ORIGINAL PAPER

RJME
Romanian Journal of Morphology & Embryology
http://www.rjme.ro/

Histochemical and immunohistochemical evidence of tumor heterogeneity in colorectal cancer

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Abstract

Introduction: Intratumoral heterogeneity implies the existence of differences between tumor cells, which can best be shown by histochemical and immunohistochemical techniques. The histological study is a mandatory step in any research aimed at characterizing tumor heterogeneity. Immunohistochemistry (IHC) also plays an important role in the differentiation of tumor types, assessing aggressiveness. *Materials and Methods*: Investigated group consisted of 50 patients with colorectal adenocarcinoma, for each were recorded clinicopathological data and harvested samples intraoperatively, which were included in paraffin blocks. We perform Hematoxylin–Eosin staining for histological grade and other indices. IHC study used Avidin–Biotin–Peroxidase (ABC), with the markers: CK7, CK20, MUC1, MUC2, Ki-67, PCNA, p53, KRAS, BCL2, PTEN, EGFR. The resulting data were analyzed by statistical methods. *Results*: Most of colorectal adenocarcinoma studied had no special histological features and had G2 grade. IHC detected in most cases the CK20+/CK7- phenotype (78%) and MUC1 (74%) protein expression. The proliferation markers (Ki-67 and PCNA) were present in all tumor mass with a variable index, which shows high intratumoral heterogeneity, but p53 and KRAS were distributed more uniformly, showing low intratumoral heterogeneity. PTEN was expressed nuclearly in 86% of the cases and EGFR in 42%. *Conclusions*: The expression profiles of cytokeratins and mucins in the colorectal adenocarcinomas are useful in defining tumor phenotypes with different prognosis and therapy. We found a significant positive correlation between KRAS protein expression and BCL2 and TP53 expression. The study demonstrated the intratumoral and intertumoral heterogeneity, expressed at phenotypic level.

Keywords: immunohistochemistry, p53, KRAS, mucins, cytokeratins, heterogeneity.

Introduction

Intratumoral heterogeneity implies the existence of differences between tumor cells, which can best be shown by histochemical and immunohistochemical techniques, because they allow not only to detect qualitative and quantitative expression differences between different areas, but also their cellular localization and relationship with tumor cellular architecture.

Immunohistochemistry (IHC) plays an important role in the differentiation of tumor types, assessing aggressiveness and metastasis origin recognition. Although molecular analysis is increasingly gaining more ground, many therapeutic protocols are still based on histological types and immunohistochemical phenotypes [1]. For these reasons, histological study is a mandatory step in any research aimed at characterizing tumor heterogeneity.

In this study, special interest was given to the expression of proliferation factor Ki-67, tumor suppressor protein p53, KRAS and BCL2 oncoproteins in colorectal cancer (CRC).

The Ki-67 protein of 395 kDa is encoded by a single gene on chromosome 10, is located in the nucleolus and may be a component of pre-ribosome [2, 3]. Ki-67 expression was limited to growth phases G1, S, M, and G2 as is mainly in cells in S/G2 + M phase.

Ki-67 protein is a marker of cell cycle and cell proliferation used to estimate the coefficient of cell proliferation in a cellular population [4]. It is an indicator of growth fraction, the number of cells that are found in active division.

p53 protein is encoded by the gene TP53, which is involved in the development of many human cancers, in which TP53 gene mutations were found. Bad functioning of p53 is required for tumor progression [5]. It can be activated by genotoxic damage, activation of oncogenes, telomere erosion, loss of stromal support and deprivation of nutrients or oxygen, cases in which this command the cell to enter apoptosis, thus removing from the proliferating cell population [6].

The KRAS (Kirsten RAS) protein is encoded by a gene on chromosome 12q [7], which was shown recently to be involved in RAS/RAF signaling pathway and is designed to activate this pathway through its GTP-asic activity [8].

