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Comparative Expression Analysis of *TP53* Tumor Suppressor and *MDM2* Oncogene in Colorectal Adenocarcinoma

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Abstract. *Background/Aim: The tumor protein 53 (TP53) tumor suppressor protein (17p13.1) acts as a significant regulator for the cell cycle normal function. The gene is frequently mutated in colorectal adenocarcinoma (CRC) patients and is associated to poor prognosis and low response rates to chemo-targeted therapy. Our purpose was to correlate TP53 expression with Mouse Double Minute 2 Homolog (MDM2), a proto-oncogene (12q14.3) and a major negative regulator in the TP53-MDM2 auto-regulatory pathway. Materials and Methods: A total of forty (n=40) colorectal adenocarcinoma (CRC) cases were included in this study. An immunohistochemistry-based assay was implemented by using anti-TP53 and anti-MDM2 antibodies in the corresponding tissue sections. Additionally, a digital image analysis assay was implemented for objectively measuring TP53/MDM2 immunostaining intensity levels. Results: TP53 protein overexpression was detected in 27/40 (67.5%), whereas MDM2 overexpression in 28/40 (70%) cases. Interestingly, in 21/40 (52.5%) cases, a combined TP53/MDM2 co-expression was detected, whereas in 6/40 (15%), a combined loss of expression was identified (overall co-expression: p=0.119). p53 overexpression was significantly correlated to grade of the examined cases (p=0.001), whereas MDM2 to stage and max diameter of the malignancies (p=0.001 and 0.024, respectively). Conclusion: TP53/MDM2 over expression is a frequent and significant genetic event in CRCs associated with an aggressive biological behavior, as a result of increased dedifferentiation grade and advanced stage/elevated tumor volume, respectively. MDM2 oncogene overactivation combined with mutated and overexpressed TP53 is observed in sub-groups of patients leading to specific gene/protein signatures – targets for personalized chemotherapeutic approaches.*

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Normal cellular microenvironment homeostasis is mediated by critical molecules (1). Among them, tumor protein 53 (TP53) is a leading regulator that enhances the normal genomic function and structural stability (2). The corresponding gene is located on the short (p) arm of chromosome 17 (gene locus: 17p13.1). TP53 gene encodes for a nuclear phosphoprotein (molecular mass of 53 kDa) acting as a central transcription factor. TP53 can enhance apoptotic cell death, also reducing cell proliferation (3). Concerning its activity, it regulates the cell cycle by providing phase arrest at the level of G1/S and G2/M checkpoints (4). Interestingly, the P53/P21/DREAM/E2F/CHR pathway is involved in cell cycle as a result of P53-mediated indirect transcriptional repression (5). This mechanism prevents DNA damage during DNA replication in the S phase. Additionally, TP53 promotes histone de-acetylation, proteolysis, apoptotic death, and negatively

regulates helicase and telomerase activity (6-8). Furthermore, TP53 acts as a strong gene transcription factor, and is involved in critical molecular pathways that provide responses to intracellular hypoxia, modify protein oligomerization, base-excision repair, glucose deficit, apoptosis regulation and mitochondrial DNA stability (9, 10).

TP53 interacts with mouse double minute 2 homolog (MDM2). The last molecule (also known as E3 ubiquitin-protein ligase), is referred as a proto-oncogene (gene locus: 12q14.3) that is responsible for the production of a nuclear-localized protein (11). The most crucial biochemical function that MDM2 regulates is the zinc ion binding to specific intracellular substrates. The molecule also demonstrates a ligase/transferase (12). MDM2 and TP53 form an auto-regulatory pathway. MDM2 binds directly to TP53, negatively modifying its transcriptional activity, promoting TP53 proteasomal degradation (13). More specifically, MDM2 binds to the TP53 N terminus inducing its ubiquitination and its permanent degradation. MDM2 oncogenic activity is mediated predominantly by gene amplification. In solid malignancies, -prominently in sarcomas and especially in liposarcomas- MDM2 overactivation is frequently correlated to a more aggressive phenotype in subsets of patients with specific genetic signatures (14). MDM2 mutations impair the ability to degrade the TP53 oncoprotein efficiently (15, 16). In the current research study, we co-analyzed TP53 and MDM2 proteins in a series of colorectal adenocarcinomas (CRCs), exploring the potential impact of their co-expression levels on clinical-pathological features of the corresponding malignant tissues.

