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Original Paper

Proto-oncogenes and p53 Protein Expression in Normal Cervical Stratified Squamous Epithelium and Cervical Intra-epithelial Neoplasia

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The aim of this study was to study the protein expression of six proto-oncogenes (epidermal growth factor receptor (EGFR), c-*fms*, c-*myc*, c-*kit*, c-*erb*B-2 and pan-ras) and one tumour suppressor gene (*TP53*), by immunohistochemical staining of normal cervical stratified squamous epithelium and cervical intra-epithelial neoplasia (CIN). Paraffin sections of 45 normal cervical specimens, 38 CIN grade one (CIN1), 37 CIN2 and 43 CIN3 were studied. An immunohistochemical (IHC) score was derived from the intensity of staining and the percentages of cells stained. In normal cervical specimens, a higher IHC score was found with EGFR and c-fms in superficial (S), intermediate (I) and parabasal (PB) cells compared with basal cells. In contrast, a higher IHC score was found with c-erbB-2 in basal cells in normal cervical specimens. Dysplastic cells in CIN had a higher IHC score with c-myc and c-erbB-2 than normal S/I and PB cells. Dysplastic cells had a higher score with EGFR than normal basal cells. However, a higher IHC score with EGFR and c-fms was found in normal S/I cells than dysplastic cells. These findings suggested that EGFR and c-*fms* were activated in more differentiated normal cells but were less active in less differentiated normal basal cells. However, EGFR was reactivated in dysplastic cells. Meanwhile, c-*erb*B-2 was activated in less differentiated normal basal cells and dysplastic cells, and was less active in differentiated normal cells. c-*myc* was activated in dysplastic cells. c-*fms* was more active in more differentiated normal cells and was not activated in less differentiated or dysplastic cells. c-*kit*, *pan-ras* and *TP53* were not activated in normal nor dysplastic cervical cells. These results suggest EGFR, c-*erb*B-2 and c-*myc* may be important proto-oncogenes in CIN and that antibodies or anti-genes targeted against them may alter the progress of CIN to invasive cancer.

Key words: ***TP53*, EGFR, c-*fms*, c-*myc*, c-*kit*, c-*erb*B-2, pan-ras, normal cervix, CIN**
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INTRODUCTION

PROTO-ONCOGENES OR tumour suppressor genes may be activated at different stages of differentiation of normal tissues or during the process of neoplasia. Cervical stratified squamous epithelium is composed of three layers of cells which differ in their differentiation status. The basal cells are the most undifferentiated type which mature through parabasal (PB) cells, intermediate (I) cells and finally superficial (S) cells. Dysplastic cells are atypical cells with nuclear enlargement and pleomorphism, dispersion of nuclear chromatin and the morphology of the cells varies according to the degree of cytoplasmic maturation. In cervix, three grades of dysplasia are defined, namely cervical intra-epithelial neoplasia (CIN) grades 1–3, which represent a progressive degree of neoplasia. Indeed, if untreated CIN3 may progress to invasive cervical cancer [1].

Epidermal growth factor receptor (EGFR), c-erbB-2, c-fms and c-kit are all members of the tyrosine kinase receptor family. EGFR is increased in cervical cancer and is prognostically significant [2]. In contrast, although 12–77% of cervical cancer is c-*erb*B-2 positive, this proto-oncogene is not prognostically significant [3, 4]. c-fms is highly expressed in ovarian cancer but has not been studied in the cervix. c-kit

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was expressed in a few gynaecological cancers [5], but again has not been reported in the cervix.

Ras is important in the transformation of human papilloma virus (HPV) immortalised cervical cell lines. Furthermore, dysregulation of *ras* occurs early in breast and colon cancer. In cervical cancer, overexpression of ras is detected in 80% of the late stage cervical cancers [6], and low-grade CIN, in which ras is overexpressed, is more likely to progress [7]. The nuclear protein c-myc plays an important role in the regulation of cell growth. Amplification or overexpression of c-myc is found in 32–44% of cervical cancer [8, 9], more commonly in CIN3, than the lower grade CINs [10]. Abnormal expression of *TP53*, a tumour suppressor gene, is common in cancers. Positive immunohistochemical (IHC) detection of p53 is found in 20–60% of cervical cancer [11, 12]. In order to help improve our understanding of the pathogenesis of cervical neoplasia, a comparison of the protein expression of these six proto-oncogenes and one tumour suppressor gene in normal cervix and CIN was undertaken in this study using an immunohistochemical staining method.

