Proto-oncogene c-myc in uterine cervix carcinogenesis*

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The biological behaviour of precancerous lesions and early stages of uterine cervix carcinoma is not always easily predictable. It is important therefore to identify new biological markers which could more reliably predict the evolution of the disease or provide important therapeutic targets. To establish the role of the proto-oncogene c-myc in uterine cervix tumorigenesis, we examined 96 tissue samples of different degrees of cervical intraepithelial neoplasia (CIN1-CIN 3), *in situ* (CIS) or invasive squamous cell carcinoma (ISCC) and control cases. Indirect immunohistochemical techniques were used to detect the c-myc expression. Significantly higher levels of Myc protein were found in keratinocytes of high-grade dysplasias in comparison to low-grade dysplasias and control cases. There was no difference between low-grade CIN and a control group of patients. The same significant changes between above mentioned groups were seen in surrounding stromal cells (fibrocytes, fibroblasts, some endothelial cells and lymphocytes). We confirm that expression of c-Myc protein is increased not only in uterine cervix cancer but also in the premalignant lesions. Problem for discussion seems to be whether increased Myc expression in stromal cells might create a more tumor promoting microenvironment which may support the growth and proliferation of transformed cells.

Key words: carcinogenesis, cervical neoplasias, keratinocytes, stromal cells, c-myc

Carcinogenesis is known to involve the aberrant expression of genes involved in cell proliferation and differentiation. In mammalian cells, several independent lines of evidence have implicated the proto-oncogene c-myc in the control of cell proliferation and entry into the cell cycle [17, 20]. This gene was discovered as a cellular homologue of the transforming oncogene of avian viruses [5, 32] and its product was subsequently found to be activated in various human cancers, including lung, breast and colon [2, 29]. The theory that c-myc acts as a central oncogenic switch in human cancers has been demonstrated by the ability of the oncogenic viral myc gene to induce rapid development of a variety of tumors in infected chickens [8]. The frequency of genetic alteration of c-myc in human cancers is high, and it has been estimated that it contributes to an one-seventh of U.S. cancer deaths [8]. The c-myc gene belongs to the myc family that includes B-myc, L-myc, N-myc and s-myc. However, only c-myc, L-myc and N-myc have neoplastic potential [20]. The product of c-myc is a protein which is usually associated with the nucleus [14]. However, immunohistochemical studies of Myc protein expression have revealed apparent differences in its intracellular distribution and have also described it's unexpected cytoplasmic locations [30, 41, 48]. Cells that express Myc fail to arrest in response to growth factor deprivation [16] and deletion of c-myc inhibits cell proliferation and leads to accumulation of cells in the G1 phase of the cell cycle [24]. Homozygous deletions of c-myc gene are even lethal during early embryogenesis [9]. Certainly, physiological expression of the gene is not sufficient for oncogenesis, because c-myc is also transcribed in proliferating normal cells [13]. Its level controls differentiation and maturation depending on stages of the cell cycle and tissue type [50]. For example, it is well known that Myc is absent or present at very low levels in resting peripheral blood lymphocytes. On the other hand, highly proliferative cells as well as tissues with high cellular turnover demonstrate increased positive staining [30]. Abnormalities involving amplification, translocation and deregu-lation of the protooncogene expression leading to constitutive or de-

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regulated expression of c-myc that becomes independent on external signals contribute to oncogenesis [19, 27]. The ability of Myc to promote cell proliferation indicates that its deregulation leads to deregulated DNA synthesis and genomic instability [7, 31]. Deregulated Myc expression is linked to increase cyclin A and increased cyclin E levels [18, 23, 35]. The expression of Myc also correlates with decrease in the level and the function of p27 Kip1 CDK inhibitor [28, 39, 42, 46]. Increased expression of c-Myc plays also an important role in the pathogenesis of cervical cancer. Amplification and/or overexpression of c-myc gene were frequently found in advanced stages of cervical cancers and were shown to be associated with tumor progression [26, 33, 36, 45] and with aggressive, poorly differentiated phenotype. Moreover, Myc overexpression was related to a 6.1 times higher risk of distant metastases suggesting that activation of the proto-oncogene may lead to the metastatic ability of tumor cells [40]. A different distribution of Myc expression has been reported in premalignant CIN lesions. While several studies have demonstrated that higher Myc expression was positively related to all stages of pre-cancerous lesions [34] others have observed higher levels of this proto-oncogene only in CIN 3 [11, 12].

