Studying Adenomatous Polyposis Coli (APC) gene at colorectal cancer patients. The role of E2F transcription factor 1 (E2F1), tumor suppressor P14 (ARF) and marker of proliferation Ki-67


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A- Conception and study design; B - Collection of data; C - Data analysis; D - Writing the paper; E - Review article; F - Approval of the final version of the article; G - Other (please specify)

ABSTRACT

Purpose: The purpose of this study is to determine the relationship between APC gene mutations and subcellular differentiation levels of E2F1, P14ARF and Ki67 proteins. Furthermore, if such connections can be used in the area of preventive health care.

Materials and methods: The 30 hours Immunohistochemistry protocol used, had samples preparation, antigen retrieval, background blocking, target detection and sample visualization. Samples were viewed and captured by light microscopy.

Results: The conducted research concern 88 patients with a range age of 56 years. 57.7% had no tobacco addiction, 3.84% were obese and 19.23% had the tendency to consume alcohol. About 31% had at least one family member with a history of cancer. Intensity and positivity of the genes vary as seen in tables.

Conclusions: By targeting specific and simultaneously multiple pathways based on molecular signatures, enables cases to be detected at an earlier stage, when there are greater chances of cure as treatment is more effective. A plan for early detection of cancer is a key component within an overall cancer control plan. An early diagnosis program is far more cheap and easy leading to appropriate treatments which finally reduce death rates and suffering due to cancer.

Keywords: APC, colorectal cancer, E2F1, P14ARF, Ki67

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INTRODUCTION

Cancer is one of the most serious health problems in developed countries today. It’s the second leading cause of death after heart diseases. Colon cancer is the third most commonly diagnosed cancer worldwide, accounting for 10% of the estimated 18.1 million new cancer cases recorded in 2020. In addition, it is the second leading cause of cancer-related death in women and the third leading cause of death in men, with 1,065,960 deaths worldwide in 2020. In human-based studies, the proportions of patients with colorectal cancer in stage II and III are about 40% and 30% respectively, with survival rates in these stages being 50-80% and 30-60% separately [1].

The APC gene encodes a large-sized protein, with many different functional regions, which plays an important role in Wnt signalling pathway, in cell attachment and communication, as well as in the functional integrity of the cytoskeleton. Inherited mutations in the gene are the causative agent of Familial Adenomatous Polyposis Syndrome (FAP), while physical mutations in the gene are found in 60-80% of sporadic cases in colon cancer. Mutations lead to, in most cases, the production of mutated and usually non-functional protein [2].

The protein encoded by E2F1 gene belongs to the E2F family of transcription factors. They play an important role in the control of the cell cycle, as well as in the function of cancer proteins. It is also a target of tumor-forming proteins that transform small DNA molecules. This protein is preferably bound to the pRB retinoblastoma protein in a special way. It can mediate both cell proliferation and dependent or non-dependent apoptosis from p53 [3].

The INK4a / ARF (9p21) gene encodes two unique proteins, p16INK4a and p14ARF, which both play important roles in the cell cycle. These proteins are welded in the same position on the exon 2. An important cyclin-dependent substrate is the Rb protein of retinoblastoma, which is phosphorylated at the end of the G1 phase, allowing it to exit this phase. Rb protein reduces cell proliferation by inhibiting the action of E2F1 transcription factors, which activate the transcription of genes required for DNA replication. When Rb is phosphorylated by cyclin D and E-dependent kinase during the G1 phase of the cell cycle, Rb cannot block the E2F1-dependent transcription and the cell can proceed with DNA synthesis in the S. phase [4].

The Ki67 non-histone cell proliferating antigen is a protein that is encoded in humans by the MKI67 gene. Among other things, it is associated with the transcription of ribosomal RNA. It is a reliable cell proliferation index, as its role in cell division is located in the cessation of cell proliferation. Another function of the protein is to stabilize the mitotic spindle by ingesting the Hklp2 factor during mitosis. The expression of the Ki67 antibody has also been associated with various types of malignancies proving the presence of mitotic activity. It is expressed in all phases of the cell cycle except the G0 phase [5].

While the APC and E2F1 genes have been studied in isolation in recent years, at the same time as the P14ARF and Ki67 genes, no sufficient research has been held. This gap in knowledge and practice is being filled in by the present research, methodically attempting through laboratory procedures and observation with optical microscopes, to prove any relationship between these genes and weather this relation can be used in human cancer prevention through early tests.

A resent research in the genetic identity of Greek Familial Adenomatous Polyposis, performed a cohort of twenty five FAP families revealing eighteen mutations of APC in twenty families (80%) [6].

