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Expression of p21^{ras} in normal and malignant human tissues: Lack of association with proliferation and malignancy

(immunohistochemistry/monoclonal antibody/oncogene/synthetic antigen)

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ABSTRACT **Proteins encoded by cellular *ras* oncogenes (p21^{ras}) are expressed in a wide variety of malignant tumors, including carcinomas, lymphomas, and neuroectodermal tumors. The function of p21^{ras} in these tumors and the distribution and role of p21^{ras} in corresponding normal tissues are unclear. This immunohistochemical study examined the relationship between p21^{ras} expression and malignant transformation, cellular differentiation, and proliferative activity *in vivo*. p21^{ras} was found to be widely expressed in normal tissues, but within those tissues expression was often sharply restricted to cells at specific stages of differentiation; terminally differentiated cells generally showed stronger reactivity with antibodies to p21^{ras} than did rapidly proliferating cells. Fetal and adult tissues had corresponding patterns of p21^{ras} expression, and the distribution of p21^{ras} in neoplasms paralleled the pattern in normal tissue from which they were derived. Thus, p21^{ras} seems to play a role in many fully differentiated cell types, and levels of p21^{ras} expression do not correlate with proliferative activity in normal cells or, in contrast to past reports, with the transformed phenotype.**

Transfection of high molecular weight DNA extracted from cultured human tumor cell lines or from tumor tissues can induce transformation of mouse 3T3 cells if the tumor cell DNA contains an activated form of one of the three known cellular *ras* oncogenes (1–4). The genetic changes leading to *ras* activation are commonly point mutations resulting in single amino acid substitutions in the encoded 21-kDa proteins (p21^{ras}) (5–7). Since *ras* activation has been detected in a proportion of human tumors but not in normal cells, it has been speculated that mutations affecting *ras* may play a role in the development of this group of tumors. In addition, studies with cultured cells have suggested that overproduction of normal p21^{ras} can also induce a transformed phenotype (8, 9) and amplification of *ras* genes and overexpression of normal p21^{ras} have been described for both tumor cell lines and tumor tissues (10–12). A recent series of immunohistochemical studies has suggested that high expression of p21^{ras}, independent of mutational activation, is a common finding in human carcinomas and is associated with a poor clinical prognosis (13–16). However, the results of immunohistochemical studies from independent laboratories have been inconsistent, and different patterns of p21^{ras} expression have been described not only for neoplasms but also for normal tissues (13–21). We have previously found by immunoblotting procedures with monoclonal antibodies that p21^{ras} is expressed in tissue extracts of a variety of human tissues, including brain, muscle, and skin (P.G.C. and H.L.N., unpublished observations). However, this approach using whole tissue extracts is bound to provide misleading information if cells within a given tissue show substantial differences in p21^{ras} expression at distinct stages of differentiation or between subsets of specialized cells. We now report the results of a comprehensive immunohistochemical study of p21^{ras} expression in normal and transformed human tissues.

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MATERIALS AND METHODS

Tissues. Tissues were obtained at autopsy or from surgical specimens. Normal adult and fetal (14 weeks gestational age) tissues from the following organs were tested: esophagus, stomach, small and large intestine, liver, kidney, urinary bladder, testis, ovary, skin, lung, adrenal gland, peripheral nerve, and skeletal and smooth muscle; adult but not fetal tissues were tested from pancreas, salivary gland, prostate, uterus, breast, cartilage, thyroid gland, brain, spinal cord, heart, and lymph node.

Monoclonal Antibodies. Antibody 142-24E05, raised against a synthetic polypeptide comprising amino acids 96–118 of p21^{ras}, a highly conserved region of the Ha-ras, Ki-ras, and N-ras proteins, detects p21^{ras} in a wide variety of established cell lines and tissues (ref. 22 and unpublished observations), and Fig. 1 shows the results of a representative immunoblotting experiment with antibody 142-24E05 on a human epidermal tissue extract. Antibodies Y13-259 and Y13-238, which were raised against viral p21^{ras}, also react with proteins encoded by the human *ras* genes (23). Ascites fluid containing antibody 142-24E05 (1:2000 dilution), purified antibodies Y13-238 and Y13-259 (50 μg/ml), and unrelated control antibodies were used for this study.