The aim of this study was to analyse tissue and cellular distribution of Myc protein in our cohort of cervical lesions to establish at which stage of neoplastic lesion is c-Myc activated.

Material and methods

Tissue samples from 86 patients with diagnosis of cervical intraepithelial neoplasias (16 CIN1, 26 CIN 2, 17 CIN 3), 27 *in situ* carcinomas (CIS) or invasive squamous cell carcinomas (ISCC) and 10 control cases of normal cervical mucosa were studied. The specimens were obtained by punch biopsy, cervical conization, amputation or total hysterectomy. Blocks of tissues were fixed in formalin and embedded in paraffin using routine methods. Indirect immunohistochemistry was performed on 5-μm sections. Endogenous peroxidase was inhibited using 0.5 per cent hydrogen peroxide. Primary mouse anti Myc monoclonal antibody, clone 9E11, IgG2 (Novocastra) was applied in a dilution of 1:150 and incubation was performed overnight at 10 °C temperature. Secondary biotinylated anti mouse antibody and the streptavidin/horse-radish peroxidase conjugate and/or EnVision detection system (Dako) were applied for detection. Peroxidase activity was developed and visualised by 0.5% H_2O_2 and 3,3'-diaminobensidine (DAB). As positive controls sections of Myc positive non-small lung carcinoma specimens were used. For negative controls, the samples were taken through the procedure with omission of primary antibody. Evaluation of Myc expression was based on assessment of the intensity of staining and the amount of positive keratinocytes and stromal cells in areas with the highest dysplastic changes and the adjacent stroma in an approximate field of 0.5 mm^2. The degree of intensity was estimated as negative (no immunostaining), weak (definitive but weak staining) or strong (intensive staining easily seen under low power magnification). Cases with weak but definitive staining were count as positive and included to analysis. The quantity of positive cells was evaluated on a semi-quantitative four-grade scale (0 = 0–5%, 1 = 5–25%, 2 = 25–50%, 3 = 50–100%). Pearson's chi-square test was used to examine associations of Myc expression both in keratinocytes and stromal cells with a grade of lesions.

Results

Immunohistochemical results showed that Myc protein was regularly expressed in basal keratinocytes of normal squamous cervical epithelium (Fig. 1) and columnar endocervical epithelium (Fig. 2). Granular cytoplasmic staining of the oncogene product with distribution differing from perinuclear staining, observed predominantly in keratinocytes to diffuse cytoplasmic staining was detected in columnar endocervical epithelium and epithelium of the endocervical glands. In pre-malignant and malignant epithelium, mainly perinuclear, but also diffuse cytoplasmic staining was observed. There was an evident increase of Myc expression in high-grade CIN and in cancers (Fig. 3), where 84% CIN 2, 76% CIN 3, and 96% CIS and ISCC (Fig. 4) were formed by c-Myc positive cells (Fig. 6). On the other hand the majority of CIN 1 cases (71%) were negative (except the basal cell layer). The differences between high-grade and low-grade CIN lesions (p=0.006) and low grade lesions and CIS/ISCC (p=0.0004) were statistically significant. No significant differences were found between groups CIN 2 and CIN 3. We failed to find any significant differences between low-grade CIN and the control group of samples. On the other hand we demonstrated a significant correlation between Myc expression in stromal cells and the stage of the lesion (p=0.0001) (Fig. 7). We observed very intensive Myc staining in fibrocytes, fibroblasts, some endothelial cells and peripheral blood lymphocytes surrounding the lesions in the groups of CIN 2, CIN 3 and carcinomas (Fig. 5). As in advanced dysplasias and carcinomas unequivocally preponderate cells with moderate or very intensive staining, our evaluation in which weakly stained cases were count as Myc positive and included to analysis does not affect obtained significant statistical results.