BCL2 protein is encoded by a 25-kDa oncogene that inhibits apoptosis named bcl2. BCL2 overexpression

results in a 14–18 translocation. BCL2 family members play important roles in tumor initiation and progression [9], but also in response to chemotherapy [10], their level of expression being a cancer prognostic factor. It has been developed antitumor chemotherapy that target BCL2 family members [11].

Aim

The primary objective of the study was to assess the expression profiles of proliferation factor Ki-67, tumor suppressor protein p53, KRAS and BCL2 oncoproteins in colorectal cancer. Secondary objectives were evaluating the intratumoral and interpatient heterogeneity and linking various expression patterns with histological type and degree of differentiation.

Materials and Methods

Study design

Investigated group consisted of 50 patients with colorectal adenocarcinoma: 27 (54%) men and 23 (46%) women, with a mean age of 59.7 years (41–79 years). These cases were recorded in a database to facilitate comparison of results. For each case, clinicopathological data were recorded: age, sex, tumor location, histological type and tumor grade. All samples investigated were taken intraoperatively, fixed in formalin and included in paraffin.

The immunohistochemical and histological study was performed in the Laboratory of Pathology from the "Victor Babeş" National Institute for Research and Development in Pathology and Biomedical Sciences, Bucharest, Romania.

Histological study

Paraffin blocks were sectioned with microtome at 3–5 μm, the microscope slides were stained with Hematoxylin–Eosin (HE) and examined with a Zeiss Axiostar plus microscope with a specialized Olympus camera, with a resolution of 5 megapixels.

Histological grade was assessed on the following histological standard criteria: the proportion of the glands within the tumor, compared with solid areas, or nests, or cords of tumor cells without lumen. Well-formed glands were present in over 75% of well-differentiated tumors in 25–75% of the moderately differentiated ones and in 25% of those poorly differentiated. In well-differentiated adenocarcinoma, tumoral glands are tapestried with cells that retain nuclear polarity. In the case of poorly differentiated adenocarcinomas, the tumor was predominantly composed of solid areas with tumor cells that have lost their initial nuclear polarity and also showed marked nuclear pleomorphism. In cases where the same tumor areas were identified with varying degrees of differentiation, grading was done according to the lowest level, even though the respective area was more limited comparing with the slide area.

For immunohistochemical study of colorectal mucosa, we used 4-μm sections obtained from paraffin blocks included, which were spread on glass slides pretreated with poly-L-Lysine then, for a better grip of sections on slides; they were left overnight in the thermostat at 37^0C.

Slides were processed using the three-stage method Avidin–Biotin–Peroxidase (ABC). We used as IHC markers: cytokeratins (CK7, CK20), mucins (MUC1, MUC2), Ki-67, PCNA (proliferating cell nuclear antigen), p53, KRAS (Kirsten-RAS), BCL2, PTEN (phosphatase and tensin homologous), EGFR (epidermal growth factor receptor).

For paraffin included, formalin-fixed tissues the most widely used monoclonal antibody for Ki-67 is MIB-1, which recognizes nuclear antigen; its reactivity is not influenced by the time of fixation.

Analysis of TP53 gene status can be made by direct observation of p53 protein in tissues by immunohistochemical techniques. IHC staining was performed with specific monoclonal antibodies anti-p53 (CM1, Novocastra, Newcastle, UK). We evaluated immune reactivity to p53 depending on the percentage of positive tumor cells. It were taken into account only the tumor cells with nuclear stain, cytoplasmic stain was not considered.

We aimed to investigate the immunohistochemical expression of KRAS in adenocarcinomas of the colon and to compare it with other studies based on similar methods. We used a monoclonal antibody with specificity for proteins KRAS, NRAS and HRAS.

For quantification of tumor or marked cells index, we used the manually count method on the computer images, with the aid of specialized software. In some cases, the assessment was made with the optical microscope using a magnification of 400× and an ocular micrometer consisting of a 10×10 grid. Immunohistochemical staining of slides was assessed regarding the pattern of intracellular distribution. We identified a pattern restricted to the nucleus and a predominantly cytoplasmic pattern.

