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ORIGINAL ARTICLE

Evaluation of combined telomerase and c-myc expression in non-small cell lung carcinomas using tissue microarrays and computerized image analysis

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Summary

Purpose: *Telomerase activation plays a crucial role in tumorigenesis by sustaining cellular immortality. It consists of two main components which include a RNA subunit (h-TERC) and a catalytic protein subunit (h-TERT). Similarly, amplification or deletion correlating with overexpression of c-myc is a common event in various neoplasias, including non-small cell lung carcinoma (NSCLC). Because c-myc activates telomerase by inducing expression of its catalytic subunit, our aim was to correlate the expression of these two proteins with the biological behavior in NSCLC.*

Materials and methods: *Using tissue microarrays technology (TMA) we evaluated by computerized image analysis (CIA) the results of h-TERT and c-myc immunohistochemistry (IHC) in 40 NSCLCs, which were cored and re-embedded into one TMA block.*

Results: *Co-overexpression (moderate or high levels of NLI: Nuclear Labeling Index) of h-TERT and c-myc was observed in the majority of cases and found to be statistically significant (p=0.001). The results showed also strong association between c-myc and h-TERT overexpression correlating with stage (p=0.001 for both of them), but not with grade (p=0.206 and p=0.313, respectively).*

Conclusion: *Our combined study showed that there is a strong correlation between the activation and expression of these two genes and maybe this co-deregulation could be used as a prognostic factor for the evaluation of biological behavior in NSCLCs.*

Key words: c-myc, immunohistochemistry, non small cell lung carcinoma, telomerase, tissue microarrays

Introduction

NSCLC comprises more than 80% of lung cancers, and complete surgical resection of primary tumors in early-stage disease is the only potentially curative treatment [1]. According to WHO histological classification of lung tumors, NSCLCs include predominantly adenocarcinomas (AC), squamous cell carcinomas (SCC) and large cell carcinomas (LCC) [2]. NSCLC is the final result of a multistep genetic deregulation. Many published studies showed that bronchial and brochioloalveolar epithelia accumulate chromosome or specific gene instabilities and these genomic damages are responsible for their neoplastic and finally malignant transformation [3]. Overactivation of oncogenes, such as K-ras and c-myc or of positive gene regulators, such as cyclin D1, correlated with inactivation of suppressor genes, such as p53 and p16, are genetic events responsible for the malignant progression [4]. Genetic instability is detected in a variety of chronic inflammation-depended cancers. In many examined cases, chromosomal instability has been detected in normal-appearing sporadic epithelia adjacent to cancer

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cell subpopulations, evidence of early genetic damage probably related to oxidative stress.

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 a 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 [5].

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. So, telomerase expression plays a role in cellular senescence, as it is normally repressed in postnatal somatic cells resulting in progressive shortening of telomeres. Overactivation of telomerase leads to cell immortalization and this genetic event has been detected in the majority of cancers [6].

C-myc proto-oncogene -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, implicating in the control of cell differentiation and apoptosis [7]. 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 [8]. The myc protein activates transcription as part of a heteromeric complex with MAX. But the most important observation is the direct telomerase activation by the c-myc gene. This gene activates telomerase by inducing expression of its catalytic subunit, h-TERT. So, h-TERT is a target of myc activity and some pathways linking cell proliferation and chromosome integrity in normal and neoplastic cells have already been confirmed [9].

TMA technology saw the light in the late 1990s as one of the most promising and powerful research tools in modern Pathology. The first description of TMA technique was published in 1998 [10]. Since then, many multitumor studies have been published using TMAs, in order to correlate immunohistochemical or molecular results with biological behavior of the examined neoplasms. A much more widespread use of TMAs is expected in the near future. The number of institutions having TMA manufacturing facilities is increasing rapidly worldwide.

The range of TMA applications appear to be very broad. Depending upon the required type of analysis, several different kinds of TMAs may be manufactured. Prevalence TMAs contain tumor samples from one or several tumor entities. Progression TMAs contain samples of different stages of one particular tumor type, allowing associations between gene or protein alterations. Prognosis TMAs contain samples from tumors with available clinical follow-up data, allowing the estimation of the prognostic significance of molecular features [11].

