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ORIGINAL ARTICLE

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Preferential localization of c-*kit* product in tissue mast cells, basal cells of skin, epithelial cells of breast, small cell lung carcinoma and seminoma/dysgerminoma in human: immunohistochemical study on formalin-fixed, paraffin-embedded tissues

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Abstract Eighteen hundred and eighty-four cases of human solid tumours and 833 samples of normal human tissues, formalin-fixed and paraffin-embedded, were examined immunohistochemically for expression of c-*kit* oncogene product using polyclonal antibody against synthesized c-*kit* peptide. Seminoma/dysgerminoma and small cell lung carcinoma (SCLC) show preferential c-*kit* expression at 92% and 36% frequency, respectively, whereas only sporadic cases of cervical carcinoma and non-SCLC lung carcinoma show c-*kit* positivity. A normal tissue counterpart positive for c-*kit* product is detected in the testis (spermatocyte) and ovary (oocyte) but not in the lung or the cervix. In contrast, normal epithelial cells of the breast, skin basal cells and tissue mast cells harbour c-*kit* product, but transformed cells of the former two are largely deficient in the c-*kit* protein. One hundred and thirty-nine neuroendocrine tumours and 39 non-pulmonary small cell carcinomas were all negative, except for two cases of neuroblastoma. This indicates a distinct character for SCLC in c-*kit* expression. The c-*kit* product may be a useful marker in diagnostic pathology of seminoma/dysgermona and SCLC among human solid tumours, and in distinction of SCLC from non-pulmonary small cell carcinoma.

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Introduction

The c-*kit* proto-oncogene represents the cellular homologue of v-*kit*, an oncogene derived from the acute feline retrovirus HZ4-FeSV: Hardy-Zuckerman 4 feline sarcoma virus HZ4-FeSV (Besmer et al. 1986). It codes for a transmembrane tyrosine kinase receptor protein, structurally related to platelet derived growth factor/colony stimulating factor-1 receptors (Yarden et al. 1987). Its ligand is stem cell factor (SCF; Anderson et al. 1990; Huang et al. 1990; Martin et al. 1990). Abnormality in the c-*kit* gene produces white coat colour, sterility, macrocytic anaemia and mast cell deficiency in the mouse (Geissler et al. 1981). In situ hybridization analysis of c-*kit* mRNA in mouse embryos has revealed that its specific signals have been detected in numerous tissues and cells with the following time course of development: placenta, neural tube, sensory placodes, embryonic gut, blood islands in yolk sac and liver, branchial pouches, primordial germ cells, hair follicle cells, melanoblasts, craniofacial area, spinal cord and central nervous system (Orr-Urtreger et al. 1990). The c-*kit* transcripts are detected in the oocyte of both immature and mature ovarian follicles, but not in the male germ line (Orr-Urtreger et al. 1990). c-*kit* expression is also detected in normal adult and fetal lung, and in erythroid and pigmented tumour lines of the mouse by Northern blot hybridization and/or in situ hybridization (Andre et al. 1989; Nocka et al. 1989; Orr-Urtreger et al. 1990). These data suggest that c-*kit* gene expression is involved in cell differentiation and/or proliferation of various murine tissues.

In humans, there are two immunohistochemical analyses of c-*kit* expression in normal and transformed human non-lymphoid tissues using cryostat sections

(Natali et al. 1992a; Matsuda et al. 1993). In these two reports, astrocytes, renal tubules, parotid cells, thyrocytes, breast epithelium, spermatogonia, adrenal medullary cells and melanocytes were positive for c-*kit* receptor protein. The gene product in human solid tumours is expressed in seminomas, lung tumours, phaeochromocytomas and melanomas of low invasiveness. In addition, glioblastoma in vivo (Natali et al. 1992a) and in vitro (Yarden et al. 1987), two haematopoietic cell lines (Andre et al. 1989), small cell lung carcinomas (SCLCs) in vitro and in vivo (Sekido et al. 1991) and normal breast epithelium (Natali et al. 1992b) have been reported to express the c-*kit* transcripts. The present study was performed to elucidate a more precise cellular localization and distribution of c-*kit* gene product in human normal tissues and various solid tumours using routine pathological specimens.