MATERIALS AND METHODS

The protocol and procedures followed, when applying human antibodies, where according to the protocols of National Institute of Environmental Health Sciences and Thermo Fisher Scientific.

Immunohistochemistry was used for the search of abnormal cells, such as those found in cancerous tumors. The determination of the optimal dilution was performed by testing the antibody, in various dilutions on known tissues. The slides were placed on a thermal plate which has been heated to 60°C, so that paraffin melts and antigen retrieval takes place. The second step was tissue hydration and first tissue revelation. Paraffin was rinsed with xylene. Then they were rinsed and rehydrated with decreasing concentrations of ethanol and TBS solution was prepared. Tris-buffered saline (TBS), is a common pH-stabilizing biological buffer due to its low toxicity. During washing with TBS, the slides were constantly immersed in liquid, so that the sections remained hydrated. The duration and number of washes, performed each time, were inversely proportional to the antibody.

Next step was the discovery of antigenic sites and detachment of permanent bonds. The detection of incisions was performed by using a microscope and simultaneously using citrate solution. During the procedure pH was always measured, citrate was gradually added and plastic basin with H2O2 was also put together so that the tissue does not dry out due to evaporation. The citrate was rinsed with TBS. Then the inactivation of endogenous peroxidases was occurred. The slides were incubated in hydrogen peroxide (3% H2O2) for 12-15 minutes. Everything was performed in the dark because the H2O2 is photosensitive. Then, the sections were washed with TBS. Primary antibodies were applied in an indicative dilution. The antibodies
were stored at °C according to the manufacturer. Then followed the non-special signal blocking. A dark box was used and each tile was separately soaked into H2O. After that was placed on the incision 50-100 λ Ultra-V Block and the tile was incubated for 7 minutes. Slides were then immersed in a TBS bucket. Finally, the diluted antibody was added. Slides were placed overnight for 16 hours at 4°C.

Next day, slides were rinsed with TBS 1x and incubated with secondary antibody for 10 minutes in room temperature. HRP Polymer incubation was held for 15 minutes. This stage was done in the dark. Diluted diaminobenzidine (DAB) solution was prepared for color development. The volume was mixed in an Eppendorf tube, with a ratio of 1 pure DAB:100 DAB substrate and microscope observation was performed. This stage required darkness. DAB was inactivated with distilled water, the sections were counterstained with haematoxylin and dehydrated with increased concentrations of ethanol and xylene. Finally, closing with DPX glue with a drop on the slide and the cover was placed.

RESULTS

A total of 88 cases of neoplasia were included. There were 24 cases of colorectal cancer, 16 orthosigmoid adenocarcinomas, 9 rectal adenocarcinomas, 10 blind adenocarcinomas, and 29 neoplasm. Patient histopathological features are shown in Table 1 and surgical methods are presented in Table 2.

Table 1. Histopathological features of patients

<table>
<thead>
<tr>
<th>Histology</th>
<th>N</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenocarcinoma</td>
<td>59</td>
<td>58.66</td>
</tr>
<tr>
<td>Neoplasm</td>
<td>29</td>
<td>41.34</td>
</tr>
</tbody>
</table>

Table 2. Type of surgery

<table>
<thead>
<tr>
<th>Type of surgery</th>
<th>%</th>
</tr>
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<tbody>
<tr>
<td>Right hemicolecotomy</td>
<td>28</td>
</tr>
<tr>
<td>Low anterior resection</td>
<td>16</td>
</tr>
<tr>
<td>Low anterior rectal resection</td>
<td>16</td>
</tr>
<tr>
<td>Sigmoidectomy</td>
<td>16</td>
</tr>
<tr>
<td>Right colectomy</td>
<td>8</td>
</tr>
<tr>
<td>Left hemilectomy</td>
<td>4</td>
</tr>
<tr>
<td>Mercury collar resection</td>
<td>4</td>
</tr>
<tr>
<td>Abdominal rectal resection</td>
<td>4</td>
</tr>
<tr>
<td>Sphenoid resection of liver volume</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>N = 88</td>
</tr>
</tbody>
</table>

The mean age of the participants was estimated to be 67.27 ± 13.2 (mean ± standard deviation) with a range of 56 (34-90) years. 50% of the participants were women. The majority of patients (57.7%) had no tobacco addiction. 3.84% were obese and 19.23% had a tendency to consume alcohol. About 30.77% had at least one family member with cancer history. The symptoms and clinical features of the patients who participated in the study are not included. 26.92% of patients had a history of high blood pressure and 38.46% of patients had a history of appendicectomy surgery, although these rates were not considered significant in drawing conclusions. Finally, 45.15% of patients were treated before surgery.

