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Clinical Significance Of hTERC And C-Myc Genes Amplification In A Group Of Egyptian Patients With Cancer Cervix

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Abstract

Background

Cervical cancer is the second most common cancer in women worldwide after breast cancer. Cervical cancer is a preventable disease. The implementation of cervical cancer screening programs has greatly decreased the morbidity and mortality, as precancerous lesions and early invasive cervical cancer could be detected and treated effectively. The detection of hTERC gene amplification was suggested as a possible diagnostic marker for use in routine cytological screening.

Objectives

The present study was designed to detect genomic gains of the hTERC and C-MYC genes using FISH technique and to investigate the relationship between genes amplification and the clinical data of the patients.

Patients and Methods

The current study was carried out on twelve cases with cervical cancer at different grades (three cases were grade I, six cases were grade II and three cases were grade III). Interphase FISH analysis using LSI probe, Cervical Cancer probe hTERC (3q26) & C-MYC (8q24), was successfully performed on 12 patients with cancer cervix.

Results

Interphase FISH analysis revealed positive hTERC gene amplification in all cases of cancer cervix (100%). However C-MYC gene amplification was detected in four cases only (33.3%). Statistical analysis of the data revealed significant correlation between hTERC amplification and grading. Also, there was significant correlation between C-MYC amplification and grading and highly significant correlation between C-MYC amplification and hTERC amplification. On the other hand hTERC and C-MYC genes amplification showed an inverse correlation with the ages of the patients.

Conclusion

The present study highlights the importance of using hTERC and C-MYC genes FISH probes for cases with cancer cervix or pre-malignant lesions as a sensitive technique. This method provides an easy and effective applicable approach which helps in the diagnosis and prognosis, as an increased copy number is associated with a more advanced grade that could be detected in the early stages of the disease.

Keywords

Cancer cervix, Telomerase, hTERC amplification

Introduction

Cervical cancer is the second most common cancer in women worldwide, after breast

cancer⁽¹⁾ with 500,000 new cases and 250,000 deaths each year⁽²⁾. It is unique among human cancers because it was the first cancer discovered to be the direct effect of an infectious agent. Numerous epidemiologic and laboratory studies have confirmed a strong causal association between human papilloma virus (HPV) infection

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and the development of premalignant and malignant lesions of the uterine cervix⁽³⁾. The implementation of cervical cancer screening programs has decreased the morbidity and mortality of the disease, as precancerous lesions and early invasive cervical cancer could be detected and treated effectively⁽²⁾.

Telomerase activity has been extensively studied as a diagnostic marker and/or as a prognostic factor in many kinds of malignant tumors. Some studies suggested an association between telomerase activity and disease outcome; others do not find this association⁽⁴⁾. The expression of telomerase may play a crucial role in the development of cervical cancer. It seems that there is a progressive increase of telomerase activity in association with the advanced stage of cancer cervix⁽⁵⁾.

Telomerase activation may be a relatively early event in cervical carcinogenesis, and this activation may be associated with the initiation and progression of cervical lesions. Telomerase activity and expression of its components may serve as biomarkers in diagnosis and prognosis of cervical neoplasias⁽²⁾.

To highlight the importance of human telomerase RNA gene (hTERC) and the C-MYC genes amplification in cancer of the cervix, the present study was designed to detect the presence of genomic gain of the hTERC and C-MYC genes in patients with cervical carcinoma using FISH technique and to correlate the genes amplification with the patient's clinical data.

Patients and methods:

Patients: Twelve patients with cancer cervix participated in this study. The ages of the patients were ranged between 35 to 72 years old. All patients were complaining from vaginal bleeding and cervical masses. The samples were collected from National Cancer Institute, Cairo University and an informed consent was taken from patients. The research was performed according to guidelines of the ethical committees of Al Azhar University Faculty of Medicine, Cairo, Egypt.

All patients were married and multipara, the general condition was mild to moderate in

severity with pallor due to bleeding. Abdominal examination revealed no masses. By inspection the vaginal examination revealed cauliflower masses, the masses were friable and bleed on touch. Also, there was cervical enlargement and limited mobility of the cervix varied in severity.