Materials and Methods

Study group and tissue specimens. A series of forty (n=40) archival, formalin-fixed, and paraffin-embedded CRC tissue specimens were obtained covering a broad spectrum of grades of differentiation and stages. Concerning the corresponding patients, nineteen (n=19, 47.5%) were female (mean age=64.5 years), whereas the rest (21, 52.5%) were males (mean age=67.2 years). The whole lab procedure took place in the First Department of Pathology, School of Medicine, National and Kapodistrian University of Athens. The Medical School, National and Kapodistrian University of Athens, Athens, Greece ethics committee consented [Reference ID Research Protocol: 219/13-12-2019 (Research ID: 1920012595-11/12/19] to the use of these tissues stored in coded form in the laboratory of Pathological Anatomy for research purposes, according to World Medical Association Declaration of Helsinki guidelines (2008, revised in 2014). The selected tissues were initially fixed in 10% neutral-buffered formalin. Hematoxylin and eosin (H&E)-stained slides of the corresponding samples were reviewed by two independent pathologists for the final histopathological diagnosis confirmation and classified according to the histological typing and grading criteria of the World Health Organization (WHO) (17).

Immunohistochemistry assay (IHC). Ready-to-use anti-p53 (clone DO7, Dako, Glostrup, Denmark; dilution at 1:40) and anti-MDM2 (clone IF2, Novocastra, Newcastle, UK; dilution at 1:40) mouse monoclonal antibodies were used in the examined tissues. IHC protocols -based on the selected antibodies- were carried out on 4μm tissue sections. The slides were initially deparaffinized in xylene and rehydrated in graded ethanol solutions. Following this stage, the slides were immunostained for the markers based on the EN Vision+ (Dako) protocol by using an automated staining system (I 6000; Biogenex, Fremont, CA, USA) This specific assay is based on a soluble, dextran-polymer system that avoids an endogenous biotin reaction.

Following peroxidase blocking, the tissue sections were incubated by applying the primary antibody for 35 min at room temperature. After this stage, incubation with horseradish peroxidise-labelled polymer-HRP (Dako) LP for 30 min was performed. The antigen-antibody binding was visualized by applying the 3-3, diaminobenzidine tetrahydrochloride (DAB) chromogen (Dako). At the final phase of the IHC process, the tissue sections were slightly counterstained by hematoxylin for 30 secs, dehydrated and mounted. Normal colon tissues expressing the markers were used as positive controls. For negative controls, the primary antibody was omitted. According to the antibody manufacturers, a predominantly nuclear and peri-nuclear staining pattern was considered an acceptable expression pattern for both (Figure 1a).

Digital image analysis assay (DIA). In order for TP53/MDM2 protein expression levels to be quantitatively and objectively evaluated, we implemented a DIA assay using a semi-automated system (hardware: Microscope CX-31, Olympus; Digital camera, Sony, Tokyo, Japan; Windows XP/NIS-Elements Software AR v3.0, Nikon Corp, Tokyo, Japan). The corresponding digital algorithm precisely calculated the corresponding staining intensity levels (densitometry evaluation) in the examined malignant cells. Ten (n=10) areas of interest per tissue section were identified (five high-power optical fields at ×400 magnification) and filed in a digital database as colored snapshots. Measurements were performed by implementing a specific macro (nuclear expression for malignant cells). Normal tissue sections (control) were measured independently and compared to the corresponding values that were extracted from the malignant tissue sections. A broad spectrum of continuous grey scale values (0-255) at the RedGreenBlue (RGB) pattern was available for discriminating different protein expression levels (Figure 1b, c). According to the DIA software, the staining intensity values that progressively decrease to 0 represent a continuous overexpression of the protein. In contrast, staining values that increase to 255 reflect a progressive loss of its staining intensity.

Statistical analysis. The statistics software package IBM SPSS v25 (Armonk, NY, USA) was used. Quantitative variables were presented as mean±standard deviation, while the qualitative variables were presented in frequency tables. To evaluate the relationship between qualitative and quantitative variables, due to the small number of subjects in each group, the nonparametric Mann-Whitney and Kruskal-Wallis tests were applied. To evaluate the relationship between independent qualitative variables, where appropriate, the chi-square (x^2) and Fisher exact tests were applied. Statistical significance (p) was evaluated in pairs and differences <0.05 were considered statistically significant.