Immunohistochemical Procedures. Fresh tissues were embedded in OCT compound (Tissue-TEK II, Miles), quick-frozen in isopentane that had been cooled in liquid nitrogen, and stored at −70°C until used; 5-μm sections were cut, mounted on gelatin-coated slides, air-dried, and fixed in cold acetone. The avidin-biotin immunoperoxidase procedure was carried out as described (24). Sections of paraffin-embedded tissues were tested after treatment with pepsin as described (24).

RESULTS

In our immunohistochemical analysis of p21^{ras} expression in normal and malignant human tissues, antibody 142-24E05 (raised against a synthetic peptide) and antibodies Y13-238 and Y13-259 (raised against viral p21^{ras}) gave identical results in both frozen and paraffin-embedded tissues. Figs. 2–4 show examples of immunoperoxidase assays with normal and tumor tissues and Tables 1 and 2 summarize our typing results for p21^{ras} tissue distribution. To confirm the specificity of the immunostaining seen with antibody 142-24E05, we have determined that all 142-24E05 staining is abolished by

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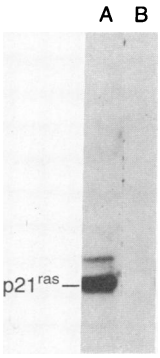

FIG. 1. Immunoblotting analysis of human epidermal tissue extract with anti-p21^{ras} monoclonal antibody 142-24E05. Lane A, antibody 142-24E05; lane B, antibody 142-24E05 previously incubated with the synthetic peptide used for antibody production.

incubation of antibody with the appropriate synthetic peptide (Fig. 2).

Table 1 shows that a wide range of normal tissues express p21^{ras}, but, within tissues, p21^{ras} was often sharply restricted to cells at specific stages of differentiation. These patterns of p21^{ras} expression generally corresponded for fetal and adult tissues, and the following is a description of the most characteristic patterns of p21^{ras} expression.

In the lining epithelia of the gastrointestinal tract the following patterns were seen: In the fundus of the stomach, p21^{ras} is detected in parietal cells but not in chief cells, undifferentiated cells, or mucous cells of the glands (Figs. 2*C* and 3*A*); in addition, weak immunoreactivity is seen in the surface epithelium. In the antrum of the stomach, which is devoid of parietal cells, immunoreactivity is restricted to cells of the middle portions of pyloric glands; no reactivity is seen in the cells at the bottom or in the upper portions of these glands (Figs. 2*D* and 3*B*) except for weak reactivity with the surface epithelium. Finally, in the small and large intestine, cells throughout the glands of Lieberkühn lack detectable p21^{ras}, whereas cells of the surface epithelium (absorptive and goblet cells) show immunoreactivity. In addition to p21^{ras} expression in the mucosa, strong immunoreactivity was found with ganglion and smooth muscle cells throughout the gastrointestinal tract. The stratified squamous epithelia of esophagus, cervix uteri, and epidermis (Figs. 2*A* and 3*D*) show p21^{ras} expression in all cellular layers. Tubular epithelial cells of the kidney vary in p21^{ras} expression (Fig. 3*F*), whereas urothelium, ductal and acinar epithelial cells of the mammary gland (Fig. 3*E*), and the glandular epithelium of the prostate uniformly express p21^{ras}. Hepatocytes (Fig. 3*C*), thymocytes, and lymphocytes also express p21^{ras}. No immunoreactivity was seen in the stroma of these tissues.

Neural cells of the brain, spinal cord (Fig. 3*G*), peripheral nerves (Fig. 3*H*), and autonomic plexus all show very strong p21^{ras} immunoreactivity. In the brain, strong p21^{ras} staining was also detected in the epithelium of the choroid plexus (Fig. 3*I*). Similarly, endocrine cells in several organs show prominent p21^{ras} expression. Thus, Leydig cells in the testis show strong immunoreactivity (Fig. 3*L*), whereas the nonendocrine components, Sertoli cells and germ cells, show only weak reactivity. Strong reactivity is seen in most cells of the

Table 1. Expression of p21^{ras} in normal human tissues

Tissue	Cell types with prominent p21^{ras} immunoreactivity
Stomach	Glandular epithelium, smooth muscle
Small intestine	Surface epithelium, smooth muscle
Colon	Surface epithelium, smooth muscle
Liver	Hepatocytes
Testis	Leydig cells
CNS, PNS	Neurons, choroid plexus
Kidney	Tubular epithelium (subpopulation)
Lymph node, spleen	Lymphocytes
Bladder	Urothelium, smooth muscle
Mammary gland	Epithelium
Prostate	Epithelium
Heart	Cardiac muscle
Skeletal muscle	Skeletal muscle
Pancreas	Epithelium, endocrine cells
Thyroid gland	Endocrine cells
Skin	Epidermis, sweat glands

Expression was determined with monoclonal antibodies 142-24E05, Y13-259, and Y13-238 by the avidin-biotin immunoperoxidase procedure. CNS and PNS, central and peripheral nervous systems.