Starting from the heterogeneity based on the distribution and intensity of staining, in order to avoid bias and difficulties in assessing the results, in most cases we used a semiquantitatively score (corresponding to staining intensity and percentage of reactive nuclei), which allowed classification into three groups of immune reactivity: low, medium and high. These values were adjusted by index values distribution, so that the set of index values were divided into three equal parts by the two threshold values.

Statistical analysis

Before applying the methods of statistical analysis of differences between groups, it was examined the normality of the distribution by Kolmogorov–Smirnov and Shapiro–Wilk tests, as well as the equality of variances by the Levene's test.

Differences in frequency between groups were analyzed using *chi*-square, or in case of small values of expected frequencies (as in most cases), Fisher's exact test.

For quantitative variables (*e.g.*, IHC index markers), it was used the univariate ANOVA analysis and Student's tests to evaluate the significance of the differences between groups. Pearson's correlation coefficient was used to establish the link between quantitative variables (IHC index) and nominal or ordinal ones that can be numerically encoded.

Statistical calculations were performed using SPSS 17.0 software package (SPSS Inc., Chicago, IL, USA).

Results

Overall, the statistical analysis of clinical and pathological parameters of the patients showed an increased incidence of disease in patients over 55 years in urban areas (Table 1).

Table 1 – *Distribution of patients with colorectal cancer according to clinical-pathological parameters*

Variable	Value	Percentage
Age		
≤55 years	12	24%
>55 years	38	76%
Gender		
Females	23	46%
Males	27	54%
Residence		
Urban	35	70%
Countryside	15	30%
Tumor location		
Proximal	31	62%
Distal	19	38%

Hematoxylin–Eosin staining has allowed assessment of microscopic grading of adenocarcinomas studied: well, moderately and poorly differentiated (Figure 1), the predominant grade (58%) was G2 (moderately differentiated followed by poorly differentiated (18%). Histologically, most adenocarcinomas (41 cases) had no special features, nine were mucinous, eight were colloids and one with signet ring cells.

We tried to make a correlation between histological grading and tumor location. We found that from the nine poorly differentiated adenocarcinomas, six were located in the distal colon. Considering the 31 cases studied with this location, the association is not statistically significant (p=0.552), as shown by other studies [12].

IHC investigation of cytokeratins expression has detected CK20 in most cases (80%) with focal pattern in tumor cells and of CK7 in eight cases (Figure 2A, Table 2) with diffuse pattern. In 78% of the tumors, it was present the CK20+/CK7- phenotype. CK20-/CK7+ phenotype was identified in five (10%) cases located in the proximal colon. Also in our study we found three (15.78%) cases with the expression of both cytokeratins (CK20+/CK7+), all located distally.

Table 2 – *Immunohistochemical expression of CK7, CK20 keratins and correlation with MUC1 and MUC2 mucins expression in colorectal adenocarcinomas*

	No. of cases	CK7	CK20	MUC1	MUC2
Histological type	50				
Tubular-papillomatous	40 (80%)	++	+	++	-/+
Tubulous-villous	1 (2%)	++	+	-/+	-/+
Signet ring	1 (2%)	+	+	++	+
Mucinous	8 (16%)	+	+	-/+	++

-/+: Marking absent or present in more than 30% of tumor cells; +: Marker present in 30–60% of tumor cells; ++: Marker present in over 60% of tumor cells.

Expression of membrane (MUC1) and secretory (MUC2) mucins had the MUC2+/MUC1- pattern of expression in normal colorectal mucosa, this phenotype was found in 18% cases, which shows mucin expression alterations. We also identified expression patterns as MUC1+/MUC2- in 38% cases, MUC1+/MUC2+ in 36% cases and MUC1-/MUC2- in 8% of cases (Figure 2B).

