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Immunohistochemical investigation and Northern Blot analysis of c-erbB-2 expression in normal, premalignant and malignant tissues of the corpus and cervix uteri

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Summary. Seventy specimens of normal endometrium ($n=13$) and cervix ($n=12$), endometrial hyperplasia ($n=4$), cervical dysplasia ($n=20$), endometrial ($n=11$) and cervical carcinoma ($n=8$) and uterine metastases of mammary carcinomas ($n=2$) have been analysed for c-erbB-2 expression with immunohistochemistry employing a monoclonal anti ERBB-2 antibody and Northern-blot hybridization using single stranded RNA probes. In comparison with the c-erbB-2 mRNA expression level found in normal samples, two advanced and poorly differentiated endometrial adenocarcinomas (FIGO IV) and two ductal mammary carcinomas which had metastasized to the uterus, together with three carcinomas in situ of the cervix, showed c-erbB-2 enhanced transcription level. All other endometrial samples including adenomatous hyperplasia and nine endometrial carcinomas (FIGO I), and all other lesions of squamous epithelial origin displayed transcriptional activities at or below the baseline level. Immunohistochemical study of ERBB-2 protein expression showed staining in most samples, although different in distribution and intensity. Staining of endometrial glands was seen in unevenly distributed cells or cell clusters. In contrast, for endocervical glands, labelling was observed distinctly on basally located cells (reserve cells) and at the subapical side of luminal cells. Faint labelling of the basal cell layer was also observed in squamous epithelia. It was more pronounced in severe cervical dysplasia and carcinoma in situ. In carcinomas of glandular origin, dedifferentiation was accompanied by an increase in cytoplasmic labelling, whereas the intensity of staining was not related to differentiation in squamous cell carcinomas. While data derived from Northern blots are suggestive of c-erbB-2 overexpression to indicate an advanced and dedifferentiated state of tumours of glandular origin, staining with an anti-ERBB-2 antibody occurred in both normal and atypical squamous and glandular epithelia and may indicate regular proliferation and/or differentiation-associated events.

Key words: Cervical mucosa – Endometrium – C-erbB-2 RNA – Protein expression

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Introduction

Proto-oncogenes are normal cellular genes, the function of which may be influenced by rough structural genomic aberrations (translocation/deletion), point mutations, and/or dysregulations, via amplification or heterologous control mechanisms. Among more than 50 different proto-oncogenes coding for proteins with functions of growth factors, growth factor receptors, cytoplasmic second messengers or regulators of gene expression, the most frequently discussed are proto-oncogenes linked to particular human neoplasms and their biological outcome. Among them, c-abl/bcr (Shtivelman et al. 1985) in chronic myelogeneous leukaemia, N-myc in neuroblastoma (Seeger et al. 1985) and c-erbB-2 in mammary and perhaps in ovarian carcinomas (Slamon et al. 1989) are regarded as prognostic indicators. So far, the function of the c-erbB-2 gene product is unknown, although molecular and structural relationships with the epidermal growth factor receptor are suggestive of its involvement in cell growth and differentiation (Schlechter et al. 1985; Semba et al. 1985; Coussens et al. 1985; Bargmann et al. 1986; Yamamoto et al. 1986; Stern et al. 1986).

Changes of the normal functional state of the c-erbB-2 gene are observed in human adenocarcinomas without any structural deviations of the gene itself (Lemoine et al. 1990). Most of the current knowledge on the functional state of the c-erbB-2 gene is derived from studies on mammary tissues. In mammary carcinomas, Slamon et al. (1989) showed that c-erbB-2 gene amplification nearly always raises the transcriptional activity, but in contrast, overexpression can take place without amplifi-

cation in a considerable percentage of cases (10%). These results underline the importance of studies at the mRNA and protein level as most reliable indicators of functional abnormalities of the c-erbB-2 gene.

To our knowledge, investigations on amplification and expression of the c-erbB-2 gene in the female genital tract are concentrated on ordinary ovarian adenocarcinomas (Slamon et al. 1989). Since uterine epithelia represent the appropriate in vivo model to study putative relationships of c-erbB-2 activation and the sequelae of hormone-dependent cellular differentiation, our studies were devoted to normal endometrium and functional disorders and normal cervical epithelia in addition to uterine premalignancies and invasive carcinomas. Major insights about c-erbB-2 activation in mammary and ovarian cancer emerged from filter hybridization techniques which, however, do not reflect the individual cell level. We wanted to compare data derived from northern-blot analysis and topochemical observations. A major prerequisite of these in situ investigations were specific and formalin-resistant c-erbB-2 antibodies which are now available for this immunohistochemical analysis (Van de Vijver et al. 1988).