Our results confirmed preferential expression of c-*kit* oncogene product in seminoma/dysgerminoma and SCLC. This is not shared with neuroendocrine tumours and non-pulmonary small cell carcinomas, except for a few cases of non-SCLCs. Ovarian carcinoma, skin carcinoma and cervical carcinoma are also mostly without expression suggesting a specific growth regulatory mechanism in seminoma/dysgerminoma and SCLC which operates sporadically on other carcinomas through a c-*kit*-ligand system. In normal human tissues and organs, positive reaction products were detected in the basal cells of skin, epithelial cells of the breast, spermatocytes and oocytes, and tissue mast cells.

Materials and methods

Anti c-*kit* protein antibody was raised in rabbits by subcutaneous injections of 14 mer peptide positions 963 to 976 amino acid residues at the C′ terminus of the c-*kit* protein (Yarden et al. 1987). As a carrier protein, thyroglobulin (Sigma, St. Louis) was used. The antisera were fractionated to immunoglobulin (Ig) G through protein A AvidGel (Bio Probe International, Tustin, Calif.) and specifically purified through antigen-bound, activated thiol AvidGel F affinity chromatography (Bio Probe).

A SCLC cell line SY with overexpression of c-*kit* mRNA and a c-*kit*-negative adenocarcinoma cell line HS were used for Western blotting. Two cell lines were supplied from Dr. Ueda (Aichi Cancer Centre, Nagoya, Japan). Both cell lines, used at cell concentration $10^5/\mu l$ were sonicated for 30 s in lysis buffer [0.1% sucrose monolaurate, 0.01% ethylenediaminetetraacetic acid, 2 mM PMSF: phenylmethylsulfonyl fluoride (PMSF) and 0.25% sucrose in phosphate-buffered saline (PBS)]. After sonification, the samples were centrifuged at 10,000 rpm for 30 min and resultant supernatant was admixed with sample buffer [1 M TRIS-hydrochloric acid pH 7.8, 18.75 ml; 2-mercaptoethanol, 15 ml; glycerol, 30 ml; sodium dodecyl sulphate (SDS) 6.9 g; 0.1% bromophenol blue, 3 ml; distilled water to 100 ml] at 2:1 ratio. Protein concentration was determined for each SDS extract. Various amounts of protein were run on SDS-PAGE and then blotted onto nitrocellulose membranes. The membrane blots were incubated with affinity purified c-*kit* antibody followed by anti-rabbit goat IgG Fab' conjugated with horseradish peroxidase. Peroxidase reaction product was visualized by Konika Immunostain HRP1S-50B (Konica, Tokyo).

Tissue of the 2717 cases examined was fixed with 10% neutral formalin and paraffin-embedded. All tissue was obtained at surgery of biopsy, except for some autopsy cases of SCLCs and non-neoplastic gonads. Tables 1–3 show the list of tissues employed in this study. Fixed SY cell line (10% formalin for 1–7 days) was used as a positive control.

For immunohistochemistry, the avidin-biotin-peroxidase complex method (Hsu et al. 1981) was used. Sections cut 4 μm thick were deparaffinized, and pretreated with 0.3% hydrogen peroxide-methanol to block endogenous peroxidase activity, and with 5% skimmed milk (Yukijirushi, Sapporo) in PBS (pH 7.4, 0.01 M) to avoid non-specific adsorption of Ig. The sections were then incubated in the antibody at concentration of 1 μg/ml IgG at 4° C, overnight. After washing, they were incubated with the sec-