Using TMA technology, we evaluated by CIA the results of h-TERT and c-myc IHC in order to possibly find statistically significant correlations with biological behavior in NSCLCs. To our knowledge, this is one of the earliest combined TMA and CIA studies involving these two markers in NSCLCs. Another previous study examined HER2/neu expression and gene alteration in NSCLCs using TMAs.

Materials and methods

Forty formalin-fixed and paraffin-embedded archival tissue samples of histologically proven NSCLC were studied: 29 AC, including 2 bronchioloalveolar carcinomas, 9 SCC and 2 LCC. Most of them were diagnosed early by fine needle aspiration (FNA), using the ThinPrep method (Cytyc, USA; Figure 1). All corresponding H&E slides were reviewed by two pathologists for confirmation of diagnosis and classification according to WHO grading and staging criteria. The tissue samples were referred to 31 male (mean age 57 years) and 9 female (mean age 62 years) patients. Clinicopathological data are demonstrated on Table 1.

Table 1. Clinicopathological data of NSCLCs cases (n=40)

	Histological type			
	AC	*SCC*	*LCC*	*Total*
Sex				
Male	22	7	2	31
Female	7	2		9
Grade				
I	4	2		6
II	12	6		18
III	13	1	2	16
Stage				
I	6	1		7
II	12	4		16
III-IV	11	4	2	17

AC: adenocarcinoma, SCC: squamous cell carcinoma, LCC: large cell carcinoma

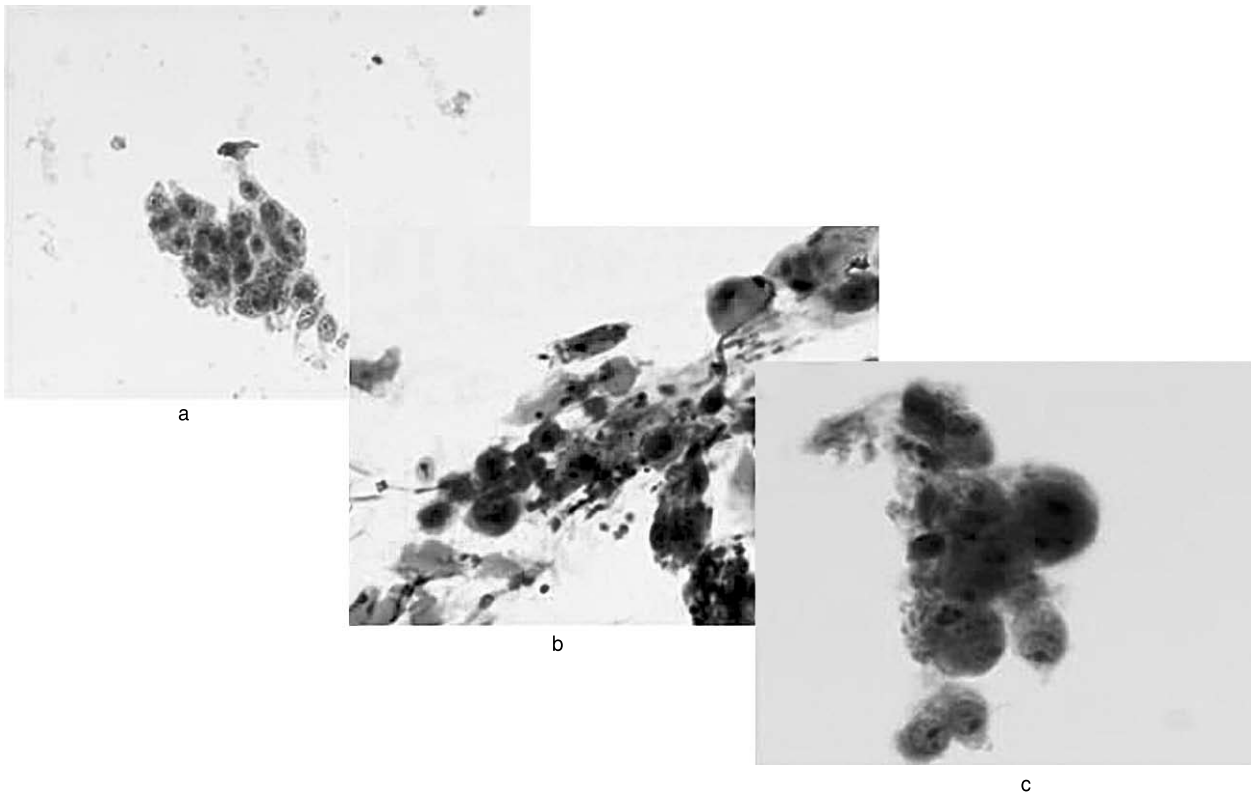

Figure 1. NSCLC histological types: a. adenocarcinoma, b. squamous cell carcinoma, c. large cell carcinoma (ThinPrep slides: original magnification ×40).