Materials and methods

Material was derived from excisional and cone biopsies and gross surgical specimens following hysterectomy (for various benign disorders and endometrial carcinomas) and the Wertheim operation.

Seventy specimens of normal endometrium ($n=13$), normal cervix ($n=12$), endometrial hyperplasia ($n=4$), cervical dysplasia ($n=20$), endometrial adenocarcinoma ($n=11$), squamous cell carcinoma ($n=8$) and uterine metastases of mammary carcinomas ($n=2$) have been included in this study. Part of the material was immediately frozen in liquid nitrogen and stored at $-80°$ C until being further processed for RNA extraction and northern-blot analysis. All tissues were submitted to routine diagnosis and consecutive immunohistochemistry after being fixed in formalin and embedded in paraffin.

RNA extraction of biopsies was performed according to the method of Chirgwin et al. (1979) employing caesium chloride after homogenization of the biopsies in liquid nitrogen.

The MKN 7 cell line (cultured from a gastric adenocarcinoma, Yamamoto et al. 1986) with c-erbB-2 overexpression was used as an external control. Cells were cultivated in RPMI 1640 medium supplemented with antibiotics and 10% fetal calf serum. After harvesting them, the RNA was extracted according to the method of Perbal (1988) for cell cultures.

Denatured total RNA samples (15 µg per well) were separated on a 1% agarose gel containing 6% formaldehyde and transferred to a biodyne A membrane (Pall BNNG 3050). The filter was submitted to hybridization with a ^{32}P-labelled single-stranded RNA probe (specific activity at least 10^8 cpm/µg plasmid) obtained with a plasmid carrying a promoter for the SP6 polymerase and a cDNA fragment corresponding to part of the external and transmembrane domains of the ERBB-2 protein (Amersham RPN 1323). The filters were exposed to Kodak XAR 5 films at $-80°$ C.

A monoclonal mouse antibody (3B5/OP 15, Dianova, Hamburg, FRG) against c-erbB-2 gene products was used for immunohistochemistry. It recognizes the amino-acid sequence 1242–1255 which belongs to the inner domain of the transmembranous protein (van de Vijver et al. 1988). Incubation with primary antibodies diluted at 1:50 in phosphate buffered saline (pH 7.4) and consecutive avidin-biotin-peroxidase detection (ABC, Vectastain, Burlingame, UK; Brumm et al. 1989) was done on paraffin material. For controls, primary antibodies were omitted and/or replaced by antibodies of different specificities (for example, filamentous proteins).

In addition to the gross and subcellular qualitative analysis of ERBB-2 stainings, the number of stained cells and intensity of the label were scored as follows: a negative reaction (−) was defined when less than 5% of cells of a stained area where strongly decorated. For a positive reaction (+), strongly stained cells of a labelled area had to range between 5 and 20% and/or medium reaction between 15 and 50%. Overexpression (++) corresponded to a strong staining affecting more than 20% of cells accompanied by a medium reaction in at least 50% of cells.

Results

Northern-blot hybridization with the c-erbB-2 probe resulted in an autoradiographical signal located at 4.6 Kb. This signal was observed in normal tissues, uterine premalignancies and carcinomas as well as metastases. The additional band at 2.3 Kb observed in the MKN 7 cell line (Yamamoto et al. 1986) was not seen in the tissues examined.

13 cases of normal endometrium at proliferative or secretory state were studied (Table 1). Six of them were completely negative. Among seven other cases, weak ERBB-2 staining was seen in glands of the stratum basale (outer rim and at luminal sites). In three cases at late secretion stage (and in one perimenopausal proliferative endometrium) stainings of the uppermost glands including the covering superficial epithelial cell layer was observed. ERBB-2 was frequently seen in the cytoplasm near to the basal membrane and at luminal sites of the glands (Fig. 1a). The subcellular analysis revealed a granular cytoplasmic staining pattern. Endometrial stromal cells were always negative. However, antibodies reacted with smooth muscles of vessel walls and of the myometrium itself.