Methods:

1. Peripheral blood samples were taken for conventional chromosomal study to detect any chromosomal aberrations;
2. Tissue biopsy. From each patient, two biopsies were taken each 1x1 cm. One sample was fixed in 10% formalin and processed for paraffin embedded tissue for histopathological evaluation. The other sample was immersed in RPMI media for tissue culture;
3. Tissue preparations for histopathological study. Slides prepared from paraffin section were stained using two types of dye. Slides stained with haematoxylin and eosin were examined to detect the grades of cancer. Another slide for the same patients was stained with Methyl green pyronin dye to evaluate the optical density of the nucleolus. The slides were analyzed using specific software program for imaging system to evaluate the nucleolus optical density;
4. Tissue preparation for FISH study. Biopsies were transferred into sterile containers with culture medium consisting of 10 ml (RPMI1640) medium and 0.5 ml penicillin and streptomycin. About 1x1 cm of the biopsy was minced into pieces of 2–3 mm. Minced tissue was incubated overnight at 37°C in a culture tube with 5 ml culture medium and 1 ml collagenase solution at a concentration of 0.8%. The cell suspension was subsequently centrifuged at 1000 rpm for 10 min and washed twice in warm culture medium. Then, the pellet was transferred to a 25-cm² filter cap flask with 5 ml (RPMI 1640) culture medium and 0.25 ml penicillin and streptomycin, and 0.5 ml amphotericin B, and 1 ml fetal bovine serum. The flask was incubated for 5 days in CO₂ incubator at 37°C with

humidified air saturated with 5% CO₂. The culture fluid was then transferred to a tissue culture tube, subjected to hypotonic solution (0.05 mmol/l KCl) and fixed in methanol/acetic acid (3/1). Then the pellet from the resulting suspension was dropped onto microscope slides, which were stored at -20°C until applying FISH procedure.

5. Fluorescence in situ hybridization analysis. The fluorescence in situ hybridization (FISH) technique was carried out according to the manufacturer's instructions of Poseidon Kreotech Repeat free cervical cancer probe which designed to detect the amplification (copy number 3 or more are considered amplified) at 3q26 (hTERC) critical region 1 (red); and 8q24 (cMyc) critical region 2 (green). Both sites are potential integration sites for the HPV (human papilloma virus) and have been described to be amplified in early stage cancer cervix⁽⁶⁾. Slides were examined by using an applied imaging system, i.e. an Olympus BX51 microscope (Olympus, Tokyo, Japan) with a fluorescent attachment and equipped with filter sets. For each case 50 interphase nuclei were

studied. Positive amplification if 3 or more signals were detected. Copy numbers were counted for each case.

6. Statistical analysis. The collected data were tabulated and analyzed using the SPSS program.

Results

The detailed patients' data are shown in Table 1.

I - Histopathological results:

1. Results of H&E stain. Examination of specimens of cancer cervix patients revealed the presence of three grades I, II and III.

Grade I (Fig. 1): Three specimens out of twelve were diagnosed as a well differentiated non keratinizing squamous cell carcinoma grade I (GI). The patients were 35, 40 and 45 years old with positive family history. One case in this group revealed patchy areas of CIN III (carcinoma in situ) in between GI. These patches showed epithelial changes without basement membrane invasion.

The invasive parts of GI showed invasion of basement membrane by these abnormal epithelial

Cases No.	Range of hTERC amplification	Mean of hTERC amplification	%of cells with hTERC amplification	C-MYC amplification	Grading	Age	Family history	Optical density mean of methyl green pyronin stain
1	3-8	4.5	80%	-ve	GI	45 years	+ve	0.235
2	3-13	5	61%	+ve	GI	35 years	+ve	0.348
3	3-7	4	68%	-ve	GI	40 years	+ve	0.213
4	3-7	4	52%	-ve	GII	37 years	+ve	0.214
5	3-5	3	58%	-ve	GII	56 years	-ve	0.256
6	3-10	4	69%	-ve	GII	72 years	-ve	0.279
7	3-4	3	41%	-ve	GII	60 years	-ve	0.246
8	3-6	3	60%	-ve	GII	51 years	+ve	0.277
9	3-6	3	55%	-ve	GII	56 years	-ve	0.200
10	3-12	5	70%	+ve	GIII	54 years	-ve	0.308
11	3-20	6.8	60%	+ve	GIII	42 years	+ve	0.354
12	3-16	6	65%	+ve	GIII	48 years	-ve	0.341
Control cases	-	-	-	-	-	45 years mean	-	0.200 mean

