significantly with histological type and grade of dysplasia (Table 1).

In 81.8% of HP, Ki-67 expression was seen in the lower third of the bowel glands, and in the rest of the cases, it extended up to the lower half of these. Consequently, Ki-67 expression in the majority of HP was G0 (59%). In most of the SSA/P and TSA cases, Ki-67 expression was increased (71.4%, and 66.6%, respectively); distribution in the crypts was predominantly disorganized in SSA/P (71.4%) and TSA (100%), extending over the lower half segment (Figure 1a). In TA and TVA, Ki-67 expression was predominantly increased; it was more intense in the upper third of the crypts (Figure 1b).

The proportion of cases with increased Ki-67 expression increased with the grade of dysplasia (WD: 50%, LGD: 95.1%, HGD: 96.1%). Ki-67 expression does not show statistically significant correlations with localization, age and gender ( $p > 0.05$ ).

### P53 immunoeexpression

Similar to Ki-67, the expression of p53 was increased in the majority of A/P (G1 in 60.6%, and G2 in 31.3%,

respectively), with a statistically significant correlation with histological type and dysplasia (Table 1).

In 77.2% of HP, p53 expression was seen in the lower third of the crypts, and in the rest of the cases, it extended up to the lower half of these. In most of HP, p53 expression was increased (G1 in 50%, and G2 in 13.6%, respectively), and absent in about one third of the cases only (G0 in 36.6%). In all SSA/P and TSA cases, p53 expression was increased, extending beyond the lower half of the crypts, and showing a disorganized distribution in TSA. Also, in all TA and TVA cases, we noticed increased p53 expression, with a similar distribution to Ki-67 expression (Figure 1c).

The proportion of cases with increased p53 expression increases with the grade of dysplasia (WD: 75%, LGD: 100%, HGD: 100%). P53 expression does not show statistically significant correlations with localization, age and gender ( $p>0.05$ ).

### Ets-1 immunoexpression

The expression of Ets-1 was increased in the majority of A/P (G1 in 47.4%, and G2 in 9%, respectively), and it was absent in 43.4% of the cases; there was a statistically significant correlation with histological type and dysplasia (Table 1).

In most of HP (95.4%) and SSA/P (71.4%), Ets-1 expression was missing, while in most of TSA cases, it was increased (G1 in 33% and G2 in 33%) (Figure 1d). In case of TA and TVA cases with increased expression are also predominant (G1: 76.9%, G2: 3.8%, and G1: 56% and G2: 17%, respectively).

The proportion of cases with increased Ets-1 expression increases with the grade of dysplasia (WD: 15.6%, LGD: 78%, HGD: 73%). Ets-1 expression does not show statistically significant correlations with localization, age and gender ( $p>0.05$ ).

In 10% of the cases (one HP case, one TSA, three TA, five TVA), in addition to the nuclear expression we observed a weak cytoplasmic reaction as well. In most of these cases, Ets-1 expression was present in less than 10% of the cells, Ki-67 and p53 expression was moderately increased; APAF-1 expression was decreased, and PTEN expression was maintained.

### APAF-1 immunoexpression

Relative to the adjacent normal tissues, APAF-1 expression was decreased in 37.3% and absent in 62.6% of the investigated A/P, showing a statistically significant correlation with histological type and grade of dysplasia (Table 2).

In all HP cases, APAF-1 expression was absent or present in less than 10% of the tumor cells, and it was more enhanced in the upper third of the crypts. Of the seven SSA/P cases, APAF-1 expression was absent in five and decreased in two, with an unequal and inhomogeneous distribution. Of the three TSA cases, this marker was not expressed or present in less than 10% of the tumor cells in two cases, and it showed decreased expression with diffuse distribution in one case. In the majority of TA (69.2%) we observed decreased expression, while in most of TVA expression was absent or present in less than 10% of the tumor cells (60.9%) (Figure 1e). The distribution of APAF-1 expression in TA and TVA was diffuse.