Regular endocervical and exocervical mucosa was evaluated in 12 cases (Table 1). The endocervical stainings concerned basally located cells (reserve cells) and the subapical part of the luminal cylindrical epithelia (Fig. 1b). Exocervical squamous epithelia showed discrete decoration of basal keratinocytes except for three cases which were completely negative (Fig. 4a).

Of 13 normal endometrial tissues, 10 showed detectable expression of the c-erbB-2 mRNA by Northern blotting. Only slight differences were noticed in these tissues. The highest transcription level observed was considered as baseline level and results for tumours were compared with it. C-erbB-2 gene expression was also seen in normal cervical mucosa and was used as reference for the cases of cervical lesions.

Three simple glandular hyperplasias, one being associated with an endometrial polyp, and one adenomatous hyperplasia were examined (Table 1). ERBB-2 staining was observed in all of them. Whereas two simple glandular hyperplasias were strongly labelled (Fig. 2a), staining of the remaining simple glandular hyperplasia and one adenomatous hyperplasia was moderate in terms of number of cells and intensity. In addition to stained cells scattered randomly within otherwise negative glands,

Table 1. C-erbB-2 protein expression in analysed tissues

Diagnostic		*N*	–	+	++
Normal endometrium		13	6	7	0
Hyperplasias		4	2	2	0
Adenocarcinomas	total:	11	2 (18%)	5 (46%)	4 (36%)
	G I	7	2	4	1
	G II	3	0	1	2
	G III	1	0	0	1
normal cervical mucosa		12	3	9	0
cervical dysplasias	total:	20	2 (10%)	14 (70%)	4 (20%)
	CIN I/CIN II	3	2	1	0
	CIN III	17	0	13	4
SCC	total:	8	2 (25%)	4 (50%)	2 (25%)
	G I	2	0	1	1
	G II	2	1	1	0
	G III	4	1	2	1

CIN: Cervical Intraepithelial Neoplasia; SCC: Squamous Cell Carcinoma; scoring: see text