Discussion

Presented study concern evaluation of Myc protein expression in a range of tissues obtained from normal, dysplas-

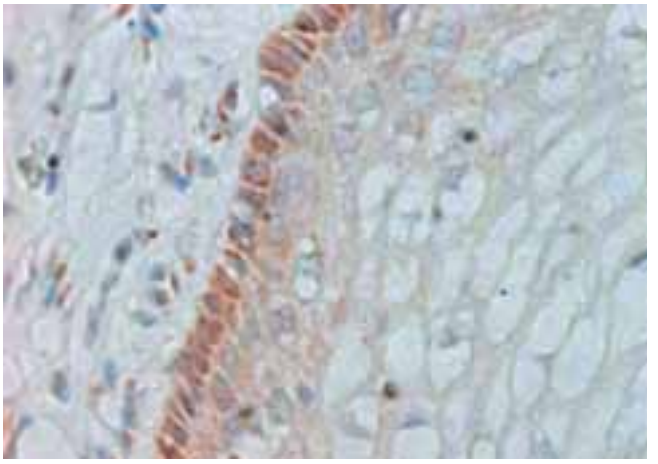

Figure l. c-Myc oncoprotein was regularly expressed in the basal cells of normal squamous cervical epithelium. Original magnification x 400.

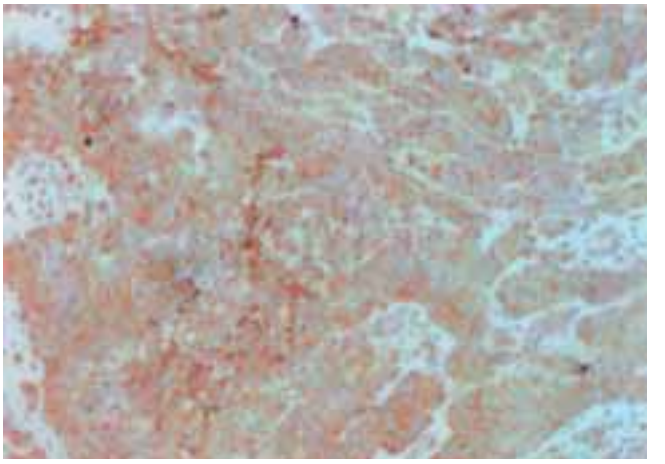

Figure 4. Most of the carcinomas were c-Myc positive with predominantly diffuse cytoplasmic staining. Original magnification x 100.

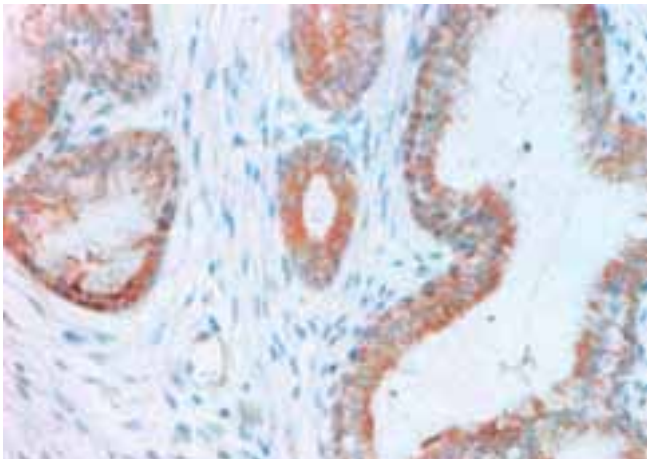

Figure 2. Cytoplasmic c-Myc expression was found in columnar endocervical epithelium and epithelium of endocervical glands. Surrounding normal stroma is negative. Original magnification x 200.