**Table 2 – Immunoexpression of APAF-1, PTEN**

	APAF-1			PTEN		
	G0	G1	G2	G0	G1	G2
<i>Histological type</i>						
HP	22	0	0	2	6	14
SSA/P	5	2	0	2	1	4
TSA	2	1	0	0	0	3
TA	8	18	0	3	6	17
TVA	25	16	0	2	16	23
	$P<0.05$			$P>0.05$		
<i>Grade of dysplasia</i>						
WD	29	3	0	4	7	21
LGD	15	26	0	4	10	27
HGD	18	8	0	1	12	13
	$P<0.05$			$P>0.05$		

HP: Hyperplastic polyps; SSA/P: Sessile serrated adenomas/polyps; TSA: Traditional serrated adenomas; TA: Tubular adenomas; TVA: Tubulovillous adenomas; WD: Without dysplasia; LGD: Low-grade dysplasia; HGD: High-grade dysplasia.

The proportion of cases with missing APAF-1 expression increases with the grade of dysplasia (WD: 90.6%, LGD: 36.5%, HGD: 100%). APAF-1 expression does not show statistically significant correlations with localization, age and gender ( $p>0.05$ ).

### PTEN immunoexpression

In the majority of studied A/P, PTEN expression was kept (61.6%), decreased in 29.2% and absent in only 9% of the cases. Expression of this marker does not show statistically significant correlations with the studied clinico-pathological parameters (Table 2).

In most of the histological types, PTEN expression was maintained, and only the proportion of cases with decreased or absent expression was variable (HP: 27.2%, and 9%, respectively; SSA/P: 14.2%, and 28.5%, respectively; TA: 23%, and 11.5%, respectively; TVA: 39%, and 4.8%, respectively) (Figure 1f). In the majority of HP and SSA/P cases, the intensity of PTEN expression in the crypts demonstrated a decreasing trend in distribution in the deep to superficial direction; otherwise, the expression was diffuse. In the majority of TA and TVA with LGD, PTEN expression showed a decreasing trend in intensity in the superficial to deep direction, otherwise it was diffuse as well; cases with HGD and TSA cases showed predominantly diffuse expression.

Although PTEN expression does not show statistically significant correlations with the grade of dysplasia, the proportion of cases with decreased or missing expression increases in parallel with the previous (WD: 34.3%, LGD: 34.1%, HGD: 50%).

### Correlations

Analyzing the correlations between the studied markers, we observed statistically significant positive correlations between p53 and Ets-1 ( $CC=0.3$ ,  $p=0.001$ ), and p53 and Ki-67 ( $CC=0.5$ ,  $p<0.0001$ ).