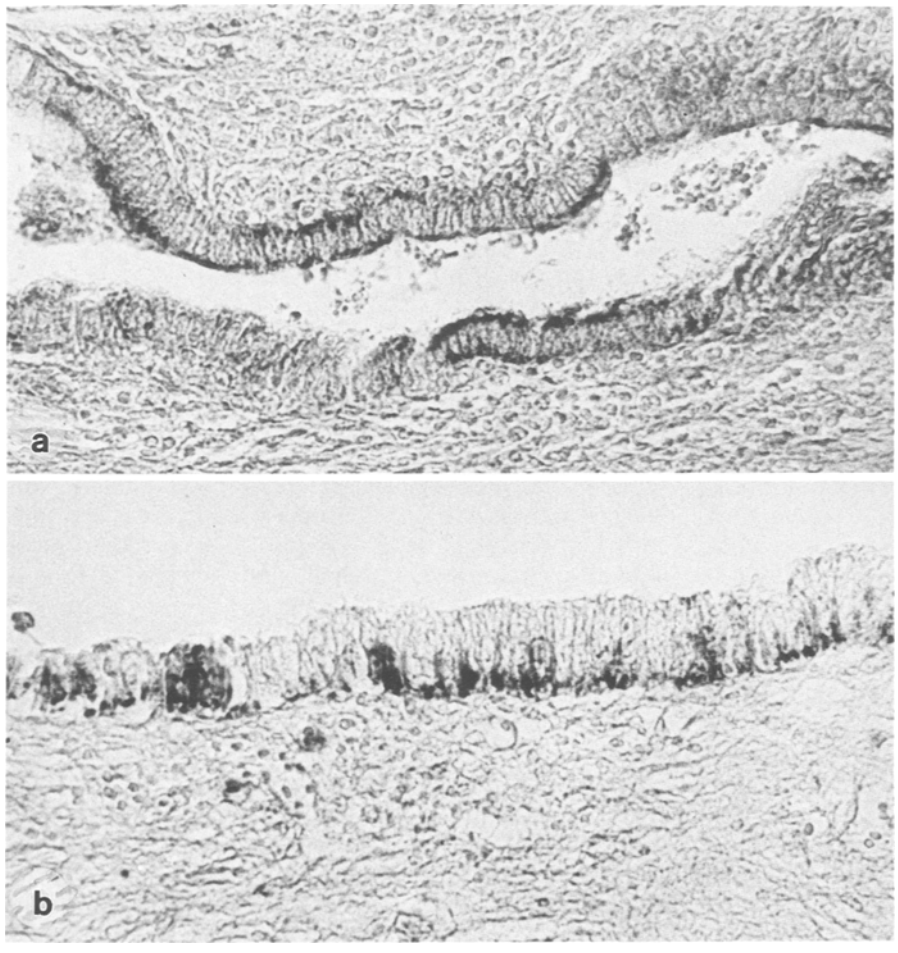

Fig. 1a, b. Immunohistochemical detection of the ERBB-2 protein in **a** normal endometrial mucosa at late secretory phase (distinct basal and apical staining of glandular cells, ×320) and in **b** normal endocervical mucosa (weak granular cytoplasmic staining, predominantly located at the epithelium/storma interface, ×320)

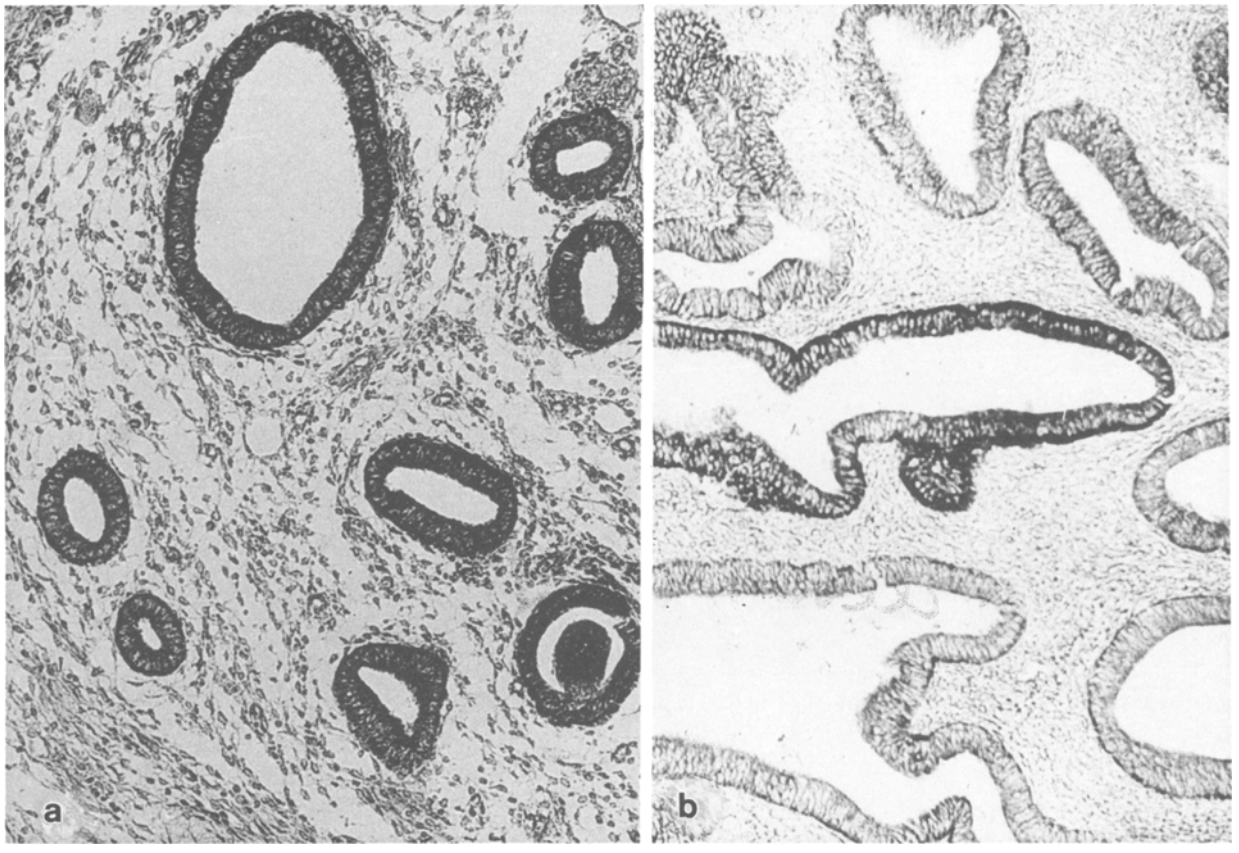

Fig. 2a, b. Immunohistochemical detection of the ERBB-2 protein in simple endometrial hyperplasia, ×80. **a** Strong labelling of the glands. Stromal cells are negative. **b** Heterogenous staining pattern in different areas with pronounced label at the epithelium/stroma interface

completely labelled glands were observed (Fig. 2b). At the subcellular level, granular cytoplasmic staining was concentrated at the epithelium/stroma interface and next to the luminal cell membranes.