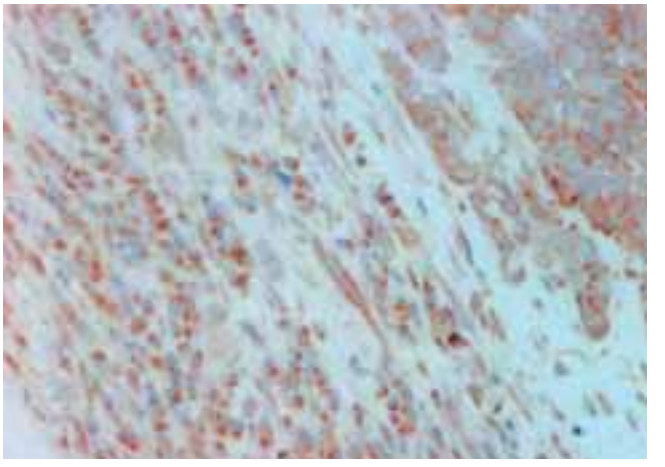

Figure 5. Intensive c-Myc staining in fibrocytes, fibroblasts and peripheral blood lymphocytes surrounding the neoplasis lesion. Original magnification x 200.

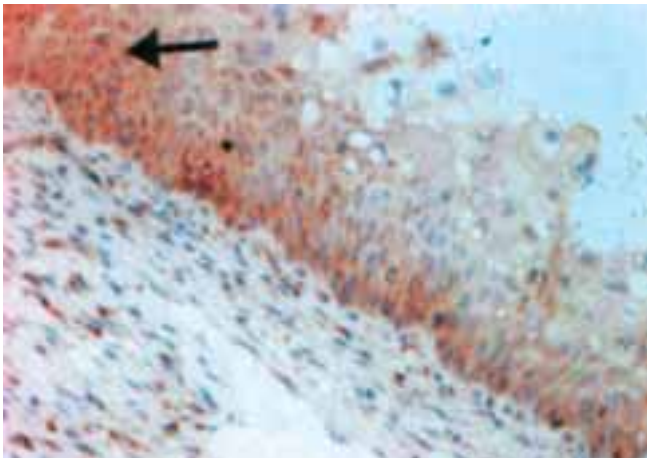

Figure 3. High grade dysplastic lesions (arrow) exhibited intensive granular perinuclear or cytoplasmic c-Myc staining. Original magnification x 200.

tic, and neoplastic conditions of the cervical mucosa in an attempt to elucidate the role of the oncoprotein in uterine cervix carcinogenesis. Myc protein was widely distributed in different tissues and is predominantly localized in nuclei of cells [15] where its positivity is related to proliferation rate. Cytoplasmic staining has been identified less frequently in convoluted renal tubules, hepatocytes or salivary glands [1, 30]. Several studies using paraffin-embedded formalin fixed material have reported a predominantly cytoplasmic intracellular distribution [21, 22] and it has been questioned whether fixation procedures can affect the results of immunostaining. Even elution of Myc from nuclei in relation to fixation procedures might lead to altered distribution. These facts might explain either the perinuclear or the cytoplasmic distribution which we have demonstrated in our study. However we should not exclude the possibility of altered distribution of the oncoprotein associated with neo-

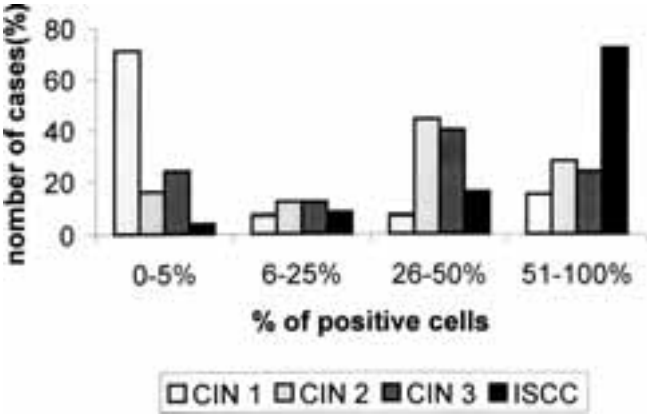

Figure 6. Relationship between c-Myc expression in cervical keratinocytes and the diagnosis.