Table 1 : The detailed data of the cases

N.B Mean of amplification = total signals in normal+amplification/no. of examined cells

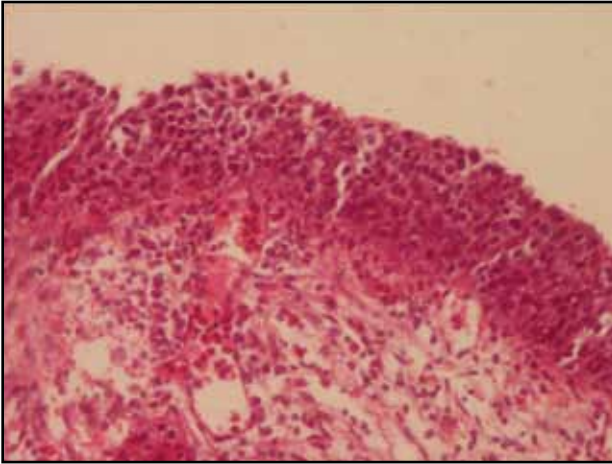


Fig. 1: A photomicrograph of a section showing grade I carcinoma. Notice the pleomorphic epithelial cells which are irregularly arranged. Notice the mitotic figures, the invasion of the B.M and the presence of these epithelial cells in the C.T. stroma. (H & E x 400).

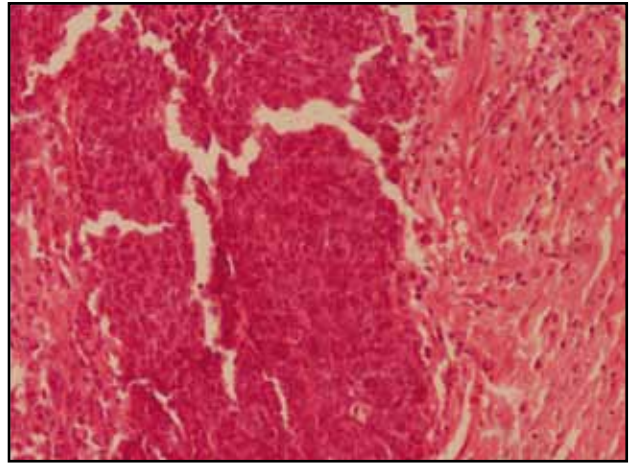


Fig. 3: A photomicrograph of a section in a cervix showing poorly differentiated squamous cell carcinoma GIII. Notice the overcrowded pleomorphic cells with many mitotic figures. No evidence of keratin. (H&E x400)

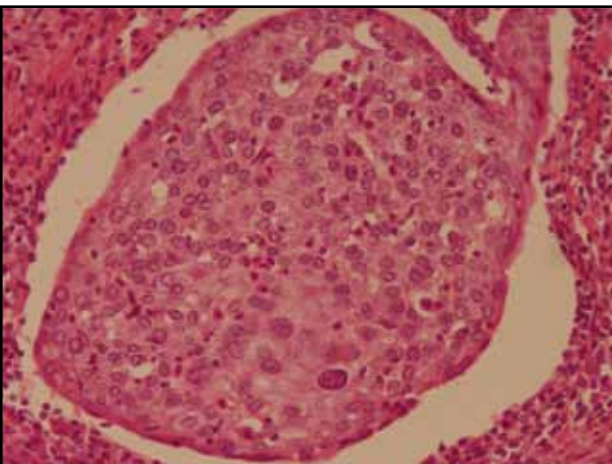


Fig. 2: A photomicrograph of moderately differentiated non keratinizing squamous cell carcinoma of human cervix GII showing abnormal neoplastic cells forming patches or sheets invading cervical stroma which is infiltrated by many rounded cells. (H & E x 200)

cells which were also scattered between stromal C.T forming sheets or units of cells invading the stroma. The nuclei of these abnormal epithelial cells were large, irregular and hyperchromatic.