MATERIALS AND METHODS

Paraffin blocks of 45 normal cervical biopsies, 38 CIN1, 37 CIN2 and 43 CIN3 biopsies were retrieved for immunohistochemical staining. All samples were reviewed to confirm the diagnosis. Normal cervical samples showed mainly ectocervical epithelium with only occasional inclusion of the immature metaplastic epithelium. CIN was graded morphologically using the criteria described in the literature [13, 14]. In normal cervical stratified squamous epithelium, positive staining was assessed in the different cell layers, namely superficial and intermediate (S/I), parabasal (PB) and basal cell layers. For CIN lesions, apart from the normal cells which lie adjacent to the CIN, cells showing dysplastic features in the CIN were assessed. The majority of the normal epithelium adjacent to the CIN was mature stratified squamous epithelium. Finally, the stromal tissue was assessed to determine the degree of background staining.

Immunohistochemical staining

Paraffin sections of 4 μm thick were dewaxed and rehydrated in preparation for immunohistochemical staining. Endogenous peroxidase was blocked using 3% hydrogen peroxide in methanol. The sections were pretreated with 0.36% urea and processed in a microwave oven for 5 min for antigen retrieval. Immunohistochemical studies were performed according to the instructions provided by the manufacturer of the ABC immunoperoxidase kit which included a secondary biotinylated horse anti-mouse or anti-rabbit Ab (Vector Laboratories, Burlingame, California, U.S.A.). Antibodies were diluted in 10% fetal calf serum in phosphate buffered saline (PBS). Monoclonal antibody for p53 protein, DO7 (Novocastra Lab., Newcastle, U.K.) was used at a dilution of 1/50. p21 pan-ras antibody, Ab-3, OP40 (Oncogene Research Products, Calbiochem, Cambridge, Massachusetts, U.S.A.) was used at a dilution of 1/3000. c-*fms* antibody, OA11-816 (Genosys Biotechnologies, Woodlands, U.S.A.) was used at a dilution of 1/250. c-kit AB-1 antibody, PC34 c-myc antibody, NCL-MYC (Novocastra Lab., Newcastle, U.K.) was used at a dilution of 1/700, c-erbB-2 Ab-3 antibody, OP15 (Oncogene Research Products) and EGFR antibody, E3138 (Sigma Chemical Company, St Louis, Missouri, U.S.A.) were all used at a dilution of 1/50. Positive and negative controls were included in each staining procedure.

The intensity of staining of the control slide was used as a reference for comparison with the rest of the slides to try to overcome the differences in the fixation procedures of different sections and batches of staining procedures. Sections from the same tissue blocks were used as controls. The intensity of positive staining was scored as 1–3, with 3 being the most intense staining. The percentage of positively stained cells were also assigned scores of 1–3 where 1 = < 25% positive staining, 2 = 25–50% positive staining and 3 = > 50% positivity. The final positive score was obtained by totalling the intensity and percentage scores and was called the IHC score. Slides with an IHC score for the stromal cells of more than 3 (median of the IHC score) were discarded because of heavy background staining. Hence, the numbers of samples were different with different antibodies and appear in parenthesis in Table 1.

Statistical analysis

The Mann–Whitney test and the Kruskal–Wallis test were used to compare non-parametric continuous variables between two or more groups. A P value of < 0.05 was taken as significant.

RESULTS

Normal cervical stratified squamous epithelium

The mean IHC scores of the seven antibodies in relation to the three cell groups, namely S/I, PB and basal cells are shown in Figure 1. Both S/I and PB cells showed significantly higher mean IHC scores with EGFR ($P < 0.001$) and c-fms ($P = 0.014$). However, the mean IHC score of c-erbB-2 was significantly higher in basal cells than S/I and PB cells ($P < 0.001$). No significant difference was detected with the other antibodies.