Ki-67 expression was detected in all studied cases (Figure 3); the average index for Ki-67 positivity was ranged between 32.8% and 59.42%, while in the literature the index is ranging from 10 to 95%. Also, in all cases we identified the presence of intratumoral heterogeneity, with varying percentages of positivity areas within the same tissue sections. It was a large number of cells positive for Ki-67 in the glandular structures of the carcinoma.

We established a statistical significant relationship between the percentage of positivity for Ki-67 and the histological grade of differentiation (ANOVA analysis for the difference between the G1, G2 and G3 grades, p=0.014<0.05).

PCNA expression was also present in all cases investigated and we have shown varying degrees of intratumoral heterogeneity both in the tumor as a whole but also in malignant glands. Average PCNA index ranged from 15–30% in well-differentiated adeno-carcinomas, 25–60% in moderately differentiated and 50–90% in poorly differentiated ones. It was observed a correlation at the limit of statistical significance between the degree of differentiation and PCNA index (ANOVA analysis for the three degrees of differentiation, p=0.085>0.05).

TP53 expression was observed in 43 (86%) cases, relatively homogeneous in tumor mass (Figure 3), as sections at different levels presented similar indexes (small intratumoral heterogeneity). It is important to mention that TP53 overexpression was not found in normal tissue adjacent to tumor.

The absence of positivity for p53 was associated with poorly differentiated adenocarcinoma form (p<0.001), as other studies reported [13]. We also found that TP53 overexpression was associated with increased mean PCNA index and Ki-67 (Student's test, p=0.0042, p=0.0025 respectively), suggesting accumulation of p53 protein in cells that already had an advantage increased by mutations that lead to increased proliferation rate. Making a correlation with the results of molecular analysis, it was found that marking for p53 was positive in all 12 cases in which TP53 gene polymorphisms were investigated.

Molecular analysis showed that 83% (five of the six) samples with more than 50% positivity index, showed allele Pro/Pro (present in 50% of the cases studied, Fisher p=0.017).

KRAS protein had a positivity index between 5% and 95%. Immunoreactivity was considered positive when over 30% of tumor cells showed positivity. Thus, the KRAS protein expression was identified in 26 (52%) of cases studied (Figure 4A), most indexes (17 cases) hovering 50% of the cases. KRAS expression was significantly increased in poorly differentiated adenocarcinoma (six of the nine cases, p=0.037).

Bcl-2 expression was identified in tumor cells in a small number of cases (12%) and varied in intensity. The expression pattern was predominantly cytoplasmic, higher in apical region, although it was occasionally perinuclear, following the pattern of subcellular distribution of BCL2 protein (Figure 4B).

The nuclear expression of the phosphatase and tensin homologue (PTEN) protein was investigated it in 12 cases. The eight (66.67%) cases with positive immunoreactivity were well-differentiated adenocarcinomas and the four negative cases were represented by one well-differentiated adenocarcinoma and three moderately differentiated. Normal epithelium adjacent to tumor areas showed nuclear PTEN expression (Figure 5A).

Epidermal growth factor receptor protein (EGFR) was identified in 21 (42%) of the 50 colorectal adenocarcinomas, with an expression pattern that included blood vessels (Figure 5B).