Table 1 List of cases examined and results of immunohistochemistry

Human solid tumours	Positive cases/ cases examined
Pulmonary carcinoma:	
non small cell type	8/ 144
squamous cell carcinoma	7/ 86
adenocarcinoma	1/ 35
large cell carcinoma	0/ 17
adenosquamous carcinoma	0/ 5
mucoepidermoid carcinoma	0/ 1
small cell type	
autopsy	21/ 75
surgical	24/ 48
Cervical carcinoma:	
non-small cell type	9/ 227
small cell type	0/ 2
Breast carcinoma	1/ 92
Ovarian carcinoma:	2/ 28
serous papillary adenocarcinoma	2/ 11
clear cell carcinoma	0/ 9
endometrial carcinoma	0/ 4
mucinous cystadenocarcinoma	0/ 4
Basal cell carcinoma	1/ 18
Malignant melanoma of skin	2/ 14
Seminoma/dysgerminoma	50/ 56
Immature teratoma	3/ 7
Other germ cell tumours	0/ 17
Others[a]	0/ 855
Total	121/1583

[a] Carcinoma of gastrointestinal tract (355), liver (59), pancreas (31), gall bladder (7), bile duct (16), kidney (29), urinary bladder (49), prostate (159), adrenal (14), thyroid (91) and skin (45)

Table 2 List of neuroendocrine tumours examined and results of immunohistochemistry

Category	Positive cases/ cases examined
Neuroblastoma	2/ 18
Ganglioneuroma	0/ 7
Phaeochromocytoma	0/ 23
Paraganglioma	0/ 12
Medullary carcinoma, thyroid	0/ 7
Retinoblastoma	0/ 1
Carcinoid	0/ 30
Lung	0/ 10
Gastrointestinal tract	0/ 20
Ewing tumour	0/ 3
Islet cell tumour	0/ 8
Total	2/139

Table 3 c-*kit* gene product-immunoreactivity in small cell carcinomas

Origin	Positive cases/cases examined
Lung	45/123
Oesophagus	0/ 27
Stomach	0/ 2
Large intestine	0/ 2
Uterine cervix	0/ 2
Prostate	0/ 6
Total	45/162

ondary anti-rabbit IgG conjugated with biotin (Nichirei, Tokyo) for 30 min at room temperature, and then with streptavidin-peroxidase complex (Nichirei) for 30 min at room temperature. The reaction product of peroxidase was visualized by incubation with Graham-Karnovsky solution (Graham and Karnovsky 1966) containing 65 mg/ml sodium azide (Sigma) to block endogenous peroxidase activity again. Finally the slides were counterstained for nuclei by methyl green. Specificity of the immunostaining was tested with use of preabsorbed antibody with the 5 µg homologous antigen.

Results

As shown in Fig. 1, Western blotting analysis revealed a positively stained, relatively broad band [120,000–155,000 in molecular weight (MW)] including protein with MW of 145,000 in the SY extract, while no definite reaction product was detected in the HS extract. This MW is compatible with c-*kit* protein as predicted from its mRNA analysis (Yarden et al. 1987). In addition, however, a labelled band with MW of 100,000 was also observed. The nature of this protein is obscure.

Immunohistochemistry disclosed specifically labelled tumour cells in the SCLC specimen (Fig. 2a), since the preabsorbed antibody by the homologous antigen failed to label these cells (Fig. 2b). The reaction product was observed on the tumour cell membranes. The results of tumour tissue staining are summarized in Table 1. The preferential occurrence of c-*kit* product is clearly indicated in seminoma/dysgerminoma (Fig. 2c) and SCLCs, at 92% (23/25) and 36% (45/124) in frequency, respectively. Twenty-one seminomas were histologically all typical seminoma with 19 positive for c-*kit* gene product. Distribution of immunostained cells was mainly diffuse (15 of 19 cases). Three of four dysgerminomas, histologically and biologically identical to seminoma of the testis, showed diffuse positivity to c-*kit* gene product. Among SCLCs, there was a difference in frequency between autopsy (28%; 21/75) and surgical (49%; 24/49) cases. Duration of fixation in formalin does not seem responsible for this discrepancy in frequency, since SY cells fixed for 1–7 days gave the same results in immunohistochemistry. Pulmonary carcinoma of non-small cell type, mainly squamous cell carcinoma (Fig. 2d), cervical squamous cell carcinoma (Fig. 3a), malignant melanoma of skin (Fig. 3b), mammary invasive ductal carcinoma (Fig. 3c), ovarian serous cystadenocarcinoma, basal cell carcinoma and immature teratoma were only exceptionally immunolabelled by the antibody. In the immature teratoma, a few primitive mesenchymal cells revealed positivity. Metastatic deposits of SCLC (Fig. 3d) and of seminoma/dysgerminoma were positive as well as the primary tumour.