The minority (13.33%) of the participants had the pathology in the blind, while the majority was closely related to the pathology of the rectum (36.66%). All cases were classified as neoplasms or adenocarcinomas. The size of malignant neoplasms ranges from 4 to 33 cm with an average of 5.50 cm. The risk of developing colon cancer appears to be significantly different in the blood group of patients (Table 3). The largest percentage of patients (60%) were found to have type O blood type. Compared with blood type O, a moderate correlation of blood type B with colorectal cancer was observed. However, there does not appear to be a statistically significant risk of cancer associated with blood groups A and AB. In addition, it is interesting to note that 96% of patients had Rhesus positive, which is consistent with the literature on various types of cancer, including colorectal cancer.

Immunohistochemistry analysis of different types of tumors, included in the study, was performed to identify and determine the expression percentage of the APC gene (Figure 1). The positive expression and intensity of the immunohistochemical expression of the APC gene observed in the cytoplasm of 88 cases of neoplasia studied are described in Tables 4 and 5.
Table 3. Patient blood type

<table>
<thead>
<tr>
<th></th>
<th>O</th>
<th>A</th>
<th>B</th>
<th>AB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients (%)</td>
<td>62 (60 %)</td>
<td>8 (12 %)</td>
<td>15 (24 %)</td>
<td>3 (4 %)</td>
</tr>
</tbody>
</table>

Figure 1. Photograph of the positive immunoassay of the anti-APC antibodies represented by the brown color in the cytoplasm of the cells in colon adenomas (immunohistochemistry (A) 200x and (B) 400x)

Table 4. Intensity of immunohistochemical expression of the APC gene in colon adenomas

<table>
<thead>
<tr>
<th>Intensity</th>
<th>n (%)</th>
</tr>
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<tbody>
<tr>
<td>Low</td>
<td>70 (79,55%)</td>
</tr>
<tr>
<td>Moderate</td>
<td>14 (15,91%)</td>
</tr>
<tr>
<td>Strong</td>
<td>4 (4,54%)</td>
</tr>
</tbody>
</table>

n, number of cases

Table 5. Positive immunohistochemical expression of APC protein

<table>
<thead>
<tr>
<th>Positivity</th>
<th>n (%)</th>
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<tbody>
<tr>
<td>&lt; 5%</td>
<td>70 (79,55%)</td>
</tr>
<tr>
<td>5 - 33%</td>
<td>14 (15,91%)</td>
</tr>
<tr>
<td>34 - 75%</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>&gt; 75%</td>
<td>4 (4,54%)</td>
</tr>
</tbody>
</table>

n, number of cases
The expression of the E2F1 gene (Figure 2) was observed in the nucleus of the cancer cells studied. High expression of the E2F1 gene was found in 4.54% of patients (4 out of 88 patients), moderate expression in 6.82% of patients (6 patients), and poor expression in 18.18% of patients (16 patients). 70.46% of patients had zero expression. When the same cases were evaluated depending on whether they had undergone color change, 30 cases (34.1%) showed color change and 58 (65.9%) did not undergo any change.

Figure 2. Immunohistochemical staining for the E2F1 gene. The illustrations (A) 200x, (B) 200x and (C) 200x, represent the examples of positive immunohistochemical staining (IHC)
Of the total patients, 33 cases (37.5%) had a positive expression of the p14ARF gene (Figure 3), while 55 cases (62.5%) showed a negative expression of the gene. The results were analyzed based on the positivity of the immunohistochemical expression and not on the intensity of the expression, as it was low in most positive samples. In addition, no correlation could be observed between the patients’ clinical and pathological characteristics and the positivity of the immunohistochemical expression, as the number of samples was small and insufficient for statistical analysis. Of interest is the fact that 29 cases of positive expression (32.95%) also had a positive expression of the APC gene. In addition, 22 cases of positive expression (25%) of the p14ARF gene showed concomitant positive expression with the E2F1 and APC genes.

A total of 33 cases of patients (37.5%) were found with a positive expression of the Ki-67 gene (Figure 4), and 55 cases of patients (62.5%) with a negative expression of the gene. There were no statistical correlations between patients’ different clinical and pathological characteristics, such as age, sex, tumor type, or tumor location, and the expression of the Ki-67 gene.

Figure 3. Representative positive immunohistochemical staining of colon cancer cells for the p14ARF gene. (A) 200x and (B) 400x
DISCUSSION

Colon cancer is a heterogeneous population of cells, so samples of patients with similar pathological features may show different results at the laboratory level [7]. This research has shown a possible correlation between colon cancer and the positive expression of the APC, E2F1, p14ARF and Ki-67 genes. This association may help to identify possible diagnostic markers for this type of cancer.