Results

IHC results and statistical differences (p-Values) are presented in Table I. According to the DIA protein

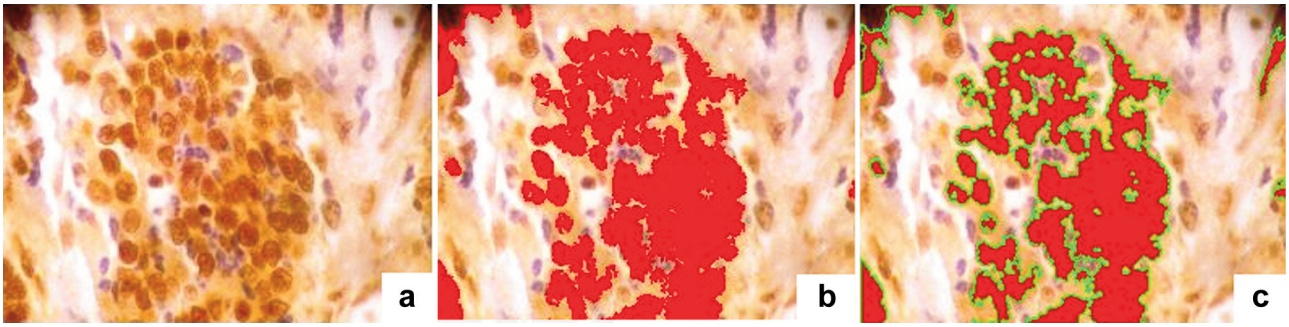

Figure 1. *Digital image analysis (DIA) of a TP53 overexpression colorectal cancer (CRC) case. (a) TP53 strong nuclear staining pattern (400×). (b) Red- painted areas are the substrate for the staining intensity level measurements inside the nuclear microenvironment. (c) Green encircled areas have been measured automatically. The extracted value of 112.70 (in the 0-225 RGB continuous grey scale spectrum of staining intensity) is the result of the objective estimation of TP53 expression. Concerning the DIA protocol, immunostaining intensity values decreasing to 0 represent a progressive overexpression of the marker, whereas values increasing to 255 show a progressive loss of its staining intensity.*

Table I. *Clinicopathological parameters and total combined P53/MDM2 protein expression results.*

Clinicopathological parameters		P53			MDM2		
CRC cases (n=40)		OE	LE	*p*-Value	OE	LE	*p*-Value
		27/40 (67.5%)	13/40(32.5%)		28/40(70%)	12/40 (30%)	
Sex				0.703			0.456
Male	19 (48%)	11/40 (%)	8/40 (20%)		10/40 (25%)	9/40 (10%)	
Female	21 (52%)	16/40 (%)	5/40 (13%)		18/40 (44%)	3/40 (8%)	
Differentiation Grade				**0.001**			**0.001**
II	21 (52%)	9/40 (22%)	12/40 (32%)		14/40 (35%)	7/40 (17%)	
III	19 (48%)	18/40 (44%)	1/40 (2.5%)		14/40 (35%)	5/40 (8%)	
Stage				0.516			**0.001**
I	7 (17%)	4/40 (10%)	3/40 (8%)		1/40 (2.5%)	6/40 (15%)	
II	12 (30%)	8/40 (20%)	4/40 (10%)		8/40 (20%)	4/40 (10%)	
III	17 (43%)	12/40 (32%)	5/40 (13%)		16/40 (40%)	1/40 (2.5%)	
IV	4 (10%)	3/40(8%)	1/40 (2.5%)		3/40 (8%)	1/40 (2.5%)	
Tumor localization				0.667			0.356
Left colon	23 (58%)	18/40 (44%)	5/40 (13%)		16/40 (40%)	7/40 (17%)	
Right colon	17 (42%)	9/40 (22%)	8/40 (19%)		12/40 (30%)	5/40 (13%)	
Max diameter				0.095			**0.024**
<5 cm	26 (65%)	15/40 (38%)	11/40 (28%)		15/40 (38%)	11/40 (28%)	
≥5 cm	14 (35%)	9/40 (22%)	5/40 (13%)		13/40 (33%)	1/40 (2.5%)	

CRC: Colon adenocarcinoma; OE: overexpression (high expression) staining intensity values ≤132 at stained cells; MLE: moderate-low expression staining intensity values >138 ≤161 at stained cells. *p*-Values in bold type refer to statistically significant correlations (≤0.05).

expression analysis, the examined colon adenocarcinoma tissues demonstrated different expression levels. In fact, TP53 protein overexpression (high expression as ≤132 staining intensity values in stained nuclei) was detected in 27/40 (67.5%), whereas MDM2 in 28/40 (70%) cases. In contrast, moderate-low expression staining intensity values (>138 ≤161) in stained nuclei were detected in the rest of the examined cases for both markers. Staining intensity values in the range of 133 and 138 were not detected. Interestingly, in 21/40 (52.5%) cases, a synchronous TP53/MDM2 expression was reported, whereas in 6/40 (15%), a progressive loss of their co-expression was detected (overall co-expression: p=0.119). TP53 overexpression was found to be significantly correlated to the grade of the analyzed cases (p=0.001), whereas MDM2 overall expression demonstrated a strong association with stage and max diameter of the malignancies (p=0.001 and 0.024, respectively).