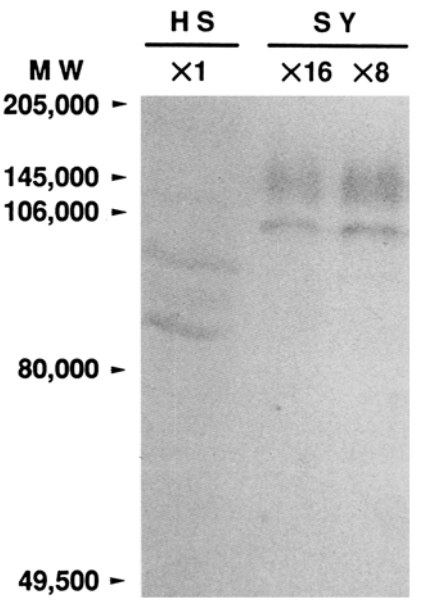

Fig. 1 Detection of protein with molecular weight (*MW*) 145,000 in the extract of small cell lung carcinoma cell line SY, identical to the molecular weight of c-*kit* protein, is possible by immunoblotting methods using the polyclonal antibody against 14 mer peptide of c-*kit* protein. Note that the immunostained band is rather wide, from 120,000 to 155,000 in MW. Another band of 100,000 in MW is also detected in the extract of SY. In the extract of adenocarcinoma cell line HS without c-*kit* gene expression, however, reaction product with MW of 145,000 is not detected at all. The number shown in the upper portion of each lane indicates dilution titre of the protein applied

One hundred and thirty-nine neuroendocrine tumours failed to show positive labelling except for two cases of neuroblastoma (Table 2). Small cell carcinomas originating from extrapulmonary sites, 39 in total, were not labelled by the antibody (Table 3). Among normal tissues employed (see Table 4) epithelial cells of both duct and acinus of the breast (Fig. 4a), spermatocytes of the testis (Fig. 4b), oocytes (Fig. 4c) and basal cells of skin (Fig. 4d) were positively labelled, in addition to tissue mast cells. Myoepithelial cells of the breast were spared. A positive result in the gonads was obtained in adult cases and negative in newborns and infants. Immature germ cells therefore seemed to be negative for c-*kit* gene product. The frequency of positive cells in gonads (mainly obtained at autopsy) was very low. The reason for this phenomenon was obscure but postmortem autolytic change or anticancer chemotherapy might be the cause. The other tissues or cells were completely negative, including the bronchial neuroendocrine cells of the lung.