The APC gene is considered a colon cancer suppressing gene and is deregulated in both stem and body cells [8]. Changes in the APC gene are premature, if not initial, in 80-85% of colorectal sporadic cancers, except for those with methyl phenytoin of CpG islets (CIMP) or myocardial infarction (microdermabrasion) instability due to lack of a DNA mismatch (MMR) repair mechanism [9]. The specialized analysis of gene sequence in human colon and adjacent mucosal adenomas reveals that APC mutations are present in precancerous lesions [10]. Genetic studies using a wide variety of models of mutated APC genes in mice have shown that mutations in this gene are responsible for intestinal oncogenesis [11]. More than 90% of mutations in the APC gene create premature termination codons, leading to consistently cut gene products [12].

Fixed expression of APC has been detected in samples of patients with familial adenomatous polyposis (FAP) and colorectal cancer. The C-terminal amino acids of the colonic proteins, present in colorectal cancer, lack the areas required for binding to the EB1 and β-catenin microtubules,
which may lead to the induction of chromosomal instability. While the loss of tumor suppression functions due to the mutant loss of the C-terminal sequence of APC is considered a critical event in the onset of colon cancer, accumulated data suggest that APC gene sevenerese exercises may be a predominant function that may contribute to the tumor. Colon tumors are known to result from pre-existing premenstrual lesions, through the acquisition of genetic changes in a specific tumor or tumor suppressor, the so-called adenoma cancer sequence. The transcriptional loss of the C-terminal sequence of the APC gene is considered essential for the onset of colon cancer through the loss of tumor suppression functions in the APC [13].

The contradictory behavior of the E2F1 transcriptional factor, which favors either cell proliferation or apoptosis, is demonstrated in a variety of carcinomas as well as in experimental models [14]. Knowing that E2F1 has been proposed as a potential treatment target, determining its action in various cancers is a major breakthrough in research in the scientific community. The lack of reports on the possible role of protein in combination with the expression of Ki67 protein in malignant carcinomas, compared to other types of carcinomas, makes such a study necessary [15].

Experiments have investigated the role of the E2F1 gene in programmed cell death. However, it has not been clearly clarified whether it acts as a tumor regulator or tumor suppressor. It has been suggested that E2F1 was a suppressive gene in colorectal cancer [16].

In this study, we examined the expression of the E2F1 gene and its relationship to tumor motility in a colorectal cancer cell specimen environment. Recording and analyzing the cases according to the state of expression of the specific transcription factor, it was observed with interest that the expression of E2F1 was associated with the proliferation of cancer cells. In this group of cells, the increased expression of E2F1 is likely to be related to the response of the p53 gene to tumor growth. Nevertheless, the bibliographic record of expression of this transcription factor has shown that the mode of action of E2F1, in different types of carcinomas, is independent of the expression of p53 protein [17].

According to research results, p14ARF protein expression levels can be an important factor in the prognosis of patients with colorectal cancer. Knowing that p14ARF protein regulates p53, the association of low p14ARF levels with possible reduced cell survival would not be surprising. The loss of ARF is known to cause resistance to certain chemotherapy treatments and inhibits radiation-related apoptosis of cancer cells [18]. Patients analyzed in such studies underwent intensive chemotherapy. In these patients, the expression levels of the p14ARF gene were not associated with induction of complete remission of the disease. However, subsequent relapses were much more common in samples of patients with low p14ARF gene expression. This may mean that low p14ARF expression is associated with a higher chance of developing resistance to certain chemotherapies [19]. It is likely that in different types of the same cancer, the expression values of the p14ARF gene vary. The increased expression of p14ARF has also been associated with aging of these cells [20]. Combined with our current findings, these findings underscore the potential of this cellular pathway to play an important role in the genesis of colon cancer cells. Therefore, the analysis of p14ARF levels may be particularly valuable in patients with normal karyotype, as the prognosis for this group of patients is very difficult to predict [19].

CONCLUSIONS

In summary, the research community is currently at a critical juncture on how it is possible to design tailored treatment strategies, taking into account the functional contribution of genetic and epigenetic changes in the regulation of regulatory mechanisms that are "signs of cancer." In this sense, the molecular "signature" of each cancer would be the description of a specific set of changes by which a particular tumor escapes these regulatory mechanisms.

Based on this knowledge, it may be possible to select appropriate combinations of therapeutic agents to reverse or rule out the functional consequences of these tumor lesions. By targeting specific and simultaneously multiple pathways based on molecular signatures, such approaches can provide greater therapeutic efficacy.

At the same time they have fewer side effects than conventional cytotoxic treatments.

One of the necessary steps in the treatment of cancer is the development of molecular markers that help predict survival, possible behavior and tumor aggressiveness.

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Conflicts of interest

There are no conflicts of interest to declare.

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