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ORIGINAL ARTICLE

c-MYC and h-TERT co-expression in colon adenocarcinoma: a tissue microarray digitized image analysis

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Summary

Purpose: c-MYC oncogene is frequently deregulated by amplification in colon adenocarcinoma. c-MYC also activates telomerase by inducing expression of its catalytic subunit (h-TERT). Furthermore, telomerase activation plays a crucial role in tumorigenesis by sustaining cellular immortality. Our aim was to evaluate the significance of c-MYC and h-TERT co-expression in colon adenocarcinoma.

Methods: Sixty paraffin embedded primary colon adenocarcinomas were cored at 1.5 mm diameter and transferred to one microarray block. Immunohistochemistry was performed using anti-h-TERT, and c - MYC antibodies. A quantitative digitized macro was performed to evaluate their expression.

Results: c-MYC and h-TERT overexpression was observed in 27 (45%) and 28 (46.6%) cases, respectively. Co-overexpression of those genes was observed in 17 (28.3%) cases and found to be statistically significant (p=0.001). The results also showed a strong association between c-MYC and grade of differentiation of the examined neoplasms (p=0.021).

Conclusion: Simultaneous c-MYC and h-TERT deregulation is a relatively frequent genetic event in colon adenocarcinoma. Because c-MYC overexpression is correlated with progressive disease – due to colon adenocarcinoma dedifferentiation - inhibition of its activity combined with h–TERT regulated expression is a new target for novel therapeutic regimens.

Key words: c-MYC, colon carcinoma, genes, image analysis, immunohistochemistry, telomerase

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Table 1. Clinicopathological characteristics and IHC results

Characteristics	*c-MYC*			*h-TERT*		
	LL (N=33)	*OE (N=27)*	*p-value*	*LL (N=32)*	*OE (N=28)*	*p-value*
	55%	*45%*		*53.4%*	*46.6%*	
Gender			NS			NS
Female (N=32)	62.5	37.5		68.7	31.3	
Male (N=28)	46.5	53.5		39.3	60.7	
Grade			0.021			NS
I (N=22)	50	50		22.3	72.7	
II (N=31)	54.8	45.2		25.9	74.1	
III (N=7)	42.9	57.1		14.3	85.7	
TNM stage			NS			NS
I (N=8)	48.5	52.5		39.4	61.6	
II (N=26)	73.6	26.4		56.3	43.7	
III(N=26)	38.5	61.5		40.3	59.7	
c-MYC vs h-TERT			0.001			

LL: loss or low expression (0, 1+), OE: overexpression (2+/3+)/ High: 72-91, Moderate: 101-119 (according to nuclear/perinuclear staining intensity levels detected by image analysis), NS: not significant. For other abbreviations see text

Introduction

Colorectal adenocarcinoma - one of the leading causes of cancer death in the Western world - arises in individuals as a result of predominantly acquired or inherited genetic imbalances [1]. Simultaneous deregulation of oncogenes, such as k-ras, c-MYC, b-RAF, EGFR, and also suppressor genes including p53, PTEN, p16, e-cadherin, is responsible for exposing the carcinogenetic process in normal colon epithelia [2-4]. Gene amplification, specific point mutations and deletions, transcriptional alterations are the main mechanisms that lead to abnormal protein expression of the corresponding genes [5].

C-MYC protooncogene - the human cellular homologue of the v myc oncogene of avian myelocytomatosis retrovirus MC29 - which is located at chromosome 8 (8q24.12-q24.13) - is found to act as a strong transcription factor, implicated in the control of cell differentiation and apoptosis [6]. Induction of this transcription factor promotes cell proliferation and transformation by activating growth-promoting genes, including the ornithine decarboxylase (ODC1) and CDC25A genes and also the E2F1, E2F2 and E2F3 genes [7]. The myc protein activates transcription as part of a heteromeric complex with MAX protein. c-MYC is also involved in direct telomerase activation by inducing expression of its catalytic subunit, h-TERT [8]. h-TERT is a target of c-MYC activity and some pathways linking cell proliferation and chromosome integrity in normal and neoplastic cells have already been confirmed [9]. Concerning colorectal adenocarcinoma, c-MYC overexpression is a common genetic event correlating indirectly with response rates to modern specific targeted therapeutic strategies [10,11].