Table 1. *Percentages of oncogenes/tumour suppressor gene with immunohistochemical staining (IHC) score* > 3 *in normal cervical epithelial and cervical intra-epithelial neoplasia (CIN)*

		Percentages with IHC Score > 3						
		p53	EGFR	c-fms	c-myc	c-kit	c-erbB-2	p-ras
Normal cervix	Superficial/intermediate	2.50 (40)*	93.50 (45)	90.60 (32)	27.30 (44)	9.80 (41)	31.00 (42)	3.10 (32)
	Parabasal	2.50 (40)	87.00 (45)	78.10 (32)	25.00 (44)	7.30 (41)	52.40 (42)	3.10 (32)
	Basal	2.50 (40)	11.10 (45)	59.40 (32)	36.40 (44)	2.40 (41)	81.00 (34)	3.10 (32)
CIN	1	0.00 (35)	37.90 (29)	77.80 (18)	44.70 (38)	3.70 (27)	94.30 (35)	7.70 (39)
	2	0.00 (34)	40.60 (32)	80.00 (15)	42.10 (19)	3.20 (31)	89.50 (38)	6.10 (33)
	3	0.00 (34)	46.20 (26)	94.10 (17)	23.50 (34)	2.70 (37)	87.50 (24)	0.00 (36)

*Number of samples in parenthesis (represents number of samples after exclusion based on a high background staining).

The percentages of samples with IHC scores greater than 3 (median of the IHC score) in the various cell groups are shown in Table 1.

Cervical intra-epithelial neoplasia

The mean IHC scores of the seven antibodies in relation to the three grades of CIN are shown in Figure 2. Only c-erbB-2 showed a significantly higher mean IHC score in CIN1 and with a decreasing trend to CIN3 ($P = 0.035$). The other antibodies showed no difference in IHC scores among the three grades of CIN.

The positive staining patterns of the c-erbB-2, EGFR, c-myc and c-fms are shown in Figures 3–6. The percentages of samples with IHC scores greater than 3 in the dysplastic cells of the three grades of CIN are shown in Table 1.

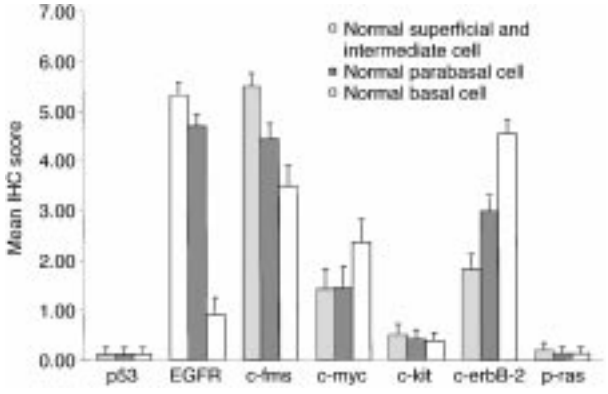

Figure 1. Mean immunohistochemical (IHC) scores of p53, EGFR, c-fms, c-myc, c-kit, c-erbB-2 and pan-ras in three groups of cells in normal cervical epithelium.

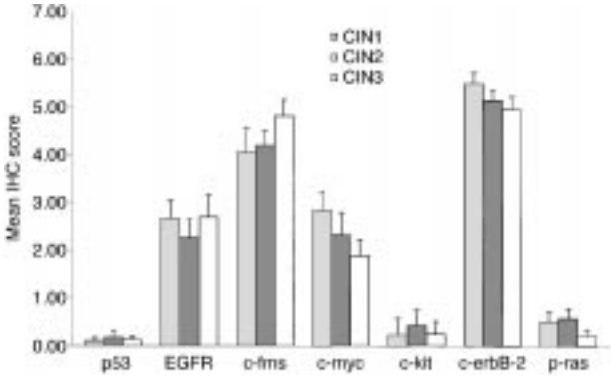

Figure 2. Mean immunohistochemical (IHC) scores of p53, EGFR, c-fms, c-myc, c-kit, c-erbB-2 and pan-ras in the three grades of cervical intra-epithelial neoplasia (CIN).

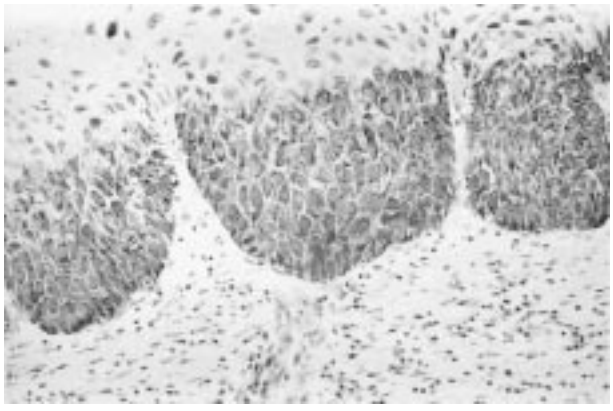

Figure 3. Immunoreactivity for c-erbB-2 antibody was mainly found in the basal layers of cervical epithelium displaying features of CIN2.