Grade II (Fig. 2): Six specimens out of twelve were diagnosed as squamous cell carcinoma of the cervix grade II (GII). This group included:

- a) Moderately differentiated keratinizing squamous cell carcinoma GII.
- b) Moderately differentiated non keratinizing squamous cell carcinoma GII

Grade III (Fig. 3): This group includes three cases and were diagnosed as cervical carcinoma grade III (GIII). This group was divided into two subgroups:

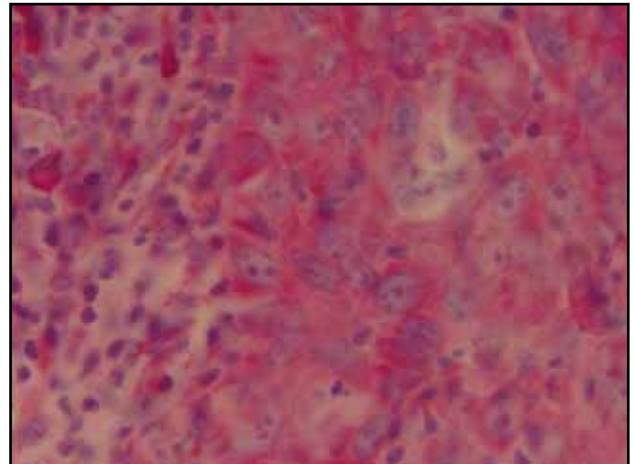


Fig. 4: A photomicrograph of a section in the human cervix showing the cytoplasm of cervical cells is rose to red, the nuclei are stained blue while the nucleoli are stained rose. Notice some nuclei contain double nucleoli. (Methyl green pyronin x 400)

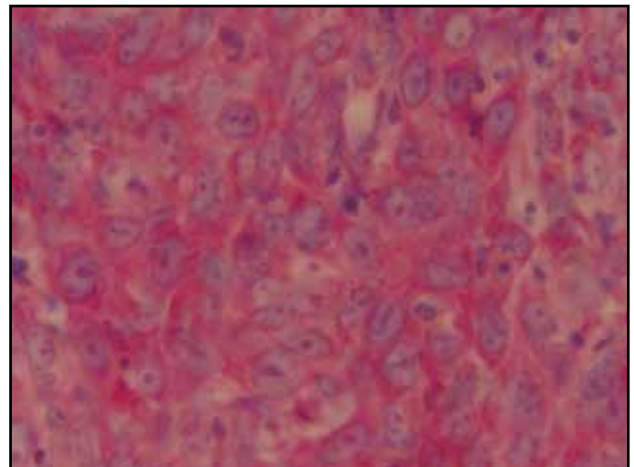


Fig. 5: A photomicrograph of a section in the human cervix showing the cytoplasm of cervical cells is rose to red, the nuclei are stained blue while the nucleoli are stained rose. Notice some nuclei contain more than 2 nucleoli (Methyl green pyronin x 400)

- a) Poorly differentiated squamous cell carcinoma GIII. This subgroup showed more atypia of the epithelial cells than the previous grades.
- b) Invasive adenocarcinoma of the cervix GIII. On examination of sections from this subgroup, there was loss of glandular pattern. The glands were ill-formed and had no basement membrane. The cells showed atypia with pleomorphic, bizarre shaped nuclei.

2. Results of methyl green pyronin stain (Fig. 4, 5). Examination of sections (control and cancer cervix) stained by methyl green pyronin stain showed that the color of the cytoplasm of cervical cells is rose to red and the nuclei are stained blue while the nucleoli are stained rose. Some nuclei contain double nucleoli. The optical density of the nucleolus was measured using image analyzer system. The results are shown in Table I.

II - Cytogenetic studies results.