TMA construction

Areas of interest were identified in H&E stained slides by a conventional microscope (Olympus BX-50). The corresponding paraffin blocks were obtained for the construction of a TMA block. Using TMArrayer 100 (Chemicon International, USA), all of the source blocks were cored twice (in order to increase the level of representativity of the samples) and 1mm diameter tissue cylindrical cores were transferred to the recipient block. So, the final block contained 40 pairs (80 cores) of cylindrical tissue specimens. After microtome sectioning and H&E staining, we observed that all of the cases were represented by at least one spot, evaluating the adequacy of the cylindrical specimens.

IHC

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 to the N-terminal and anti-c-myc mouse monoclonal antibody (clone 9E10-DAKO-dilution 1:60), which recognizes the region near to the C-terminal. The slides were deparaffinized and En Vision IHC protocol (DAKO Corp, 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 were used as positive controls for both of the markers (Figure 2).

Evaluation of IHC results by CIA

In order to evaluate the IHC results not in a qualitative way but in a more accurate and fast way, we performed CIA by using a semi-automated system with the following hardware features: Intel Pentium IV, MATROX II CARD FRAME GRABBER, CAMERA MICROWAVE SYSTEMS (640×480), microscope Olympus BX-50 and the following software: Windows 2000/Image Pro Plus version 3.0-Media Cybernetics

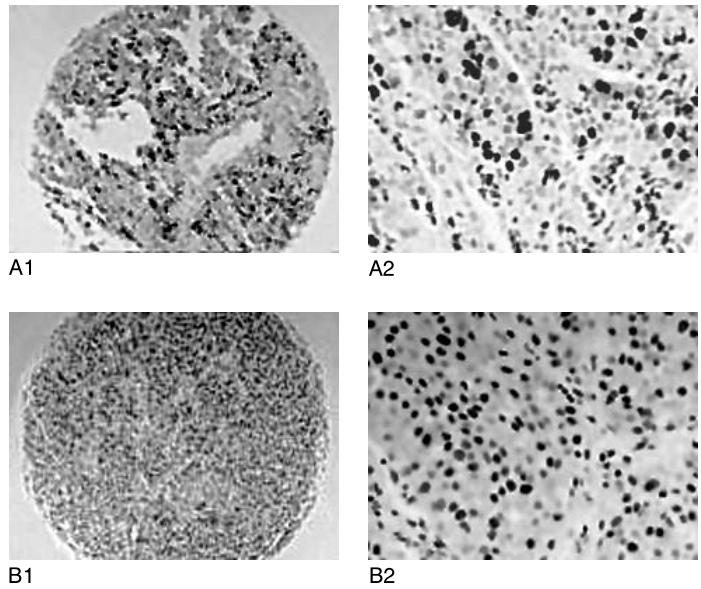

Figure 2. IHC stained TMA slides (tissue spot diameter: 1 mm) A1: h-TERT expression (original magnification ×10), A2: h-TERT expression (original magnification ×40), B1: c-myc expression (original magnification ×10), B2: c-myc expression (original magnification ×40).

1997. We calculated the NLI for each of the cases. Measurements of h-TERT and c-myc immunostained nuclei were performed in 5 optical fields per case and at magnification of 400 (40×10). In a rectangular active window on the computer screen -covering an area of approximately 16848 μm^2– each pixel contained a 24-bit value, called an RGB "TRIPLE". This RGB-triple is made up of three separate 8-bit samples. Each sample represents the level of brightness of its respective color channel: red, blue or green. Finally these brightness values represent levels within a 256-level scale (0-255). A macro (BasicProPlus) was implemented. According to this, all stained nuclei (DAB stained- dark or more light brown objects) per case in the corresponding optical fields were measured and the final number was filed in Excel sheet (Figure 3). Interpretation of NLI and total IHC results are described in Table 2.