RNA analysis showed only distant relationships with immunohistochemical results. Three of the four tissues revealed c-erbB-2 expression at or below the baseline level. Differences in the expression level could not be assigned to a particular type of hyperplasia.

Adenocarcinomas were graded as G I in seven and G II in three cases; one G III-tumour was examined (Table 1). Of G I carcinomas, two cases were negative, four showed only a moderate reaction which was also recognized in one G II tumour. The remaining adenocarcinomas (1 × G I, 2 × G II, 1 × G III) were strongly positive.

Among these cases, an outstanding intensive staining of almost all tumour cells was only observed in one G II tumour and in the G III carcinoma (Fig. 3b). These two carcinomas were staged as FIGO IV, showing tumour infiltrates across the myometrial wall, lymphangiosis carcinomatosa and parametrial infiltrates. The other positive cases displayed a heterogeneous staining pattern with labelled cells and glands intermingled irregularly with negative ones. No polar orientation of stainings was observed. At the subcellular level, granular cytoplasmic staining were again apparent.

C-erbB-2 RNA expression was detected in all endometrial adenocarcinomas studied. Enhanced transcription level could be only observed for the two cases which were staged as FIGO IV (Fig. 5, lane 6).

Three cases of CIN I/II and 17 CIN III/CIS were studied (Table 1). In CIN I/CIN II weak c-erbB-2-staining of basal layer cells was observed in only one case while being absent in the other ones (Fig. 4b). In contrast, all CIN III/CIS were found to be positive, four of them strongly (Fig. 4c). Generally, the signal decreased with epithelial differentiation. Interestingly, koilocytes were negative (Fig. 4c). The subcellular granular distribution of the label was also seen in these tissues except for intensively stained and overloaded lesions (Fig. 4d).

At the RNA level, expression was observed for all but 2 cases (one CIN II and one CIN III). A slightly enhanced transcription level was observed for three of them, all being histologically classified as carcinoma in situ.

We analysed eight cases of squamous cells carcinomas (2 × G I, 2 × G II, 4 × G III; Table 1). Among these, one G II and one G III tumour were negative. Interestingly, one G I carcinoma was strongly stained at the surface similar to some of the CIN III cases already mentioned while the invasive border of this tumour was only