1. Conventional cytogenetic analysis. Conventional cytogenetic analysis of blood samples using G-banding technique was performed for all cases yielded successful cultures with well spread metaphases in all cases. The G-banding technique was performed to detect any chromosomal instability that might be associated with patients with cancer. Karyotyping of all cases showed normal female karyotype 46, XX chromosomes, except case No.11 which showed 46,XX with few chromosomal breakage 3breaks/25 metaphase (translocation 7;14) and



Fig. 6: Part of metaphase spread shows breakage of chromosome at (10q22)

breakage involving chromosome 10 and 7 (Fig. 6, 7a, b);

2 - FISH study. After successful tissue culture (Fig. 8), all biopsies were studied at interphase nuclei by FISH using cervical cancer probe hTERT (3q26) & C-MYC (8q24) for detection of hTERT and C-MYC genes amplifications. FISH



Fig. 7 (a) : Metaphase spread and shows 46,XX,t(7,14) (p10;q10)

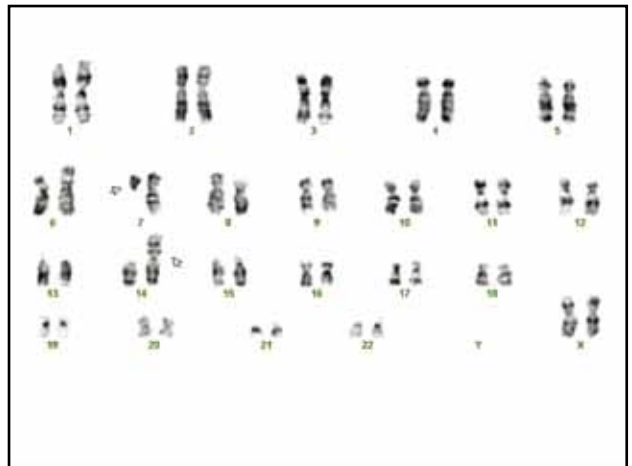


Fig. 7 (b): Karyotype shows 46,XX,t(7,14) (p10;q10)

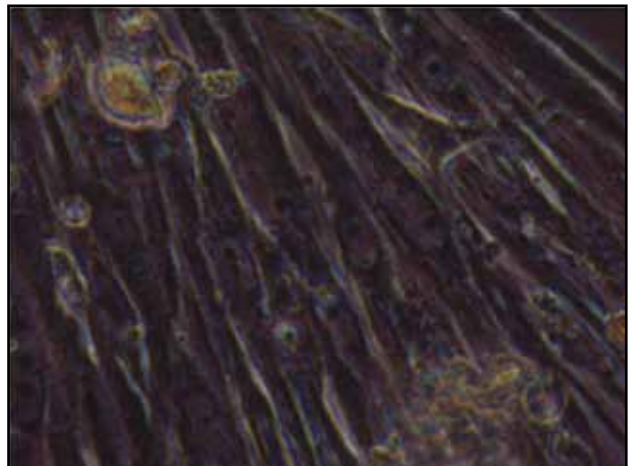


Fig. 8: Phase contrast of cultured cancer cervix cells at day 10 of tissue culture

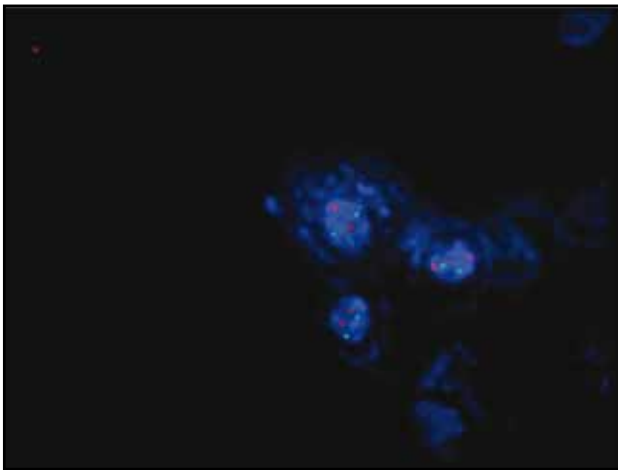


Fig. 9: Interphase FISH analysis showing negative amplification of hTERT and c-Myc genes (two red and two green signals in each cells) TERC gene at chromosome 3q26 (red), c-Myc gene at 8q24 (green), and DAPI nuclear staining (blue).