Table 2. h-TERT and c-myc IHC results in NSCLCs and statistical analysis (n=40)

IHC NLI† *(mean values)*			*p-values**		
			Grade	*Stage*	*Histological type*
h-TERT			0.313	0.001	0.829
	L				
	M	13			
	H	27			
c-myc			0.206	0.001	0.683
	L	3			
	M	14			
	H	23			

†nuclear labeling index, *Chi square test expressed as number of immunostained nuclei per optical field (n/opt f) (×40) – L: low 0-5 n/opt f, M: moderate 6-10 n/opt f, H: high >10 n/opt f

Statistical analysis

Associations between IHC, NLI and sex, histological type, tumor grade and stage were performed using chi-square test for the confirmation of independence among previous variables (SPSS Inc Chicago IL, USA v.11.0). Two tailed p-values < 0.05 were considered to be statistically significant.

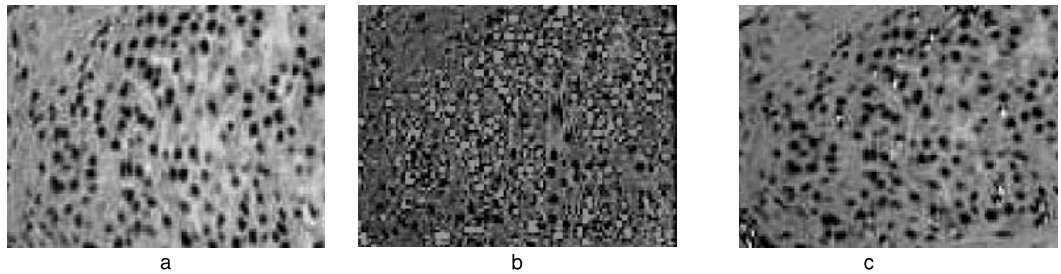

Figure 3. Computerized image analysis process. Detection (a), labeling (b) and counting (c) of stained h-TERT nuclei.

Results

IHC study showed overexpression of h-TERT in all of the tumors. High levels of NLI were observed in 27/40 (67.5%) of the cases and moderate levels in 13/40 (32.5%). Similarly, c-myc overexpression was detected in 37/40 (92%). 23/40 (57.5%) and 14/40 (35%) showed high or moderate NLI, respectively, and only 3/40 (7.5%) low NLI. Evaluating concordances between subgroups of h-TERT and c-myc expression based on the CIA levels of NLI we observed strong correlations. In cases characterized by high levels of h-TERT and c-myc expression the concordance was 86%, and similarly in the other group of co-overexpression with moderate levels of NLI it was 93% (Figure 4). Correlating protein expression status and grade we failed to find statistical significance for both of the markers (p=0.313 for h-TERT and 0.206 for c-myc). But the correlation between levels of overexpression and stage for both of the markers was statistically significant (p=0.001). The cases of LCC demonstrated the highest levels of co-overexpression of all of the studied tumors, according to the NLI Evaluation Chart. Interestingly, two of the cases demonstrating a low level NLI c-myc expression, also showed moderate levels of h-TERT NLI. But specific correlation between the markers in relation to histological type or sex failed to show statistical significance (p=0.829 for h-TERT and p=0.683 for c-myc). Finally, correlation between c-myc and h-TERT overall expression was highly significant (p=0.001)

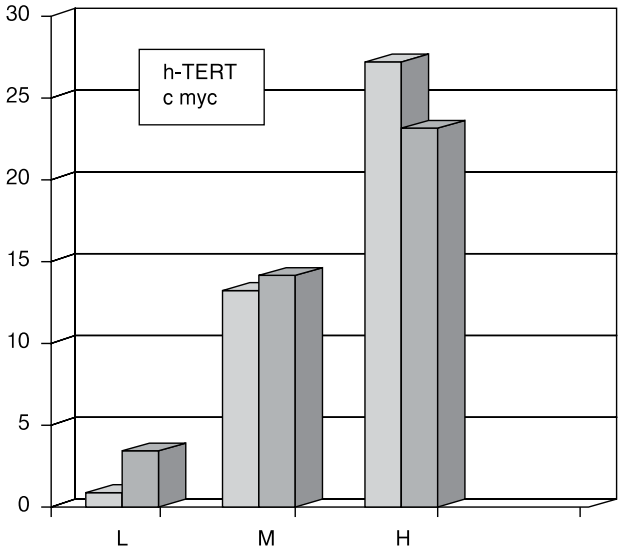

Figure 4. Histogram of combined h-TERT and c-myc IHC levels (L: low, M: moderate, H: high), showing strong concordance of their expression.