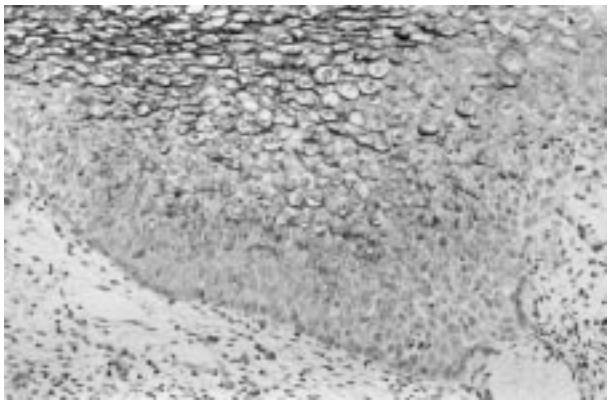

Figure 4. Immunoreactivity of EGFR antibody was mainly found in the upper layers of cervical epithelium displaying features of CIN2.

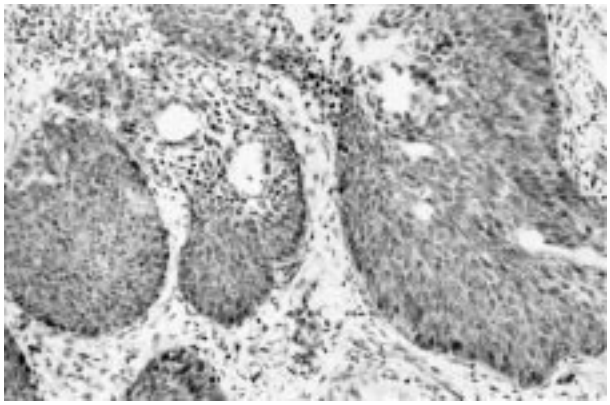

Figure 5. Uniform cytoplasmic staining for c-fms antibody was demonstrated in epithelial cells of CIN3.

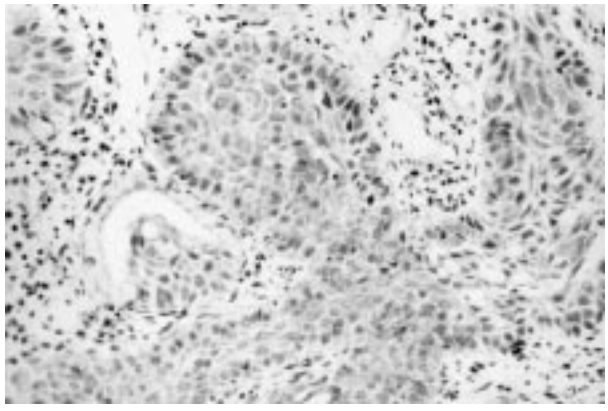

Figure 6. Uniform cytoplasmic staining for c-myc antibody was demonstrated in epithelial cells of CIN3 involving glandular epithelium.

Comparisons between normal cervical cells and dysplastic cells in CIN

The IHC scores of dysplastic cells of the three grades of CIN were grouped together as CINs and compared with the three groups of normal cervical cells using the Mann–Whitney test. Since only a few were positive with antibodies raised against p53, c-kit and pan-ras, statistical analysis was not performed with these antibodies.

Dysplastic cells had a significantly higher mean IHC score when these cells were stained for c-myc ($P = 0.009$, 0.013) and c-erbB-2 ($P < 0.001$, < 0.001) compared with normal S/I and PB cells. However, no difference was detected with the basal cells. In contrast, a higher mean IHC score was found in dysplastic cells compared with normal basal cells stained for EGFR ($P < 0.001$).

S/I cells showed a higher mean IHC score with EGFR ($P < 0.001$) and c-fms ($P < 0.001$) than dysplastic cells. PB cells showed a higher mean IHC score with EGFR ($P < 0.001$) than dysplastic cells.

There were no statistical differences in the mean scores between the three cell types in normal cervical epithelium with and without co-existing CINs. Abrupt changes from normal staining to abnormal staining were observed for sections where there was continuity seen from normal epithelium to CIN.