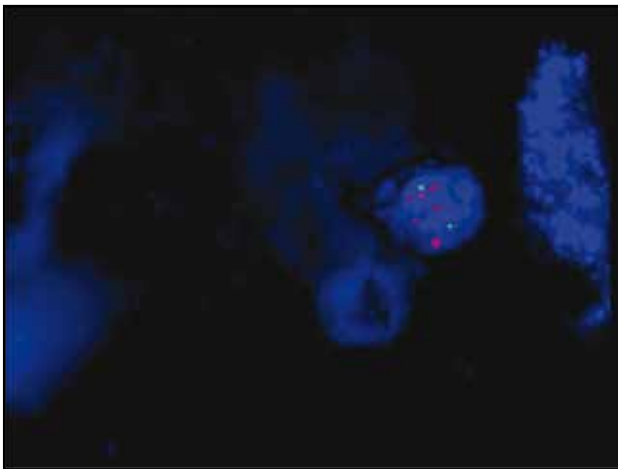


Fig. 10: Interphase FISH analysis in a case of cancer cervix shows hTERT gene amplification (6 red signals) and two green signals (No c-myc amplification).

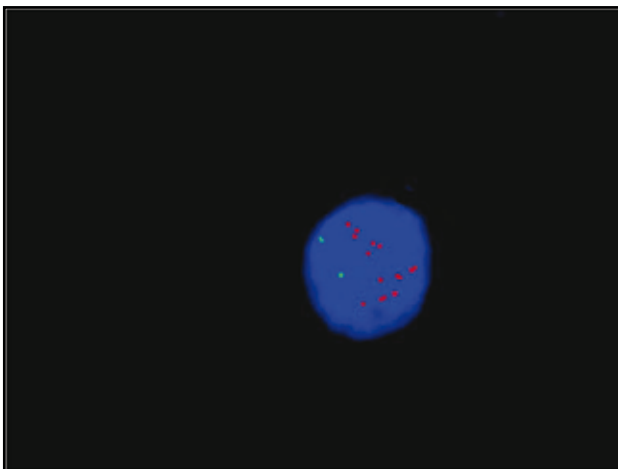


Fig. 11: Interphase FISH analysis shows hTERT gene amplification and no c-Myc amplification.

study was successfully performed on the tissue biopsy of all cases. The patient was considered to have positive gene amplification if more than 5% of cells showed genes amplification according to the manufacture quality control. However, all patients showed genes amplification in more than 50% of their cells except one case (case no. 7);

The interphase FISH analysis revealed positive amplification of hTERT gene in all cases denoted by the presence of more than two red signals (Fig. 9-12). Four cases only showed C-MYC gene amplification denoted by the presence of three green signals (Fig. 13).

The patients were classified into 3 groups according to the grades (G1, G2, and G3). The mean hTERT amplification in GI (3 cases) was found to be 4.5 ± 0.5 , the mean of hTERT amplification in GII (6 cases) was 3.33 ± 0.5 and the mean of hTERT gene amplification in GIII (3 cases) was 5.93 ± 0.9 . The statistical analysis revealed that GIII showed the highest value with marked significant difference when compared with GII and GI ($P < 0.001$ and 0.05) respectively, followed by GI which showed significant difference when compared with GII (Table II).

Positive correlation was found between hTERT amplification and c-Myc amplification ($P < 0.001$) and an inverse correlation was found between the genes amplification (hTERT and cMYC) and the age of the patients ($r = 0.42$ and -0.33) respectively ($P < 0.05$) using correlation coefficient “r” test. On the other hand, there was no correlation between hTERT amplification or grades and +ve family history ($P > 0.05$)

The mean values of optical density in GI (3 cases) and in GII (6 cases) were found to be 0.27 ± 0.7 and 0.25 ± 0.03 respectively, without statistical difference between the 2 groups ($P > 0.05$) while the mean value of optical density in GIII was 0.33 ± 0.02 and had a very high statistical significant difference ($P < 0.001$) when compared to the values of GI and GII. Also, there was very high significant correlation between the optical density and genes amplification (hTERT and C-MYC) ($P < 0.001$).