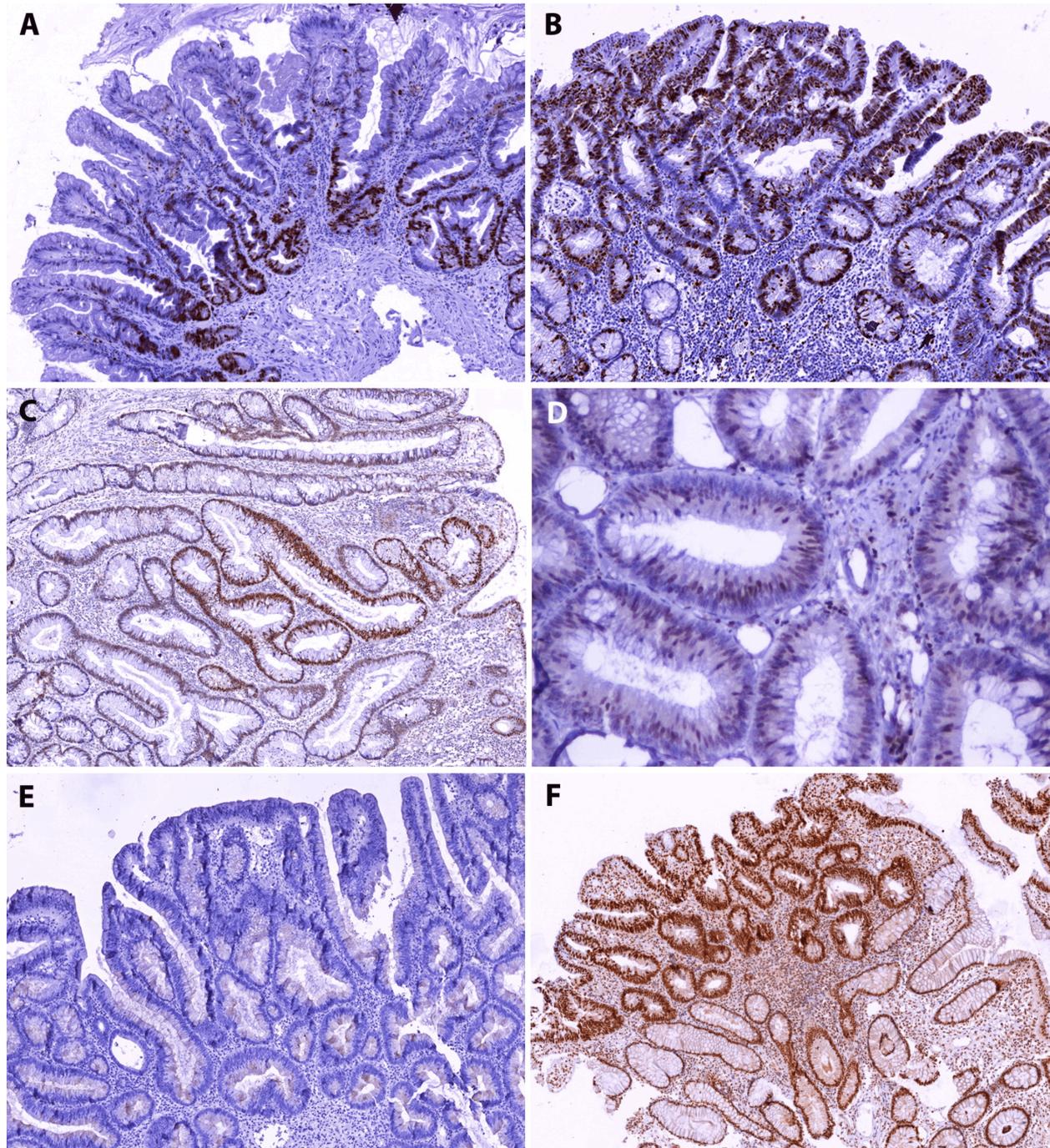
The most frequent immunophenotype in HP cases was Ets1-/Ki67+/P53+/APAF1-/PTEN+ (31.8%), along with Ets1-/Ki67-/P53+/APAF1-/PTEN+ (18.1%), and Ets1-/Ki67+/P53+/APAF1-/PTEN- (9%). In SSA/P cases, in addition to the already mentioned three immunophenotypes there is an Ets1+/Ki67+/P53+/APAF1-/PTEN+

(14.2%) immunophenotype, and the Ets1-/Ki67+/P53+/APAF1-/PTEN+ (28.5%) and Ets1/Ki67+/P53+/APAF1-/PTEN- (28.5%) are predominant. The majority of TSA, TA, TVA were Ets1+/Ki67+/P53+/APAF1-/PTEN+ (66%, 53.8%, and 39%, respectively). In TA and TVA cases, the Ets1-/Ki67-/P53+/APAF1-/PTEN+ immunophenotype is missing, although present in HP, SSA/P and TSA; in about 25% of the cases the immunophenotype Ets1+/Ki67+/p53+/APAF1-/PTEN- occurs (Figure 2a).