Discussion

Our study was designed to detect the level of correlation between telomerase and c-myc overexpression in NSCLCs based on TMAs substrate. Although we did not perform the sensitive telomeric repeat amplification protocol (TRAP) assay introduced by Kim et al. in 1994, we observed significant association between c-myc and telomerase gene expression [12]. C-myc acts as a positive regulator for telomerase reverse transcriptase (h-TERT) and its deregulation, due to gene amplification, happens rarely in these tumors (about 10%), in contrast to small cell lung carcinomas (SCLCs), which demonstrate higher amplification frequency (about 30%) [13]. Gene deletion - loss of heterozygosity - combined with mutation of the other allele or promoter hypermethylation are alternative deregulation mechanisms in order to explain c-myc IHC overexpression. But despite the type of mechanism, this is a crucial genetic event promoting malignant transformation process via h-TERT activation, which leads cells to immortality, probably escaping from the apoptotic death pathway and accumulating new chromosome or gene instabilities [14]. Combined overexpression of the two markers was observed in the majority of NSCLCs and was found to be independent of the grade of differentiation. Similarly, the concordance of co-overexpression was found to be high, based on the NLI levels (low-moderate-high), according to CIA measurements. This observation shows that deregulation of the c-myc/h-TERT molecular pathway probably is an early genetic event in malignant progression in some NSCLC cases, similarly to SCLCs. The results also indicate that c-myc and h-TERT overactivation is correlated with the stage of the tumors, which is a very important association in order to co-evaluate overexpression and biological behaviour in NSCLCs [15]. In particular, LCCs are characterized by a very aggressive biological behavior and the highest levels of c-myc and h-TERT co-overexpession that we detected, comparing the values between all the histological types of the studied NSCLCs, reflect this high genetic instability status.

Although many recently published studies have shown a direct correlation between h-TERT and c-myc gene regulation, they have also demonstrated controversial results correlating IHC expression and prognosis [16]. Our study confirmed those results by using only two 1 mm tissue cores of each of the examined NSCLCs. Despite the tumors' genetic heterogeneity, TMAs detected cancer cell subpopulations demonstrating c-myc and h-TERT overexpression, and, finally, statistically significant correlations among IHC results

and biological behavior of the examined cases were observed. Many studies have compared IHC findings on TMAs with their corresponding traditional large sections, with the vast majority of them revealing a high level of concordance of results [17]. Multiple samples were taken from the donor blocks in order to determine how many samples are needed to obtain results on TMAs that are sufficiently concordant to those observed in large sections analyses. So, those studies found that 2 or 3 samples provided more representative information than a single sample, and that adding more than 4 or 5 samples would not lead to a massive improvement of the concordance level [18]. According to our TMA management experience, 2 or 3 cores of each sample in cases of lung or pancreatic cancer are representative in order to correlate IHC or chromogenic in situ hybridization (CISH) analysis with the biological behavior of these tumors [19,20]. Finally, as many published studies have shown, CIA appears to be a fast and accurate method of IHC evaluation, discriminating levels of NLI in a quantitative way [21]. Cytological applications are considered to be easier and more convincing, especially for measurements referred to cell or nuclear geometrical features [22]. The reasons are first and foremost that segmentation is always easier to apply in cytology because of the more homogeneous background, but also that in cytology the entire nucleus is visible and not just sections of it. The last observation is critical because nuclear integrity level influences the evaluation of gene or chromosome signals, when FISH or CISH protocols are performed for the detection of numeric genetic instabilities. So, cytology and especially new techniques, such as ThinPrep method, offer rapid and more accurate results because they secure exactly the nuclear integrity.

In conclusion, our combined c-myc and telomerase (h-TERT) IHC TMA study suggests that this molecular pathway deregulation is responsible for NSCLC tumorigenesis and this genetic event could be used as a prognostic factor of the biological behavior in these tumors (correlation with stage). Finally, the high level of concordance in co-expression of these two genes, which was confirmed by the application of TMA technology – comparing with conventional IHC slide studies – is a strong evidence of their virtue in combined multitumor IHC or molecular analyses, decreasing the cost of them.

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