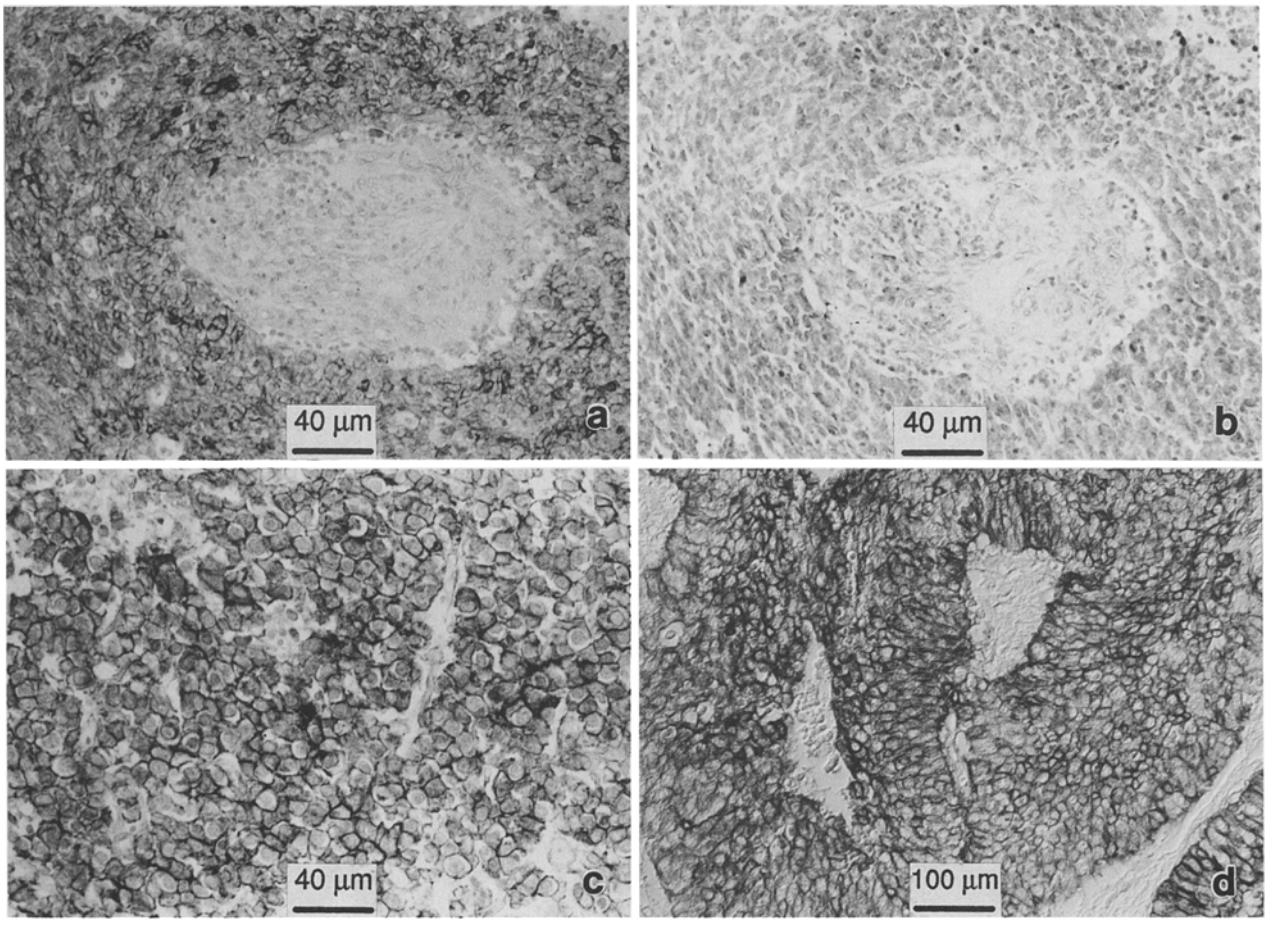

Fig. 2 a Small cell lung carcinoma (SCLC) is intensely immunostained at the cell membranes of cancer cells (× 100). **b** Complete disappearance of immunolabelling is observed with use of antibody preabsorbed by the homologous antigen (× 100). **c** Dysgerminoma cells are also labelled by the antibody at the cell membranes (× 100). **d** Pulmonary carcinoma of non-SCLC type, squamous cell carcinoma, is positive for the c-*kit* product (Romanovsky interference optics; × 40)