DISCUSSION

Normal cervical epithelium

Both EGFR and c-fms were more highly expressed in the superficial, intermediate and parabasal cells than in the basal cells, suggesting that these two proto-oncogenes were expressed in the more differentiated cells. In contrast, c-erbB-2 was more highly expressed in the less differentiated basal cells compared with the parabasal, intermediate and superficial cells suggesting that c-erbB-2 was active in less differentiated cells. However, this finding was contrary to that reported by Wang and colleagues [15] who showed EFGR in the parabasal and basal cells and c-erbB-2 in the intermediate cells. These differences may be due to the different antibodies used in the two studies. In this study, both antibodies for EGFR and c-erbB-2 were raised against the internal domain of the receptors, whereas in the other study, antibodies were raised against the external domain. In contrast to the above results, no difference in the IHC scores was noted in the staining of the three cell types with antibodies against c-myc, pan-ras, c-kit and p53. In fact, with the exception of c-myc, less than 10% of normal cells expressed the other three proto-oncogenes or tumour suppressor gene. This suggests that these genes are probably not important in the differentiation of cervical epithelium.

Cervical intra-epithelial neoplasia (CIN)

Only c-erbB-2 showed a reverse correlation with increasing grades of CIN. Though c-erbB-2 was more active in basal cells, the decrease in c-erbB-2 activity in grade 3 CIN may represent a decrease in activity with increasing neoplastic transformation. However, in another study, an increase in the amplification of c-erbB-2 was seen in CIN3 compared with normal and CIN1 and 2 cells [10]. However, since amplification may not be directly related to protein expression, these two results cannot be directly compared.

c-myc also showed a similar trend of decreased protein expression associated with an increase in the grade of CIN, although the difference was not statistically significant. A similar trend was observed by Slagle and colleagues [16] in higher grades of CIN where positive IHC staining for c-myc was detected in 23% of CIN1, 26% of CIN2 and 14% of CIN3. Moreover, Pinion and colleagues, in their study on c-erbB-2 mentioned above, also found an increase in the amplification of c-myc in CIN3 compared with normal or CIN1 and 2 [10].

In agreement with Dellas and colleagues [17], no correlation of EGFR and c-fms expression was found with the CIN grades. p53, pan-ras and c-kit all had very low IHC scores in CIN1–3 and no correlation with grades seemed apparent. However, Sagae and colleagues [7] found an increasing trend of positive IHC staining with a monoclonal ras antibody, from 18 to 54% with increasing grades of CIN.

Comparisons between normal cervical cells and CIN

When all grades of CINs were grouped together, both c-myc and c-erbB-2 showed increased IHC scores of the dysplastic cells compared to the normal S/I and PB cells. However, no difference was observed with the basal cells. This suggested that c-myc and c-erbB-2 were more active in both basal and dysplastic cells than the more differentiated cells. Slagle and colleagues [16] found no difference in the expression of c-myc in normal and CIN. In contrast, although increased expression of EGFR was detected in dysplastic cells compared with basal cells the more differentiated parabasal and intermediate cells had an even higher expression. This suggested that although EGFR was active in differentiated cells and less in undifferentiated basal cells, in the process of dysplasia, it was re-activated and its expression was higher than the basal cells. Similar findings were observed by Dellas and colleagues [17]. However, in another study, using an enzyme linked immunosolvent assay (ELISA) for EGFR, no difference was observed between normal cervix and CIN [18], although this could reflect differences in the methodology.

Pan-ras, p53, c-fms and c-kit showed no difference in expression between normal cervix and CIN. Slagle and colleagues [16] also did not show any difference in expression of ras and p53 between normal cervix and CIN. c-kit Had a low expression in normal cervix and CIN and is probably not important in cervical dysplasia. Furthermore, although c-fms had a high expression in both normal cervix and CIN, its activation or inactivation does not seem to play a role in cervical dysplasia.

In conclusion, c-erbB-2 and c-myc were active in undifferentiated basal cells of a normal cervix and in dysplastic cells of CIN, whereas, EGFR was more active in differentiated or differentiating I and PB cells of a normal cervix and less active in basal cells. However, it is re-activated in dysplastic cells. c-fms was also more active in normal differentiated cells. pan-ras, p53 and c-kit were not activated in normal cervix nor in CIN. Antibodies or anti-genes targeted against these proto-oncogenes active in CIN may alter the progress of CIN to invasive cancer.

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