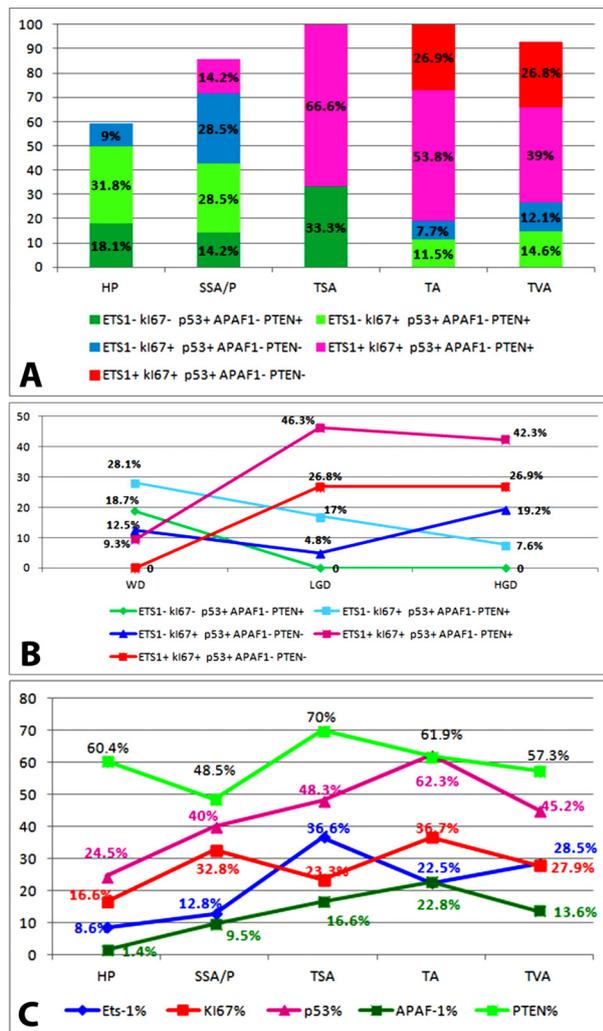
The ratio of Ets1-/Ki67+/P53+/APAF1-/PTEN+ and Ets1-/Ki67-/P53+/APAF1-/PTEN+ cases decreases, while

those of Ets1+/Ki67+/P53+/APAF1-/PTEN+ and Ets1+/Ki67+/p53+/APAF1-/PTEN- cases increases with the grade of dysplasia (Figure 2b).

In case of Ets-1, Ki-67, p53, APAF-1, the average of percent ratios of positive cells shows statistically significant differences according to histological type (Student's test,  $p < 0.0001$ ). This correlation could not be demonstrated in case of PTEN. For Ki-67, Ets-1, p53 this average was higher TSA, TA and TVA. The average of PTEN expression was decreased in SSA/P, and APAF-1 expression in HP and SSA/P (Figure 2c).



**Figure 1** – (A) Increased Ki-67 immunoexpression (G1) in sessile serrated adenoma/polyp,  $\times 100$ ; (B) Increased Ki-67 immunoexpression (G2) in tubulovillous adenoma with low-grade dysplasia,  $\times 100$ ; (C) Increased p53 immunoexpression (G2) in tubular adenoma with high-grade dysplasia,  $\times 100$ ; (D) Increased Ets-1 immunoexpression (G1) in traditional serrated adenoma,  $\times 200$ ; (E) Absent APAF-1 immunoexpression (G0) in tubulovillous adenoma with low-grade dysplasia,  $\times 100$ ; (F) Maintained PTEN immunoexpression (G2) in tubular adenoma with low-grade dysplasia,  $\times 100$ .



**Figure 2 – (A) The most frequent immunophenotypes relative to the histological type of A/P; (B) The most frequent immunophenotypes relative to the grade of dysplasia; (C) The mean percentage of positive cells relative to histological type. HP: Hyperplastic polyps; SSA/P: Sessile serrated adenomas/polyps; TSA: Traditional serrated adenomas; TA: Tubular adenomas; TVA: Tubulovillous adenomas; WD: Without dysplasia; LGD: Low-grade dysplasia; HGD: High-grade dysplasia.**

## Discussion

During the last decades, thanks to introduction of new screening methods for CRC, the histological subtypes of serrated adenomas (HP, TSA and SSA/P) were also identified [26]. Consequently, the histological classification of A/P has been renewed recently [27, 28], and CRC screening guidelines officially acknowledged in 2012 the involvement of TSA and SSA/P in CRC carcinogenesis, alongside conventional adenomas [26].