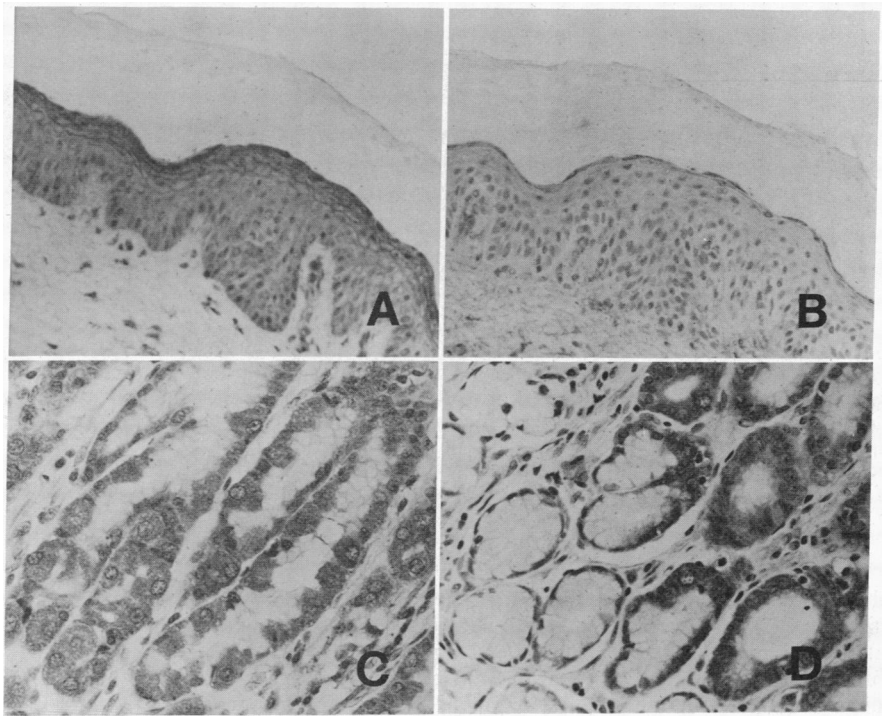

FIG. 2. Immunoperoxidase staining of human epidermis with anti-peptide antibody 142-24E05. (*A*) Reactivity throughout the epithelium. (*B*) All reactivity is abolished by incubation of antibody 142-24E05 with the synthetic peptide used for antibody production (1 mg/ml; 1 hr at 37°C) before staining. (*C* and *D*) Reactivity of antibody Y13-259 with normal mucosa of the gastric fundus (C) and gastric antrum (D). Note strong reactivity with parietal cells in *C* and middle portions of pyloric glands in *D*. (*A* and *B*, ×200; *C* and *D*, ×400.)