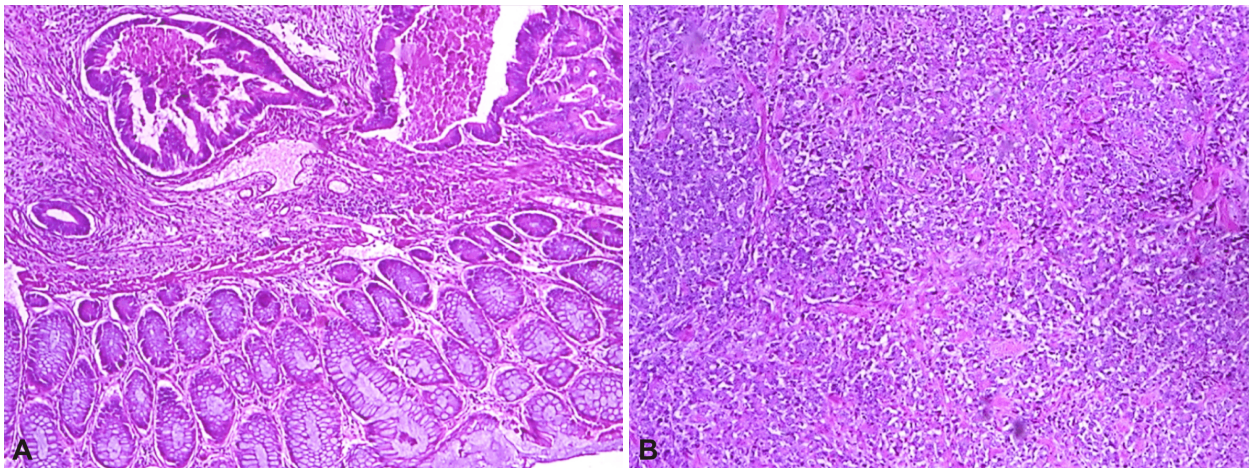

Figure 1 – ***(A) Well-differentiated adenocarcinoma with infiltration in the submucosa, adjacent to normal mucosa (HE staining, ×100). (B) Poorly differentiated colon adenocarcinoma – solid area of tumor without the presence of glandular differentiation (HE staining, ×100).***

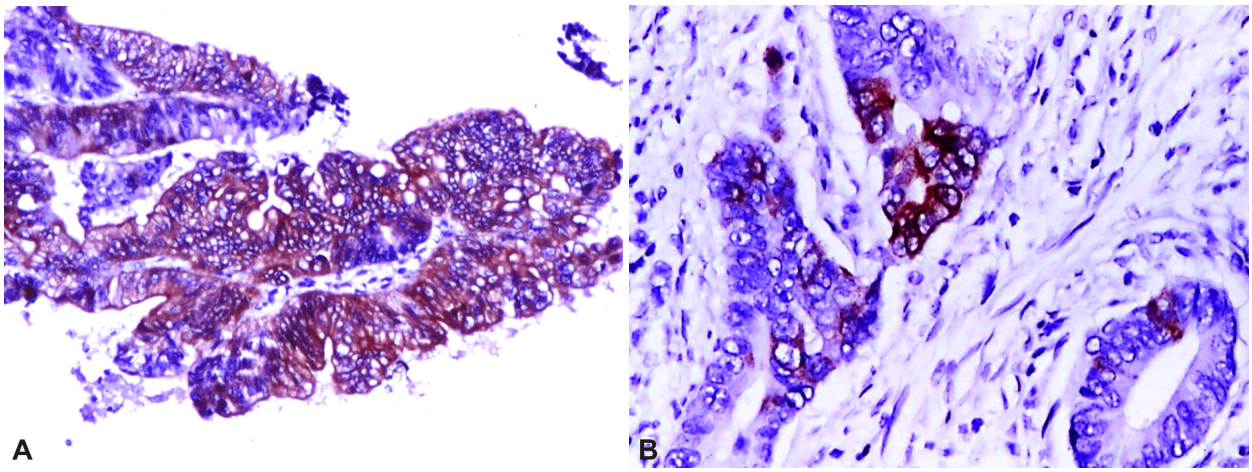

Figure 2 – ***(A) The expression level of cytokeratin 20 in a well-differentiated colorectal adenocarcinoma (IHC staining, ×100). Focal positivity in tumor cells and diffuse markings in normal epithelial cells. (B) MUC2 mucin expression in a moderately differentiated adenocarcinoma case (IHC staining, ×200). Cytoplasmic and membranar positivity.***