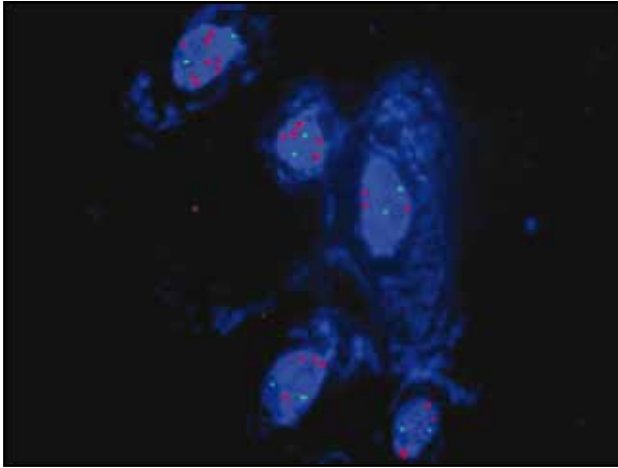


Fig. 12: Interphase FISH analysis shows hTERT gene amplification and No c-myc amplification.

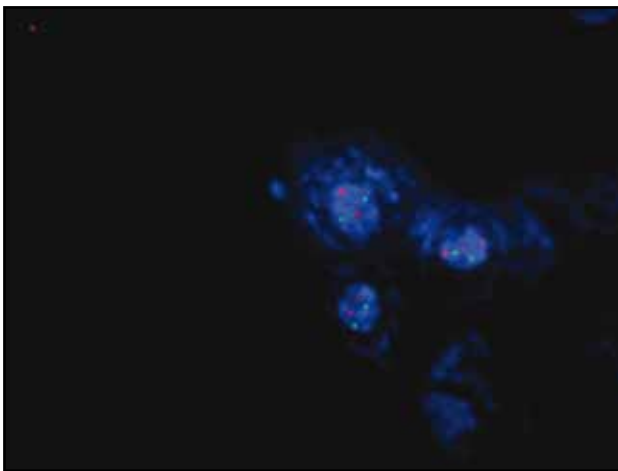


Fig. 13: Interphase FISH analysis shows hTERT gene amplification (6 red signals) and c-Myc gene amplification (3 green signals).

Discussion

The current study was carried out on twelve cases with cervical cancer at different grades (three cases were grade I, six cases were grade II and three cases were grade III). Interphase FISH analysis using LSI probe, Cervical Cancer probe hTERT (3q26) and C-MYC (8q24), were successfully performed on 12 patients with cancer cervix.

The interphase FISH analysis revealed positive hTERT gene amplification in all cases of cancer cervix (100%). The number of hTERT copies ranged from 3-20 copies. On the other hand, C-MYC gene amplification was detected in only four cases (33.3%) with 3 copies of the C-MYC gene.

In the present study, there was statistical significant correlation between hTERT amplification and grading. Although the case numbers are limited, statistical analysis suggests a strong correlation between the presence of extra copies of hTERT gene and disease progression (grading). This finding is in acceptance with a study done by Tu et al., (2009) and Li et al (2009) Fan et al (2010)^(2,7,8). They found that the rate of hTERT amplification increased as the severity of cervical disease increased. Also, another study was done by Sharma et al., (2007)⁽⁹⁾, supported our findings. This study found that patients with cervical cancer at early stages showed 68% positive telomerase activity and those at later stages showed 92% positive telomerase activity. These findings suggest that telomerase activation is a relatively early event in cervical carcinogenesis and correlates with the grade of cervical lesion and clinical staging. On the other hand only few studies have found any correlation between increased telomerase activity and histopathological changes and staging of the disease. ^(10, 11)

Also, our findings are in accordance with study done by Hopman et. al., (2006) and Sui (2009)^(6, 12). These studies found that there was increase in chromosome 3q copy number (i.e. amplification in telomerase gene TERC at 3q26 detected by FISH) with cervical carcinoma.