Discussion

The present study confirmed c-*kit* gene in a SCLC cell line SY and SCLCs in vivo, as reported by Sekido et al. (1991) and Hibi et al. (1991). In the latter paper the authors described co-expression of the SCF, a ligand, and suggested an auto/paracrine growth mechanism(s) through this ligand-receptor system. Preferential expression of the c-*kit* gene product was noticed in the seminoma/dysgerminoma group. A similar result was documented by analysis of c-*kit* mRNA expression in human seminomatous testicular tumours (Strohmeyer et al. 1991). Expression of the SCF has not yet been elucidated in seminoma/dysgerminoma. However, since mouse Sertoli's cells of the testis have been reported to express SCF mRNA (Rossi et al. 1991), and mast cell GF or steel factor (identical to SCF) stimulates growth or survival of murine primordial germ cells in vitro (Dolci et al. 1991; Matsui et al. 1991), the c-*kit*-receptor-ligand system might also potentiate growth of human seminoma/dysgerminoma cells.

The frequency of immunohistochemical positivity of c-*kit* in SCLCs is significantly higher in biopsies than in surgical specimens. The reason for this discrepancy is not clear, but duration of formalin fixation is not apparently responsible, as stated. Autolytic change at autopsy may contribute to this discrepancy. In addition, possible modification of c-*kit* expression by radiation and/or chemotherapy might cause this phenomenon in the autopsied SCLCs, which were regularly treated with these regimens. Histological subtypes of SCLC, namely oat cell type and intermediate cell type (variant), were equally immunolabelled by the antibody in spite of their differences in phenotypes and sensitivity to chemotherapy so far described (Brereton et al. 1978; Abeloff et al. 1979; Mathews and Gazdar 1981; Carney et al. 1983).

The present study also revealed expression of c-*kit* protein in a limited number of pulmonary carcinomas of non-small cell type, in squamous cell carcinomas of the uterine cervix, breast carcinomas, basal cell carcinomas of skin and malignant melanoma, while the neuroendocrine tumours and non-pulmonary small cell