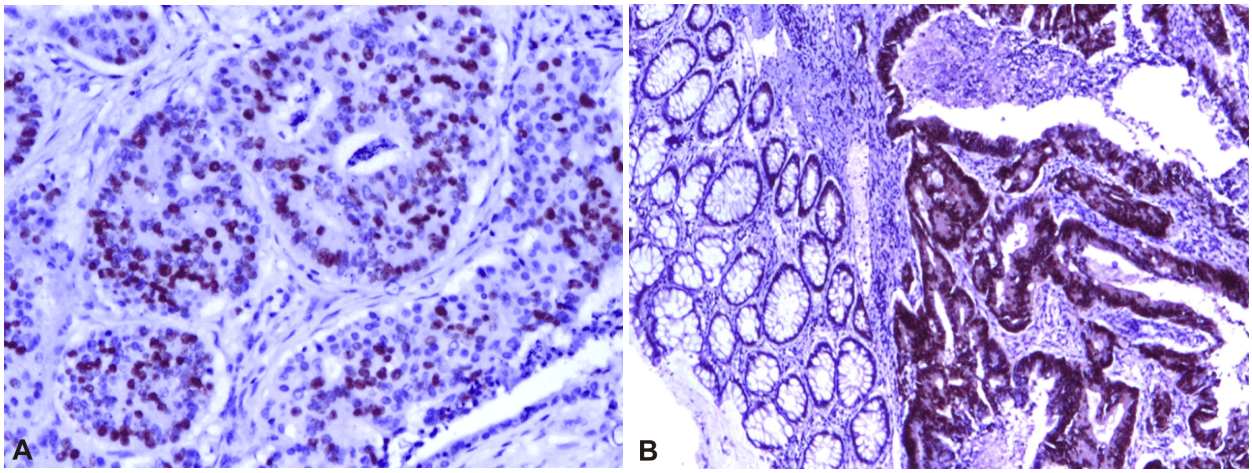

Figure 3 – ***(A and B) Ki-67 expression in a case of moderately differentiated colorectal adenocarcinoma (IHC staining, ×100). Ki-67 positivity index: 70–80% of tumor cells. Adenocarcinomas of the colon well-differentiated with labeling of p53 protein (IHC staining, ×100)***

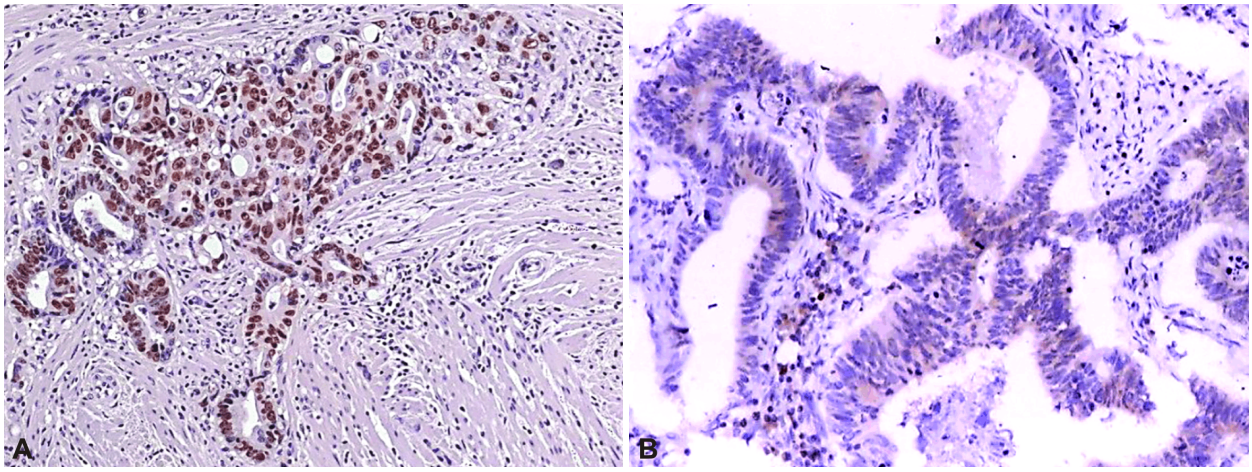

Figure 4 – ***(A) Well-differentiated adenocarcinoma of the colon with labeling for KRAS protein (IHC staining, ×100). KRAS positivity index: 80% of tumor cells. Adjacent normal tissue observed has no labeling at all. (B) Adenocarcinoma of the colon with labeling of BCL2 protein (IHC staining, ×100). Positive cytoplasmic marker. There is KRAS positivity in lymphocytes.***