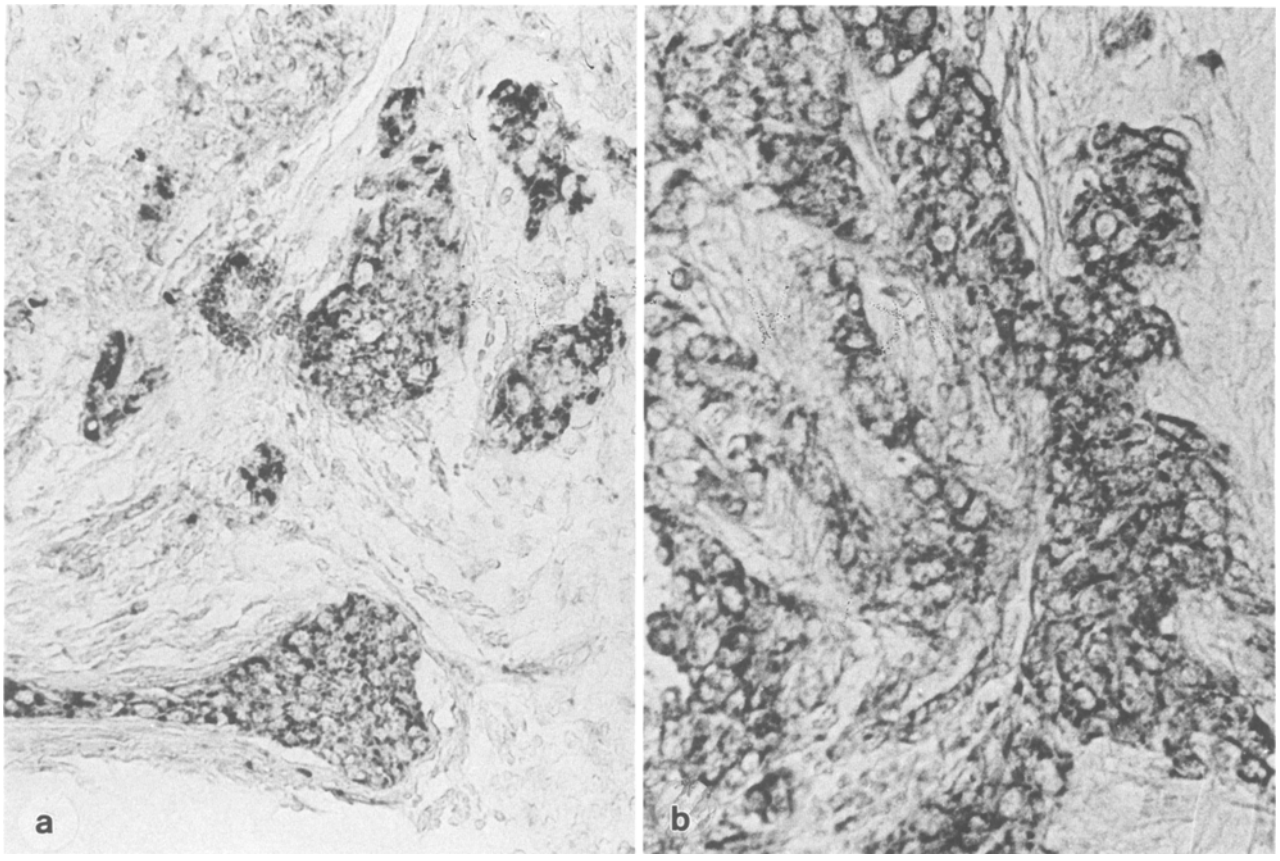

Fig. 3a, b. Immunohistochemical detection of the ERBB-2 protein in: **a** Grade III squamous cell carcinoma of the uterine cervix with coarse granular cytoplasmic staining, ×80. **b** Grade III endometrial adenocarcinoma with diffuse and membrane orientated labelling, ×320

decorated to a moderate degree and in a heterogeneous manner. This profile was also seen in the remaining cases except for one G III tumour, which was intensively labelled. The subcellular distribution of the label was similar to that in the CIN III cases.

In five cases, marginal tumour-free mucosa was seen to be negative or only weakly positive.

Seven of the eight cases of squamous cell carcinomas showed a baseline transcription level, whereas no expression was detected for one G I tumour.

Two biopsies of mammary carcinoma metastastatic to the endometrium and the cervix have been included in this study. Both of them showed strong labelling in most of the tumour cells. Subcellular investigation revealed a strong membrane-related staining pattern in addition to a diffuse label.

Both biopsies showed strikingly enhanced transcription level (Fig. 5, lanes 7 and 8) in Northern blots.

Discussion

Activation of proto-oncogenes is usually defined by biochemical means. These assess the increase of one particular mRNA or protein as a statistical value, disregarding the variable activations within the heterogeneous cellular constituents in normal and neoplastic tissues. Since purely quantitative deviations of the gene and its products are evaluated, mass tissue analysis based on Northern blots may only detect the tip of the iceberg of the biological spectrum of mRNA expression.

Our previous Northern blot studies on mammary tissues showed c-erbB-2 enhanced transcription level in 30% of invasive ductal carcinomas (data not shown) consistent with most of the recent reports on amplification and transcription (Van de Vijver et al. 1987; De Potter et al. 1989; Slamon et al. 1989; Tauchi et al. 1989; Guerin et al. 1989; Marx et al. 1990; Heintz et al. 1990). However, immunohistochemical studies also revealed c-erbB-2 expression at a high level in a variable number of cells in the remaining two thirds of mammary carcinomas and even in non-neoplastic tissues. Thus, different results are not surprising when comparing immunohistochemistry with Northern-blot results.