The current study included one case of GIII cervical adenocarcinoma, this case showed highest range of hTERT amplification 3-20 with a mean 6.8. This finding was in agreement with Anderson et al., (2006)⁽¹³⁾ who reported hTERT gene amplification in primary cervical adenocarcinomas which confirmed that the amplification of the human telomerase gene (hTERT) is a consistent cytogenetics abnormality in cervical adenocarcinomas. Therefore, application of this probe set may provide an objective genetic test for the assessment of glandular cells in Pap smears and hence for the diagnosis of cervical adenocarcinomas.

The comparison between grade I, grade II and grade III in relation to hTERT gene amplification, revealed unexpected results, as GIII showed marked increase in the hTERT

gene amplification then GI and finally GII revealed the lowest values of gene amplification. The presence of marked increased level in GIII is in acceptance with the previous reports in the literature⁽¹⁴⁾ However, the increased level of gene amplification in GI over GII needed further explanation. It could be attributed to many factors concerning the patients for instance, the young age of the patients in this group. As it was revealed by the statistical analysis there is an inverse correlation between the age of the patient and the telomerase amplification. Also positive family history was noticed in all patients in GI. So we can conclude that the positive family history and the young ages of the patients could be contributing factors for the increased hTERC gene amplification in these patients. However, there was no consistence pattern for the relation between the positive family history and the level of hTERC gene amplification and this might be attributed to the small studied sample. In contrary to our results there was no correlation between the age of the patients and telomerase activity in another study.⁽¹⁰⁾

Regarding C-MYC amplification, there was significant correlation between C-MYC amplification and grading. Also, there was a high significant correlation between C-MYC amplification and hTERC amplification. As the C-MYC gene directly activates telomerase expression by binding to the telomerase promoter, so our findings support the role of C-MYC gene for the telomerase amplification. As far as our knowledge this finding was not reported before as regards to cervical carcinoma. However, it was reported once in a study that was conducted on patients with breast cancer.⁽¹⁵⁾

It is worthy to mention that telomerase activity was present not only in cervical tumor lesions but also in pre-malignant lesions. Telomerase activity was found to be significantly higher in patients with CINs than in patients with cervicitis. Moreover, there were significant correlations between the severity of cervical lesions and the signal intensity of telomerase activity.⁽¹⁶⁾

In addition to the hTERC gene evaluation by FISH study, the optical density of the nucleolus has been investigated by staining the

nucleolus with methyl green pyronin stain. As it was reported before the morphology of the nucleolus is highly variable depending on cell activity. The pathologists have realized that hypertrophy of the nucleolus is one of the most consistent cytological features of cancer cells.⁽¹⁷⁾ In the present study, there was a high significant correlation between the optical density and hTERC and C-MYC gene amplification. This finding indicated that the RNA content of the nucleolus increased due to genes amplification that led to increased gene expression which finally will increase the enzymatic activity in the cells. So, direct visualization of the nucleolus by the imaging system could give an idea about the RNA component of the cells either increased by gene amplification or other factors such as polyploidy.

Conclusion

There was a significant correlation between hTERC amplification and the grade of the cancer in which there is an increase in the gene amplification along with the progression of the disease. As it was reported before, gene amplification is responsible for carcinogenesis even before the development of any morphological changes in the tissue.⁽¹⁸⁾ Therefore, the presence of the hTERC gene amplification could serve as a prognostic and a diagnostic indicator in patients with cancer cervix even in the very early stage and grade of the disease as it was recommended by previous study.⁽⁸⁾ From the current study, we can conclude that telomerase gene amplification and positive family history could be contributing factors for the development of cancer cervix in the young age. Applying FISH technique on cervical preparations may be an adjunct method to cytology screening for early detection of cervix neoplasm, and may determine the progressive potential of individual lesions especially in high-risk group that would lead to early detection of the disease, minimize the complications and offer a better response to the treatment.

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