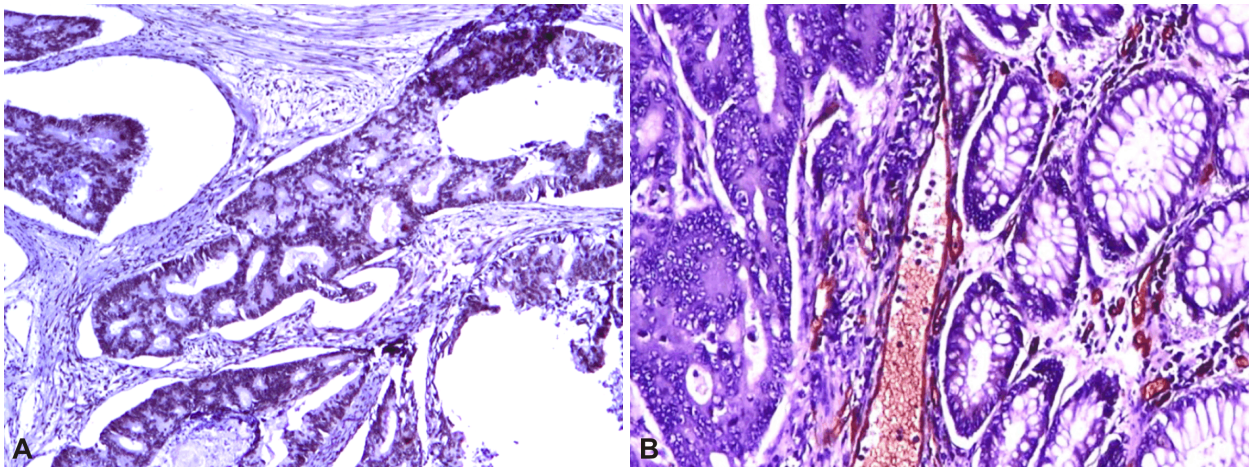

Figure 5 – ***(A) PTEN expression in moderately differentiated adenocarcinoma – nuclear labeling both in tumor cells and stromal and inflammatory cells (used as positive internal control) (IHC staining, ×100). (B) EGFR expression in colorectal adenocarcinoma (IHC staining, ×100). Negative labeling of tumor cells, positivity in blood vessels.***

Discussion

The colorectal cancer has usually a higher prevalence in older persons, which was confirmed by our study, and also in the urban areas, that was related to dietary differences [14, 15].

From our study, it was showed that 58% of colorectal adenocarcinomas are moderately differentiated and 18% are poorly differentiated. Other studies show that moderately differentiated is the most common form, with percentages up to 75% [16].

The cytokeratins expression pattern can be used to identify the origin of a metastasis. Our study confirmed the findings that CK20+/CK7- is the most frequent pattern and that CK20-/CK7+ is not found in the distal colon. Some studies of rectal adenocarcinoma report the presence of CK20+/CK7+ phenotype in up to 22% of cases [17, 18].

The mucins are frequently expressed in human cancers, and our study confirmed a high percentage of expression of MUC1 protein in colorectal carcinomas. Moreover, the expression of MUC1 and MUC2 proteins was demonstrated to be an indicator of the malignancy potential in benign tumors [19, 20].