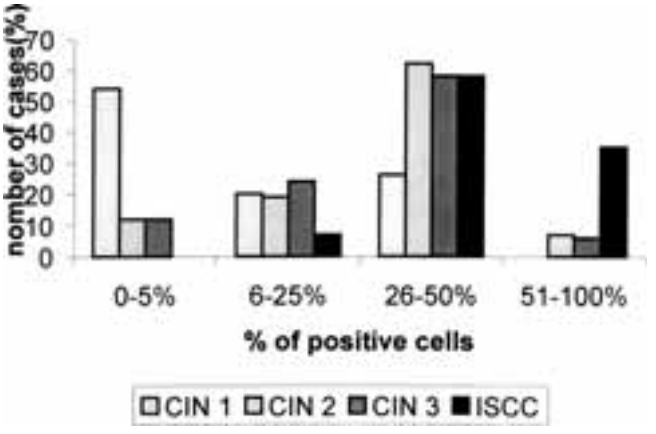

Figure 7. Relationship between c-Myc expression in mucosal stromal cells and the diagnosis.

plastic changes within pathological epithelium [41]. Several mechanisms might be proposed to explain this phenomenon. Apart from the altered c-myc structure including structural alteration of mRNA or protein and changes induced by phosphorylation, even alterations in the structure of the cytoplasmic binding factors may be involved. For these reasons mutation in the c-myc gene affecting the nuclear targeting sequence, the cytoplasmic binding factor sequence, or any of the phosphorylation sites could lead to accumulation of the protein in the cytoplasm. Alternative splicing of mRNA has been for example also demonstrated for L-myc in tumor cells [10]. The question, whether dystopic location of Myc protein has a specific role remains open. As transcription of the c-myc gene is considered to be regulated by nuclear Myc protein in a feed-back manner [38] then defects in its nuclear transport might be accounted for characteristic overexpression of the c-myc product. Keratinocytes are cells that normally lack the Myc protein. Sustained activation of the oncoprotein results in uncontrolled cell proliferation that is concomitant to loss of terminal differentiation. The deregulated Myc protein expression in cervical epithelium may be an initial stage in progression of the lesion through dysplasias towards carcinoma. This event can occur very early in tumorigenesis and may vary at different degrees of the pre-malignant stages. In our study significantly increased expression was found not only in squamous cell carcinomas but also in high-grade CIN lesions. We failed to observe higher levels of the protein in low-grade dysplasias. However, increased c-Myc level is not restricted only to dysplastic and neoplastic keratinocytes, it is constantly observed in normal mucosa in the highly proliferating zones, and even the protein content may be far higher than that found in the tumor cells [41, 48].

Currently little is understood about the biology of reactive stroma in cervical cancer. From this point of view the most interesting finding was the increase of Myc expression in stromal cells such as fibrocytes, fibroblasts and some lymphocytes in the neighbourhood of high-grade dysplastic lesions and carcinomas. Our results are in agreement with the newly recognized important function of the oncogene c-myc to induce changes in the microenvironment of neoplastic lesions [6, 44]. There are studies indicating that fibrocytes and fibroblasts from cancer patients display certain behavioral abnormalities characteristic for transformed and/or foetal cells [43] expressing growth factors and proto-oncogenes [25, 49]. Also a different fibroblastic immunophenotype has been detected and this may result in alteration of the host response to tissue damage [3]. It has been demonstrated that this new reactive stroma microenvironment even enhances tumorigenesis by supporting cancer cell survival, proliferation and migration as well as by induction of angiogenesis [47]. Hence, for example, deregulated expression the c-myc oncogene in tumor lesions is associated with a loss of expression of the intercellular adhesion molecule E cadherin, which is a prerequisite for loss of cell-cell contacts and for initiation of invasion [37]. Furthermore, protein Myc has been shown to be a key regulator of angiogenesis. Marked angiogenesis following even shortly after Myc activation has been described [37]. On the other hand, tumor size and vascularization were drastically reduced in Scid mice exhibitting loss of c-myc [4].

In conclusion, our study confirmed that Myc protein seems to have critical importance in uterine cervix carcinogenesis. We have demonstrated that not only overexpression and/or dystopic location of the oncogene accompany the development of malignant lesions, but also an increased Myc expression in stromal compartment cells might create a more tumor promoting microenvironment. These changes can be recognized even in premalignant lesions. Since the reactive stroma may have a role in early uterine cervix carcinogenesis, a therapeutic approach towards altering tumor promoting microenvironment might be an appropriate tool for modulation of disease development.

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