In the present study, we found enhanced transcription levels in two advanced endometrial carcinomas and three cervical carcinomas in situ in addition to two mammary carcinomas metastatic to the uterus. To our knowledge, c-erbB-2 amplification and/or overexpression have

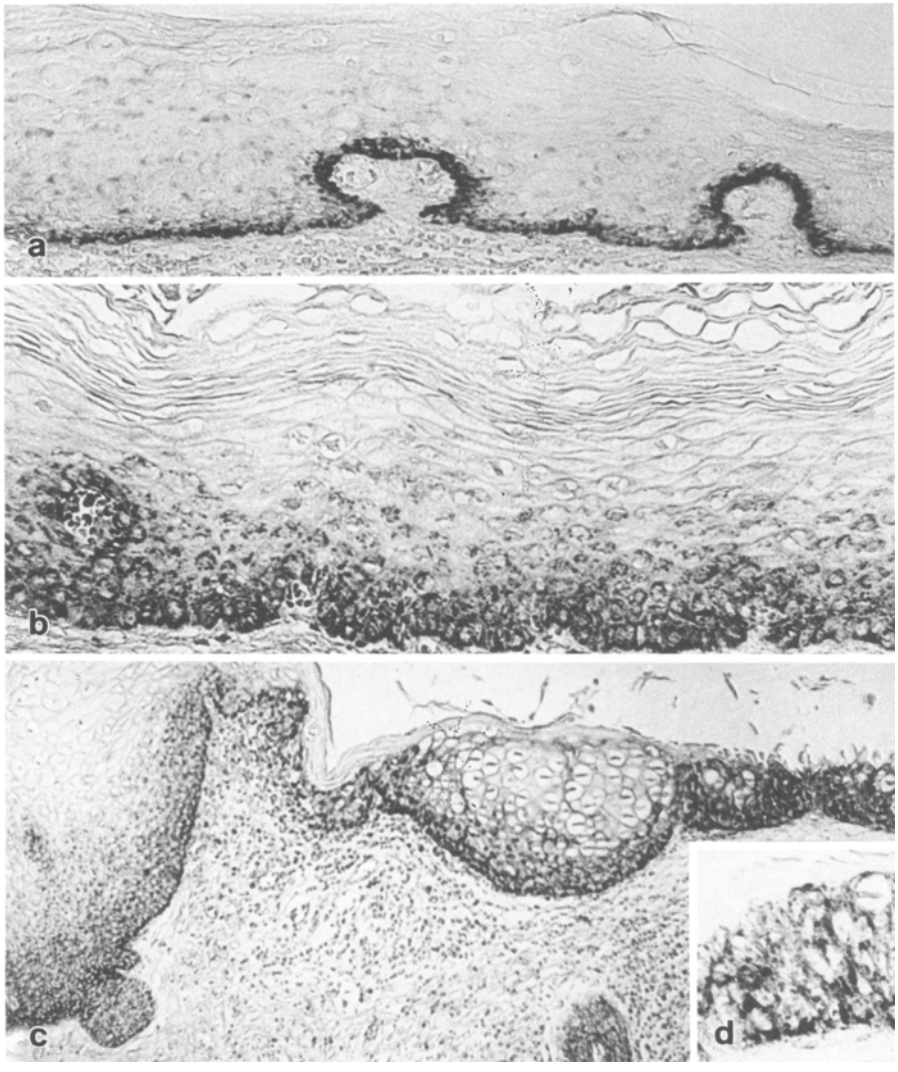

Fig. 4a–d. Immunohistochemical detection of the ERBB-2 protein in:
a Normal cervical squamous cell epithelium with coarse granular decoration of the basal layer, ×80.
b CIN I with coarse granular labelling decreasing with the differentiation of the epithelial cells, ×320.
c Cervical transformation zone with carcinoma in situ (*right side*), marginal koilocytic dysplasia (*centre*) and adjacent exocervical mucosa. ERBB-2 staining increases with loss of differentiation: full-thickness staining of carcinoma in situ in contrast to the faint label of basal/suprabasal cells of adjacent epithelia. Koilocytes negative, ×80.
d CIS showing the typical coarse granular staining pattern, ×320