The Ki-67 positivity index in our study had a narrower range (32.8–59.42%) compared with the literature (10–95%), that can be explained by the relatively small number of cases. The Ki-67 positivity was linked with the histological grade of differentiation, link that was confirmed by other authors [21].

The TP53 expression was observed in a relatively high percentage (86%) compared with other studies that give a percentage between 5% and 80% [22]. In interpreting these results, we must consider that immunohistochemically detected TP53 overexpression means an accumulation of protein in cells [23]. However, not all TP53 gene mutations lead to its accumulation, so it is possible to exist false negative results [24]. We believe that the high percentage of Pro/Pro allele detected by the molecular analysis is rather due to increased frequency of allele Pro/Pro involvement in colorectal cancer, rather than relationship between this allele and immunohistochemical expression of p53 protein [24, 25].

KRAS protein is disrupted in most human cancer, and the colorectal cancer is no exception. Our study showed KRAS positivity in 52% of the cases, percentage very close to those from literature [26], possibly due to faster replication and raised opportunity to accumulate oncogenic mutations.

The increased index for KRAS and p53 proteins in these patients suggests that mutations of these two genes are present in most tumor cells (low intratumoral heterogeneity) [27], so it is possible that a single cell clone is responsible for tumor development. In order to confirm this hypothesis, would be required more studies with double IFC stain, both for KRAS and p53, as well as analysis of double marked cells index.

Usually, BCL2 expression in colorectal cancer is considered to be a favorable prognostic factor [28].

BCL2-labeled tumor cells were located predominantly in the malignant glands from the base and superficial regions of the tumor. Although the tumor was heterogeneous labeled, the lymphocytes were constantly marked in all sections (they served as internal positive control). These results are consistent with other expression studies [29]. The fact that the BCL2 expression was not correlated with relevant clinicopathological parameters suggests that this oncogenic protein may play a role in the early stages of adenoma–carcinoma sequence, but probably its expression in established carcinomas has little significance.

PTEN protein negatively regulates the phosphoinositide-3-kinase (PI3K) signaling pathway. In colorectal cancer, the loss of nuclear expression of the PTEN protein varies markedly, as well as its association with outcome. It was shown that PTEN suppression is induced by RAS/ERK pathway [30], thus explaining why of the four cases PTEN negative, three were positive for KRAS.

EGFR is overexpressed in many types of cancer but especially in colorectal cancer. Its expression was linked with more aggressive tumors. The 42% positivity rate in our study is between the limits in the literature [31]. It is worth to mention that in our study EGFR-labeling was present in blood vessels, highlighting the microangiogenesis process in the tumor invasion front and tumor glands [31, 32].

The tumoral heterogeneity in colorectal cancer has many implications that are not discussed here. There are a lot more histological markers and even methods for immunohistochemistry [33], and there are a lot more aspects than contribute to the cancer evolution than those detected here, and that contribute to prognosis [28]. For example, the immune system, by production of cytokines [34], the tumoral angiogenesis [35] or genetic polymorphisms [36, 37].

Conclusions

The expression profiles of cytokeratins and mucins in the colorectal adenocarcinomas studied are modified compared to those observed in normal mucosa adjacent to tumor, demonstrating their usefulness in defining tumor phenotypes with different prognosis and therapy. The cell proliferation markers, Ki-67 and PCNA, were positive in all cases investigated with an index between 10% and 95%. Intratumoral heterogeneity was marked in all cases, as there are large differences within the same section index. Tumor suppressor p53 protein expression was identified in 86% of cases studied and it was limited to tumor cells. Oncogenes were relatively homogeneous expressed in tumor mass, denoting low intratumoral heterogeneity. By correlating the histological grade with immunohistochemical findings of proteins expression in tumors, it was demonstrated the intratumoral and intertumoral heterogeneity, expressed at phenotypic level.

Conflict of interests

The authors declare that they have no conflict of interests.

Author contribution

All the authors had an equal contribution to the work presented in this paper.

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Received: September 6, 2014

Accepted: February 20, 2015