Telomerase is a ribonucleoprotein enzyme that lengthens chromosome ends, which have been shortened during successive cycles of cell division. It consists of two main components, including an RNA subunit (h-TERC) that acts as template for telomeric DNA synthesis and a catalytic protein subunit (h-TERT). h-TERT gene is located at chromosome 5 (5p15.33) and its protein product acts as a telomerase reverse transcriptase [12]. Telomeres are short specific tandem DNA repeats (5-TTAGGG-3) located at the end of the chromosome. By the end of each replication cycle, human telomeres in all somatic cells undergo progressive shortening and this event functions as a tumor suppressor mechanism by preventing the abnormal, excessive replication of the DNA molecule. Telomerase expression plays a crucial role in cellular senescence, as it is normally repressed in postnatal somatic cells resulting in progressive shortening of telomeres [13]. Overactivation of telomerase leads to

cell immortalization and this genetic event has been detected in the majority of cancers [14]

In the current study we analyzed c-MYC and h-TERT protein co-expression in CA specimens based on a combination of tissue microarrays and digital image analysis.

Methods

Study group

For the purposes of this study, we used 60 formalin-fixed and paraffin-embedded tissue samples of histologically confirmed colorectal adenocarcinomas obtained by surgical resection (local or distant colectomies) between 2005 and 2007. The Department of Pathology (417 VA Hospital-NIMTS, Athens, Greece) and the local Ethics Committee gave permission to use those tissues for research purposes. Oral informed consent was obtained from each patient and the study protocol adhered to the ethical guidelines of the "World Medical Association (WMA) Declaration of Helsinki – Ethical Principles for Medical Research Involving Human Subjects" adopted by the 18th WMA General Assembly, Helsinki, Finland, June 1964, as revised in Tokyo 2004. Ten microscopically normal-appearing colon epithelia were used as control group. All corresponding hematoxylin and eosin (H&E)-stained slides were reviewed by two pathologists for confirmation of diagnosis and classification according to World Health Organization (WHO) grading criteria for colorectal adenocarcinoma. Furthermore, staging was assessed using the TNM staging system. Clinicopathological data are displayed in Table 1.

Tissue microarray (TMA) construction

Areas of interest were identified in H&E-stained slides by conventional microscope (Olympus BX-50, Melville, NY, USA). Selection of those areas was performed on the basis of tumor sufficiency avoiding sites of necrosis or bleeding. Using ATA-100 apparatus (Chemicon International, Temecula, CA, USA), all of the source blocks were cored twice (in order to secure the presence of each case in the final blocks) and 1.5-mm diameter tissue cylindrical cores were transferred from each conventional donor block to the three recipient blocks. After 3 mm microtome sectioning and H&E staining the final constructed TMA blocks contained 92% cores of tissue cylindrical specimens. Microscopically we observed that all of the examined cases were represented by at least one

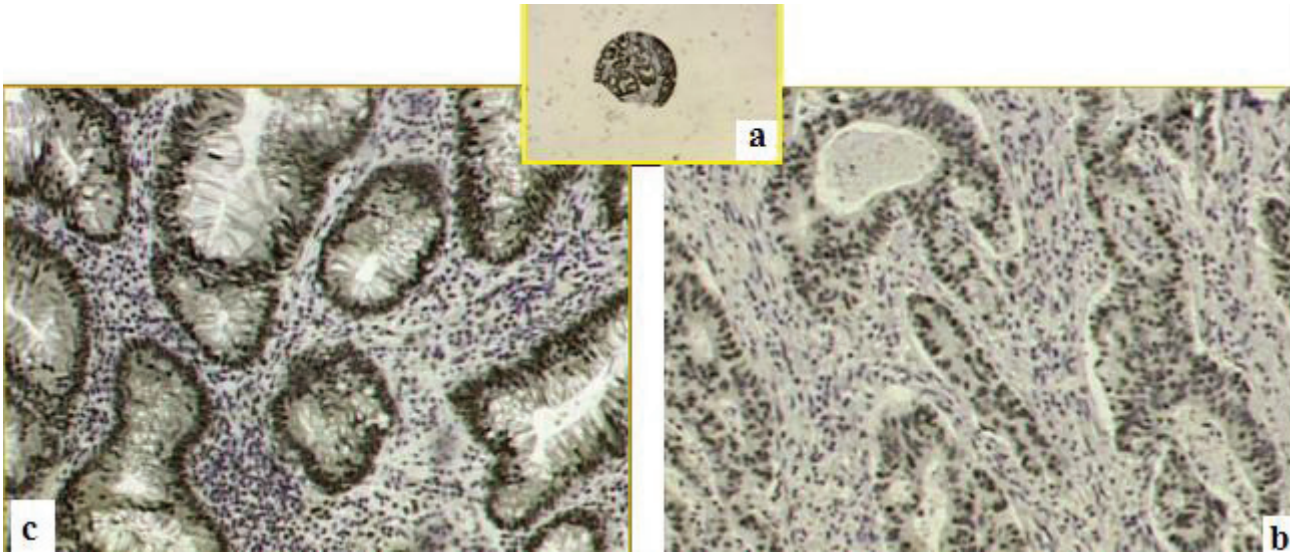

Figure 1. Overexpression of c-MYC and h-TERT in colon adenocarcinoma tissue microarray cores **(a)**. Note a significant number of overstained nuclei regarding c-MYC **(b)** and h-TERT **(c)** proteins (original magnification x10).