Ki-67 expression is increased in SSA/P relative to HP, aiding specialists in diagnosing these lesions, alongside the well-known microscopic features of SSA/P: crypt dilation, irregularly branching crypts, horizontally arranged basal crypts [27–29]. In our study, Ki-67 and p53 expression was also more frequent and asymmetric in SSA/P and TSA, compared to HP with a Ki-67 expression localized to the lower third of the crypts [27, 28, 30]. HP cases (18.8% in our study) with Ki-67 and p53 expression

extending towards the lower half of the crypts probably represent an intermediate stage between HP and SSA/P [28]. In the majority of conventional adenomas, Ki-67 and p53 expression was increased, with enhancements in the upper third of the crypts [31]. Similar to several already published studies, our result support the correlation between Ki-67 and p53 expression, and histological type and grade of dysplasia [32, 33].

During the embryonic stage, the Ets-1 transcription factor plays a role in the development of the digestive tract and of tissues derived from the mesoderm, by stimulating cell proliferation and differentiation. Expression of this marker gradually decreases during fetal life, and its reactivation in CRC correlates with tumor invasion and presence of lymph node metastases [22, 34]. Recent studies also reported increased quantities of Ets-1 mRNA in CRC [14]. The mechanism of Ets-1 involvement in cell apoptosis inhibition and development of resistance to chemotherapy is still unclear [15]. It has been demonstrated that the mutated p53 protein [35], and several carcinogenetic pathways [5, 15, 18, 20] stimulate activity and nuclear accumulation of this protein. As a result, Ets-1 expression has been reported in CRC and in breast cancer, both in nucleus, and in the cytoplasm [21, 22, 36]. In breast cancer, nuclear expression of Ets-1 was associated with a poor outcome and increased p53 expression, while cytoplasmic expression was associated with a favorable tumor phenotype [36].

In our study, Ets-1 expression was predominantly nuclear and increased in the majority of A/P cases, contrary to the results reported by Nakayama *et al.* (2001) [22]. In 10% of the cases, we observed cytoplasmic expression, together with a moderately increased Ki-67 and p53 expression, decreased APAF-1 expression, and maintained PTEN expression. In our previous publications, we could not demonstrate correlations between histological type of A/P, and grade of dysplasia and Ets-1 expression [37]. By studying a new batch of A/P cases, and using the recently introduced histological classification criteria, we observed that expression of this protein was absent in the majority of HP and SSA/P, and it was increased in most of the TSA, TA and TVA; it also showed statistically significant correlations with the grade of dysplasia. There are published studies that support the absence of Ets-1 expression in HP [38], and the correlation with the grade of dysplasia [22], but there is no data about the expression of this protein in serrated adenomas. Additionally, the mean percentage of positive cells was highest in TSA, further supporting the early involvement of this marker in serrated carcinogenesis.

The role of tumor suppressor protein p53 is to induce apoptosis through stimulation of the transcription of certain pro-apoptotic genes, like APAF-1 [12], a key regulator in the mitochondrial pathway of apoptosis. The loss of APAF-1 is followed by resistance against the apoptotic signals [39]. Decrease of APAF-1 expression in CRC correlates with left colon localization, tumor invasion, presence of lymph node metastases, advanced TNM and Dukes stage, and poor histological differentiation, and short survival [12, 39–41].

In the study of Paik *et al.* (2007), all A/P colon cases maintained APAF-1 expression, similar to the adjacent

normal epithelium [39]. We could not find other published data about the expression of this protein in A/P. In our study, APAF-1 expression was decreased or absent in all A/P cases, and correlated with histological type and grade of dysplasia. In HP cases, the distribution of APAF-1 expression was enhanced in the upper third of the crypts, in SSA/P cases it was irregular, while in TSA, TA and TVA it was diffuse. Using the same antibody and signal amplification system, Sträter *et al.* (2010) observed luminal enhancement of APAF-1 expression, suggesting that colon cells undergo apoptosis here. They mentioned that staining within the basal parts of the crypts occurs due to unspecific labeling of mucus. Probably the different immunohistochemical methods and interpretation systems described in the literature contributed to the non-conclusive results regarding the prognostic role of this marker in CRC [41].