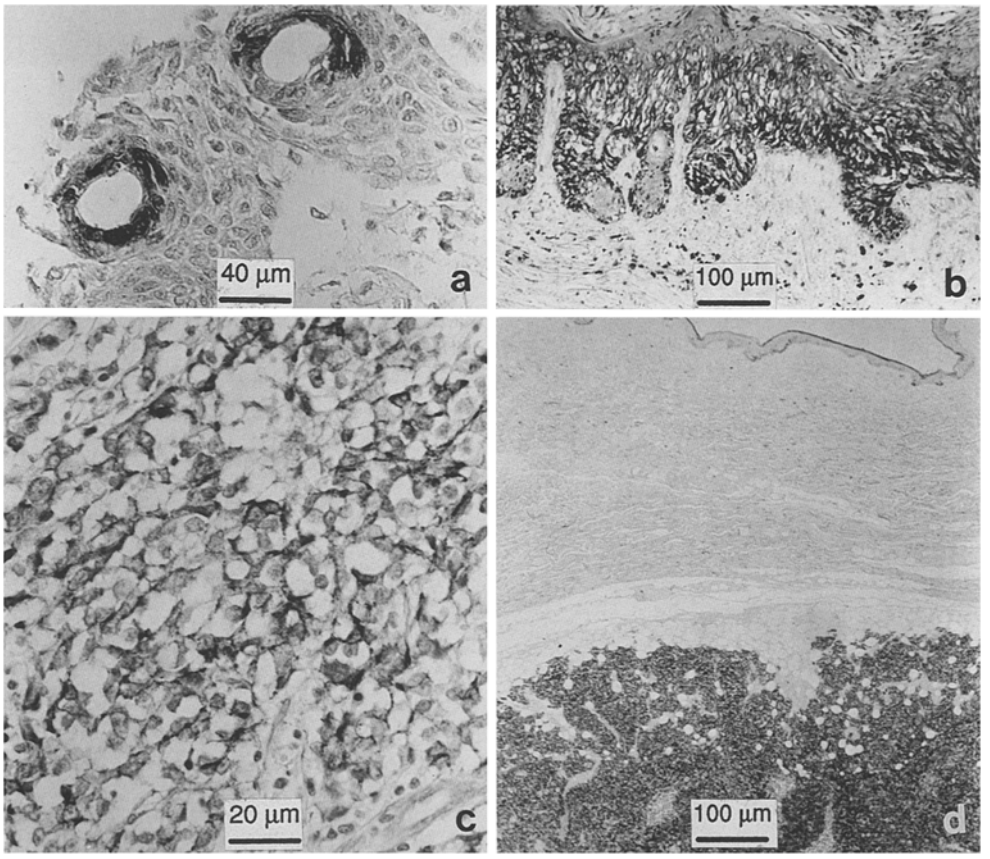

Fig. 3 Squamous cell carcinoma of uterine cervix shows positive reaction to the antibody at the basal layer of the cancer tissue (**a**, ×100). Malignant melanoma of skin (**b**, ×40) and breast carcinoma cells (**c**, ×200) are also positive. Metastatic small cell lung carcinoma in the skin is markedly immunostained by the antibody (**d**, ×40)

Table 4 c-*kit* gene product-immunoreactivity in normal human tissues

Tissue	Positive cases/cases examined
Oesophagus	0/ 65
Stomach	0/144
Colon	0/118
Lung	0/100
Heart	0/ 2
Breast	14/ 21
Liver	0/ 29
Pancreas	0/ 23
Gallbladder	0/ 3
Kidney	0/ 20
Urinary bladder	0/ 23
Prostate	0/ 26
Testis (fetus – 28 years old)	3/ 24
Ovary (newborn – 29 years old)	3/ 11
Uterine cervix	0/ 84
Thyroid	0/ 57
Adrenal	0/ 41
Skin	25/ 29
Others[a]	0/ 13
Total	45/833

[a] Eye (1), lymph node (5), parathyroid (2), thymus (1), tonsil (2) and uterus (2)

carcinoma examined, both of which tumours share a common neuroendocrine nature with SCLCs, were all negative. From these results c-*kit* gene product seems to be almost selectively expressed in seminoma/dysgerminoma and SCLC but is not common to neuroendocrine tumours and/or non-pulmonary small cell carcinomas.

In normal human tissues and cells, expression of c-*kit* gene product was confirmed in the epithelial cells of breast, skin basal cells (seemingly melanocytes), spermatocytes and oocytes. Our study failed to label renal tubules, adrenal medullary cells and thyrocytes, differing from the two previous reports (Natali et al. 1992a; Matsuda et al. 1993). The reason is not clear but difference in tissue processing (cryostat section, acetone fixation vs formalin fixation, paraffin-embedding) and the nature of the antibody used (monoclonal antibody against extracellular domain of c-*kit* protein vs polyclonal antibody intracellular domain of c-*kit* protein in the case of Natali et al. 1992a) might be responsible. In human testis, spermatogonia were not immunostained, differing from the mouse (Yoshinaga et al. 1991) and previous reports on man (Natali et al. 1992a), while transformed spermatogonia (seminoma) was regularly positive for c-*kit* gene product, as stated above. In contrast, malignant transformation of melanocytes and epithelial cells are negative for c-*kit* expression in general. This phenomenon has been confirmed at the c-*kit* mRNA level (Lassam and Bickford 1992; Natali et al.