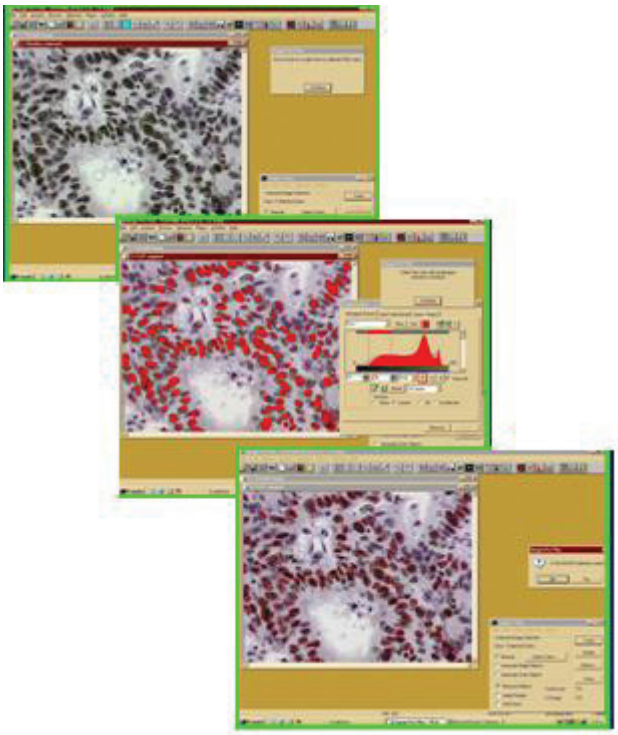

Figure 2. Digital image analysis for c-MYC and h-TERT evaluation. Red areas represent different expression levels regarding nuclear immunostain (original magnification x40).

or two tissue spots (confirmation of the adequacy of the examined specimens) (Figure 1).

Immunohistochemistry (IHC) analysis

IHC for h-TERT and c-MYC antigens was carried out on 3 μm paraffin sections of the TMA block described above. Two slides were stained for each marker. We used anti h-TERT mouse monoclonal antibody (clone 44F12-Novocastra-dilution 1:40), which recognizes the region near the N-terminal, and anti-c-MYC mouse monoclonal antibody (clone 9E10-DAKO-dilution 1:60), which recognizes the region near the C-terminal. The slides were deparaffinised and En Vision IHC protocol (DAKOCorp, Denmark) was performed using an automated IHC staining system (I 6000-Biogenex, USA). Diamminobenzidine (DAB) was used as chromogenic substrate. This IHC protocol, based on a water-soluble dextran polymer system, prevents the endogenous biotin reaction, which is responsible for the background in the stained slides. Nuclear staining pattern was considered to be acceptable for the evaluation of IHC specificity in both of the markers (c-MYC expression was observed also as pale-brown cytoplasmic staining pattern). Specimens of breast adenocarcinoma cases overexpressing these markers were used as positive controls. For negative control slides, the primary antibodies were omitted (Figure 1).

Computerized image analysis assay (CIA)

c-MYC and h-TERT protein expression levels were evaluated by a macro based on staining intensity levels measurements provided by digital image analysis. We performed CIA using a semi-automated system (Windows XP/NIS-Elements Software AR v3.0, Nikon Corp, Tokyo, Japan) (Figure 2). A digitized database was constructed including all the snapshots of the immunostained TMA cores. A macro was implemented for evaluating the slides. Measurements of staining intensity levels were performed in five optical fields per case and at a magnification of x400. All measurements were performed inside an active window. All numerical data were filed in Microsoft Excel sheets. Interpretation of staining intensity values was assessed in a range of 256 continuous values, including the RGB protocol in grey scale (0: absolute black, 255: absolute white).

Statistical analysis

Associations between variables including protein expression levels and clinicopathological parameters were performed by applying the SPSS v 20 statistical software (Chicago Inc, IL, USA). In the analysis, c-MYC and h-TERT expressions were treated as ordinal variables (0-3 scale) based on a categorization of staining intensity levels provided by image analysis; in the tables, however, the 0-1 (Loss or Low expression, "LL") and 2-3 /High-Moderate by image analysis (overexpression, "OE") categories have been merged for reasons of brevity. Concerning the associations between these variables and other ordinal or continuous variables, p-value was derived from Spearman's rank correlation coefficient. Total IHC results and also p-values are described in Table 1.