Decreased PTEN expression was reported in about 20–40% of CRC, and PTEN gene mutations (10q23) in 1–29% of CRC; these were most frequent in CRC with microsatellite instability and BRAF mutations [23, 42]. In CRC, decreased PTEN expression correlates with tumor size, young age, female gender, left localization, tumor invasion, lymph node metastases, advanced Dukes stage, poor histological differentiation and short survival [23, 25, 42–44]. There are only a small number of studies about PTEN expression in conventional A/P, but not serrated ones. PTEN expression was kept in 40–85.7% of A/P cases, although the authors worked with small number (14–44) of cases [23, 42, 43, 45].

In case of our samples, nuclear expression of PTEN was maintained in 61.6% of A/P cases, and did not correlate with clinico-pathological parameters, which was similar to data published by other authors [43]. It should be noted that in all TSA cases, PTEN expression was maintained. In the majority of HP and SSA/P cases, the intensity of PTEN expression in the crypts had a decreasing trend in the deep to superficial direction, an inverse trend in conventional adenomas with LGD, while in those with HGD, and in TSA cases, we observed diffuse expression in most of the cases.

There are several immunohistochemical studies in CRC about PTEN that returned different results according to the antibody, methods and interpretation grading they used. Using anti-PTEN clone 6H2.1 (1/100), Naguib *et al.* (2011) described cytoplasmic and nuclear reaction in normal colon tissue, maintained cytoplasmic reaction and absent nuclear expression in A/P cases, and absent nuclear and cytoplasmic reaction in CRC [42]. Waniczek *et al.* (2013) [43] used anti-PTEN clone 138G6 (1/75), and Li *et al.* (2009) [44] used anti-PTEN 6H2.1 (1/100), and they reported predominantly cytoplasmic expression, and more rarely nuclear expression. Jang *et al.* (2010) [23] used anti-PTEN clone 28H6 (1/200) and reported only nuclear reactions; Colakoglu *et al.* (2008) [45] used anti-PTEN clone Ab-4, which resulted in cytoplasmic expression. In our study, we used anti-PTEN clone PTN-18 (1/400) and a Dako EnVision/Flex HRP System, and we obtained nuclear reactions. In a previous study, we used the same antibody (1/25) and the UltraVision Labeled Polymer system (LabVision, Fremont, CA, USA) for detection, and we obtained cytoplasmic reactions [46]. Considering

that there is no international consensus, Maiques *et al.* (2014) tried to optimize the reaction conditions using anti-PTEN clone 6H2.1 in different concentrations and different antibody diluents and detection systems; they reported that the quality and type of reactions show differences not only according to the above-mentioned parameters, but also depending on the studied tissue [47].

Comparative analysis of the immunohistochemical results demonstrated that in HP cases, Ets-1 expression is mostly absent, Ki-67 and p53 expression is already increased, APAF-1 expression is decreased, and PTEN expression is maintained. The majority of SSA/P cases are characterized by the same immunophenotypes as HP cases, but PTEN expression decreases because of serrated carcinogenic pathways that are already active in these lesions. In addition to increased Ki-67 and p53 expression, conventional adenomas are characterized by increased Ets-1 expression, decreased APAF-1 expression, and maintained PTEN expression. In 2/3 of the TSA cases, we found immunophenotypes seen in conventional adenomas, and in 1/3 of the cases immunophenotypes seen SSA/P cases.

## ☞ Conclusions

APAF-1 expression decreases in early stages of colorectal carcinogenesis, in addition to increases in Ki-67 and p53 expression. These changes in serrated carcinogenesis are followed by a decrease in PTEN expression, while in the conventional adenoma–carcinoma sequence this is maintained, but instead Ets-1 expression increases. Clarification of the involvement of these markers in colorectal carcinogenesis requires other molecular biology studies.

## Conflict of interests

The authors declare that they have no conflict of interests.

## Acknowledgments

The project has been funded by the Internal Research Grants of the University of Medicine and Pharmacy of Țirgu Mureș, Romania (Individual Contract No. 24/11.12.2013 and Collective Contract No. 20/23.12.2014).

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*Received: April 10, 2015*

*Accepted: December 30, 2015*

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