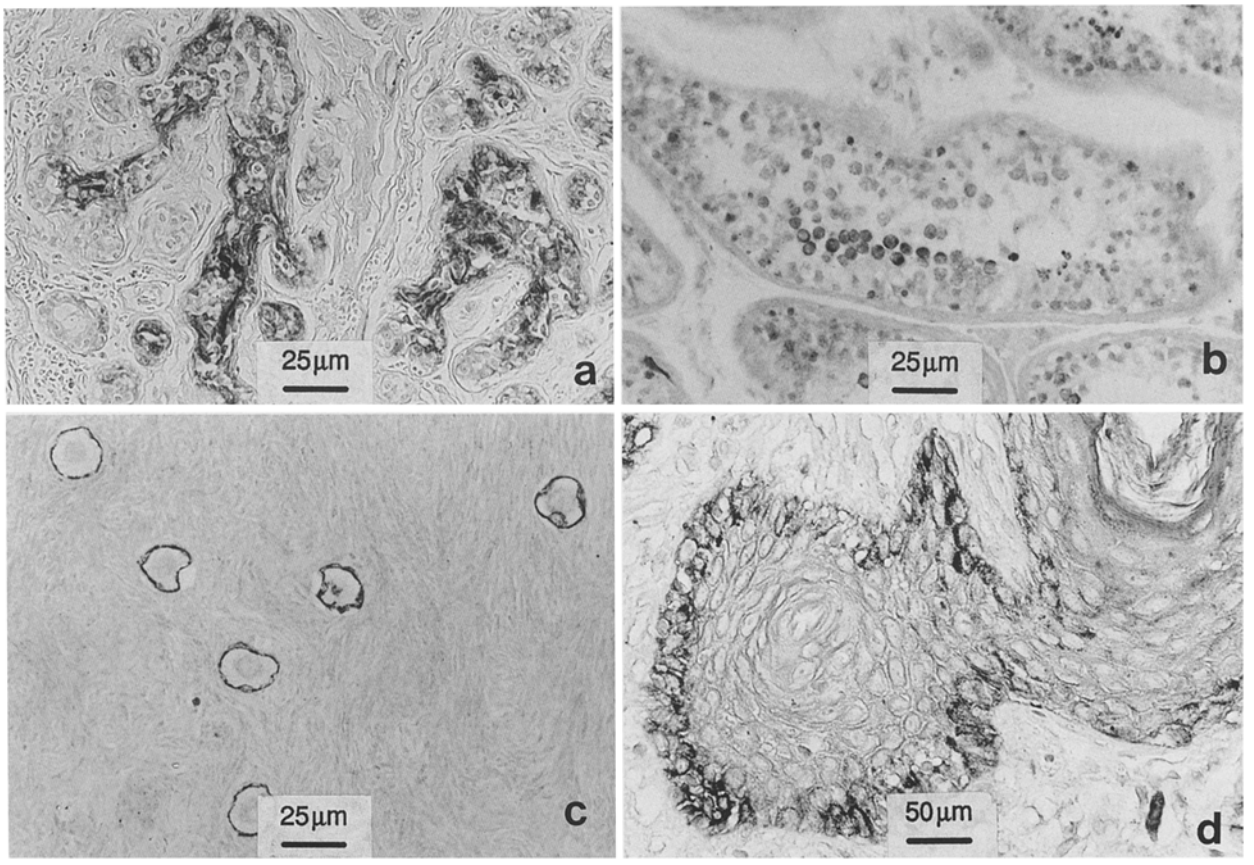

Fig. 4 In the normal breast the epithelial cells of both ducts and acini (not shown in this figure) are positive for c-*kit* product, but the myoepithelial cells are negtive (**a**, × 100). A few spermatocytes (**b**, × 100) and oocytes (**c**, × 100) reveal reaction product in the cellular membrane to the antibody. Numerous basal layer cells of skin, seemingly melanocytes, are immunolabelled by the antibody (**d**, × 200)

1992b). Although a general role of c-*kit* protein and mechanisms of up- and/or down-regulation of c-*kit* gene expression have not been clear in these tissues or cells, c-*kit* product may be a useful marker in diagnostic pathology of SCLC and seminoma/dysgerminoma among human solid tumours. It may help in the differential diagnosis of SCLC from small cell carcinomas other than those of pulmonary origin.

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