Results

IHC study showed different expression patterns of these molecules in the examined tumors. c-MYC overexpression (high & moderate expression values; 2+/3+ categories) was detected in 27/60 (45%) cases. High levels of expression were observed in 13/27 (48%) of the cases and moderate levels in 14/27 (52%). Similarly, h-TERT overexpression was detected in 28/60 (46.6%) cases. In 16/28 (57.1%) cases high expression levels were detected, whereas 12/28 (42.9%) cases showed moderate expression. Co-overexpression of these genes was observed in 17/60 (28.3%) cases. Overall, c-MYC vs h-TERT expression in the examined cases was found to be statistically significant (p=0.001). The results also showed a strong association between c-MYC and grade of differentiation (p=0.021).

Discussion

Mutations of k-ras oncogene, EGFR amplification, and c-MYC over expression via gene amplification are frequent genetic events in colorectal adenocarcinomas, correlating with prognosis and response rates to specific targeted therapeutic strategies, especially based on monoclonal antibodies (i.e. cetuximab) [15-18]. Besides these molecules that act as blockers in the external part of the receptors preventing ligand-binding and signal transduction to the nucleus, novel agents seem to demonstrate a specific activity in other protein chains, such as Wnt/β-catenin pathway. Overactivation of the β-catenin/T-cell factor (TCF) response to transcriptional signalling plays a critical role early in colorectal carcinogenesis [19]. Quite recently, magnolol, a neolignand from the cortex of Magnolia obovata, was applied in cancer cell cultures and was associated with decreased c-MYC protein expression levels in those cultures [20]. Additionally, there are early data focused on an anti c-MYC activity via ellagic acid (EA) application in experimental studies. According to experimental results, EA oral supplementation in rats reduced aberrant crypt foci (ACF) proliferation and progression to neoplasmatic transformation in colon epithelia acting as a chemopreventive agent [21]. Suppression of c-MYC at the protein level is a target of a new agent that decreases the S phase and increases G1 phase during proliferation in epithelia. Ursodeoxycholic acid (UDCA) demonstrates a chemopreventive activity in colon epithelia inhibiting not only c-MYC but also CDK4 and of CDK6 kinases [22]. Additionally, a significant anti-EGFR agent (erlotinib) was found to decrease c-MYC expression levels in rats based on an experimental study regarding a colitis-associated colon cancer model [23]. In those animals a completely reduced cell proliferation was also detected.

In our study, c-MYC overexpression was observed in a significant number of the examined TMA cores, correlated also with the grade of the corresponding tumors. Besides gene amplified-dependent c-MYC overactivation, other mechanisms include gene hypermethylation and single nucleotide polymorphism (SNP)-based utilization in pre-existing large chromatin loops [24]. Concerning also c-MYC correlation with specific chemotherapeutic agents, such as camptothecin or paclitaxel, a c-DNA microarray analysis showed that besides proliferation, its expression regulates the apoptotic process in colon cancer epithelia [25-26]. Furthermore, crucial genes including p53 and p21 are found to be targets of c-MYC activation. Interestingly, c-MYC expression levels, as well as p53 and ki 67, were found to be progressively co- decreased in surgical margins of coloctomies and normal mucosa [27].

In the current study, c-MYC overexpression was significantly correlated with h –TERT expression levels. Interaction of these molecules has been also identified in carcinomas of different histogenetic origin [28-30]. Concerning colon adenocarcinoma, h-TERT overexpression is a frequent genetic event but there are controversial results regarding its association with pathologic parameters, such as grade or stage of the examined tumors [31,32] Quite recently, a c-DNA analysis showed that specific intragenic h-TERT SNPs are correlated with colon or rectal carcinogenesis [33]. Furthermore, identification of a specific polymorphic tandem repeats minisatellite of h-TERT gene - termed MNS16A - showed a significant relation with increased risk of colorectal polyps and colorectal cancer [34]. In addition, genes that produce ATPase enzymes, such as RuvBl2, seem to induce h-TERT

nuclear activation in colon carcinogenesis [35]. Because telomerase overexpression induces carcinogenesis, some studies have proposed application of anti-telomerase targeted strategies based on chemo-agents such as flavonoids (including resveratrol and tannic acid) or even gene therapies via HDV ribozymes directed against its RNA component [36,37].

In conclusion, c-MYC and h-TERT co-deregulation is a frequent genetic event in colon cancer correlated also with an aggressive phenotype (neoplasm's dedifferentiation). Based on the molecular mechanisms of their aberrant expression, rational novel targeted therapeutic strategies should be performed to inhibit their activity.

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