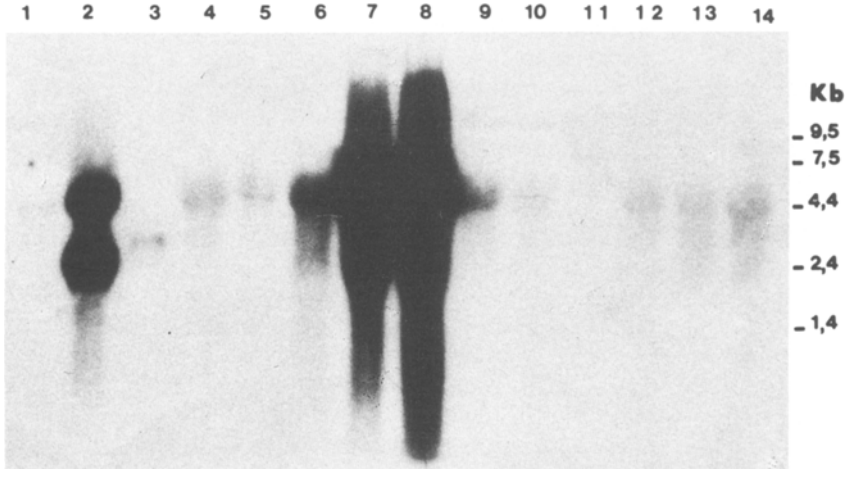

Fig. 5. C-erbB-2 RNA expression in normal and lesional uterine biopsies. 1: A 431; 2: MKN 7; molecular weight marker; 4,6: endometrial adenocarcinomas; 7: breast adenocarcinoma metastasized to the endometrium; 8: idem, in the cervix; 9: CIN III; 10,12: endometrial hyperplasias; 11,14: cervical SCC; 13: normal endometrium

not been described, as yet, in endometrial carcinomas. This part of our investigations underlines the present belief of the widespread occurrence of c-erbB-2 activation in glandular carcinomas (Yokota et al. 1986; Zhou et al. 1987). All other uterine samples showed low transcription level with only minor differences on Northern blots.

Immunohistochemical staining of c-erbB-2 was pres-

ent in most uterine tissues although being heterogeneous within and between individual cases. Stainings were limited to the epithelial compartment. Stromal cells examined at all steps of differentiation were always negative. Former in situ studies on estrogen/progesterone activities in normal uterine tissues revealed a higher content of their receptors in the proliferative phase and a decrease in the secretory phase (Lessey et al. 1988). Impact of the physiological rhythms of estrogen/progesterone activities correlating with known morphological structures (Dallenbach-Hellweg 1981) were not apparent on c-erbB-2 expression.

Marked intra- and interindividual variations of c-erbB-2 expression were also observed in glandular hyperplasia and low grade adenocarcinoma. Interestingly, in normal cervical mucosa, endocervical reserve cells and exocervical basal cells were faintly labelled, as was also seen in mild/moderate dysplasia. Differentiated intermediate and superficial cells with koilocytic changes were negative. In contrast, the enlarged basal compartment of severe dysplasia and especially the anaplastic cell population of carcinoma in situ were strongly stained. The ERBB-2 positive basal cell layer is exactly the zone of cytokeratin 19 expression which is regarded as indicator of glandular differentiation. The number of stained cells increases with the rate of anaplastic cells (the degree of CIN). Similar results were obtained earlier with antibodies against Tissue Polypeptide Antigen (TPA) which biochemically corresponds to gland-specific cytokeratins (Löning et al. 1983; Weber et al. 1984). C-erbB-2 overexpression even in these tissues may merely reflect the conserved potential of glandular differentiation in exocervical squamous epithelia and their premalignant and malignant counterparts.

While the specificity of monoclonal antibodies is generally accepted, their reactivity may not only be directed to the particular antigen they were raised for. Thus, the small antigenetic determinant detected by monoclonal antibodies may be part of larger molecules of different kind and function. De Potter et al. (1990) showed that the 3B5 antibody recognizes two antigens of different molecular size: the ERBB-2-related p185 protein and a different one (p155) of unknown structure and function. De Potter stressed the importance of differences of staining qualities related to p185 (membrane label) and p155 protein (coarse granular cytoplasmic staining). For this reason, we reevaluated all cases which showed enhanced transcription level (two endometrial carcinomas, two mammary cancers metastasized to the uterus, three cervical CIN III/CIS). We found striking membrane staining in the mammary metastases only, while the other cases revealed predominant cytoplasmic label. Furthermore, some cases of mammary adenocarcinomas with very high transcription level (estimated to 50 fold over the level observed for normal mammary glands) did not show the membrane staining pattern (data not shown). Thus, taking into account our Northern-blot experiments, we conclude that the immunohistochemical pattern of p185 protein is heterogeneous and that p185 reactivities are difficult to separate from p155 ones at the immunohistochemical level alone.

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