Discussion

Identification of unique genetic events in solid malignancies is a modern and optimal approach for oncologists to plan and apply targeted chemotherapeutic strategies to patients (18-20). Concerning the TP53/MDM2 auto-regulatory pathway deregulation, it has been implicated on a variety of solid malignancies, including CRC (21, 22). It is well known that TP53 nuclear over expression is detected in ~70-90% of solid malignancies characterized by different histo-genetic origins (23, 24). Molecular analyses based on TP53 and other genes-including the *K-RAS* oncogene - have revealed simultaneous mutations that affect these genetic markers in specific populations (25, 26). Besides *K-RAS/TP53* mutations, multigene mutations in colon carcinoma patients create specific genetic signatures that modify the corresponding response levels to targeted therapeutic regimens (27, 28).

In the current research study, we simultaneously analyzed TP53 and MDM2 proteins in a series of colon carcinoma cases, by implementing a protocol based on the combination of IHC and DIA for objectively estimating their protein expression levels. Our analysis revealed a significant co-expression of the examined markers. Concerning their impact on the clinical-pathological features of the malignancy, TP53 over expression was strongly correlated to the grade of the examined cases, whereas MDM2 to stage and max diameter of the malignancies. Concerning the modern oncological approaches, a combination of wild type P53 enhanced function and MDM2 decreased oncogenic activity should be a crucial step for handling subgroups of patients with specific genetic signatures (29, 30). Interestingly, specific mutant TP53 variants ($p53^{K120R}$) are involved in metabolic process in cancer patients, especially modulating glucose metabolism (31, 32). Furthermore, TP53 alterations combined with mucin-5 over expression and microsatellite instability (MSI) are involved in colitis-associated colorectal carcinoma, as a result of a progressive chronic inflammation-dysplasia-cancer carcinogenesis process (33, 34). Concerning new agents with anti-tumor activity that target TP53 expression, aurora-A - a key G2/M phase regulator kinase – seems to inhibit the TP53 signaling, negatively affecting the response to oxaliplatin-based treatment in CRC patients (35). Similarly, medicinal plants such as ginkgo biflavones could be used as wild type normal p53 enhancers and also MDM2 inhibitors in CRC patients with specific molecular substrates (36). Additionally, a plant substance derived from Ophiopogon japonicus, named cycloastragenol, demonstrates antioxidant, anti-inflammatory, and anti-cancer effects by inducing apoptosis through p53 and c-MYC regulation (37, 38).

Moreover, new genetic markers, such as microRNAs (miRs) and upregulated circular RNAs (circRNAs) seem to critically modify the TP53 expression levels in subsets of CRC patients (39). Two study groups reported a positive role of miR-887-3p in CRC by inhibiting cell proliferation and, in parallel, enhancing apoptotic rates due to wild type P53 activation, whereas miR-338-3p negatively affects the resistance rates to 5-fluorouracil (5-FU) in TP53 mutant CRC cases (40, 41). Concerning the potential role of TP53 and MDM2 protein expression levels as reliable biomarkers for onset, progression, prognosis and modifiers of the CRC biological behavior, there is a variety of studies that provide positive results, especially correlating *TP53* gene mutations with increased metastatic potential (42, 43). Interestingly, specific MDM2 genetic signatures, including single nucleotide polymorphisms, seem to be significant for predicting increased susceptibility risk to CRC development compared with the wild-type T allele carriers (44). Additionally, phosphatase and the tensin homolog (PTEN, 10q23) suppressor gene silencing seems to increase MDM2 phosphorylation leading to normal p53 function in an experimental model of colon carcinogenesis (45).

Conclusion

In conclusion, the TP53 tumor suppressor gene apoptotic activity antagonizes the MDM2 oncogenic activity that induces proliferation in neoplastic and malignantly transformed cells. Mutant TP53 over expression combined with MDM2 over activation are frequently observed in CRC cases correlated to an aggressive biological behavior (dedifferentiation, increased tumor dimensions, and advanced stage). Interestingly, TP53 protein accumulation in the nucleus of tumor cells -as a result of *TP53* mutations- does not necessarily combine with decreased MDM2 expression. As MDM2 directly binds to p53 and represses its transcriptional activity promoting p53 degradation, its overactivation negatively affects crucial apoptotic p53-based functions. TP53/MDM2 complex deregulation in solid malignancies, and particularly in CRC, is a target and challenge for further investigation and development of targeted anti-MDM2 strategies for an optimal oncological handling of CRC patients at the basis of specific genetic signatures.

Conflicts of Interest

The Authors declare that they have no conflicts of interest.

Authors' Contributions

AN and ET: Design of the study and article writing; DD, NK, DP, DS and KCK: review and data evaluation as academic advisors; HS, LM, DD, EF and SM: collection and management of references and published data. All Authors read and approved the final article.

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