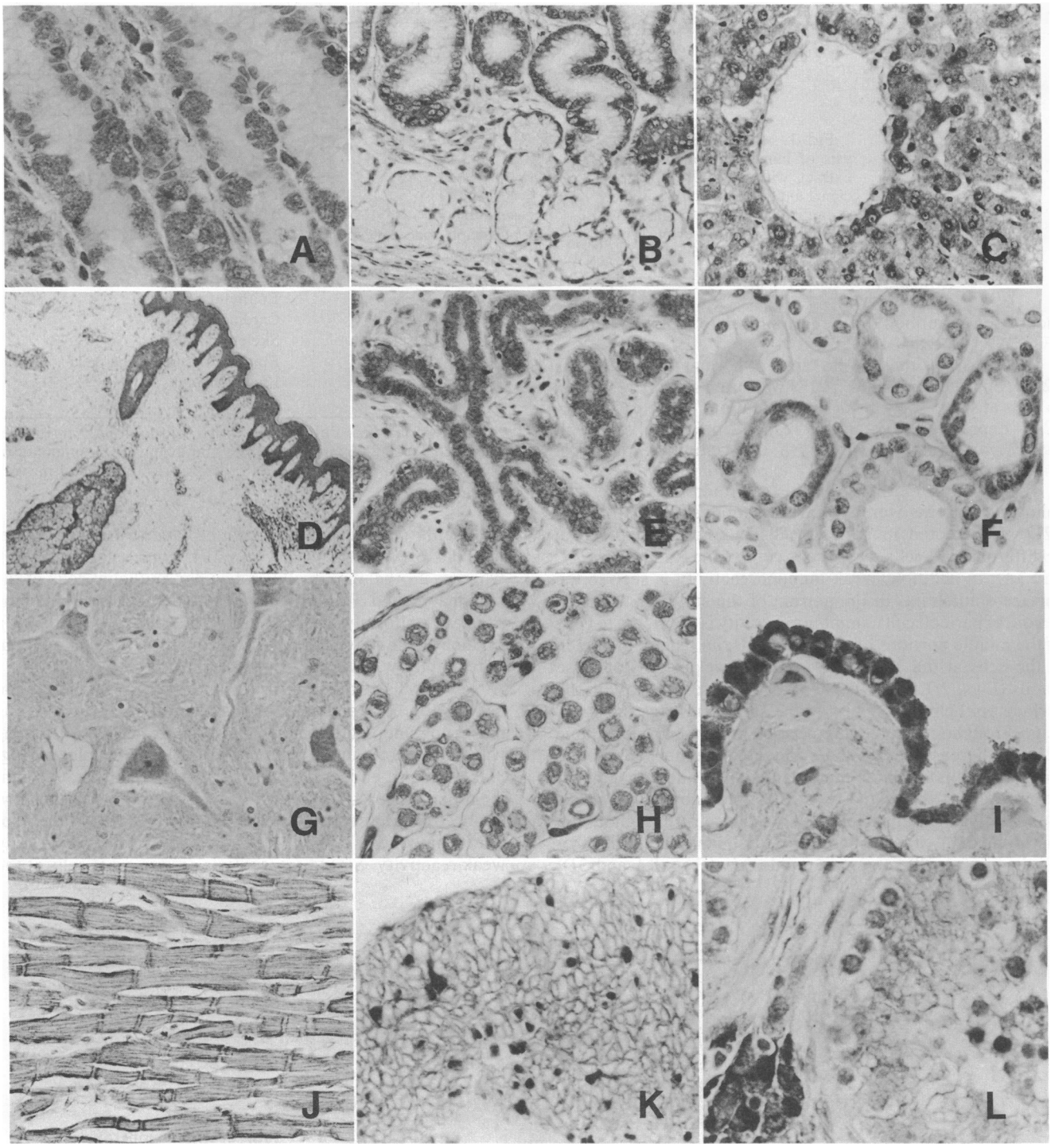

FIG. 3. Reactivity of monoclonal antibody 142-24E05 with normal human adult tissues tested by the avidin-biotin immunoperoxidase method. (*A*) Stomach, glands of the fundus with strong staining of parietal cells. (*B*) Stomach, pyloric glands of the antrum with strong staining of cells in middle portions of glands (upper) but not in bottom portions of glands (lower). (*C*) Liver, staining of hepatocytes. (*D*) Skin, strong staining of epidermis, hair follicle, and sebaceous gland (lower left). (*E*) Breast, staining of mammary gland ducts and acini. (*F*) Kidney, juxtaposition of reactive and nonreactive tubules. (*G*) Spinal cord, staining of anterior horn neurons and neuropil. (*H*) Peripheral nerve, cross section with staining of axons and Schwann cells. (*I*) Choroid plexus epithelium. (*J*) Cardiac muscle, longitudinal section. (*K*) Smooth muscle of dermis, cross section. (*L*) Testis, with strong staining of Leydig cells (lower left), weak staining of germ cells in seminiferous tubules (right and upper left) increasing in mature cells closer to lumen, and no staining of Sertoli cells. (Hematoxylin counterstain; *A*, *B*, *F*, *H*, *I*, *K*, and *L*, ×400; *C*, *E*, *G*, and *J*, ×200; *D*, ×40.)

anterior lobe of the pituitary gland and in a proportion of islet cells in the pancreas, in follicular epithelial cells and C cells in the thyroid gland, in adrenal cortical cells, and in scattered cells in the glands of the gastric antrum and small intestine (possibly representing cells of the diffuse neuroendocrine system of the gastrointestinal tract). All three types of muscle tested show $p21^{ras}$ expression: smooth muscle of blood vessels, skin (Fig. 3*K*), and viscera; skeletal muscle; and cardiac muscle (Fig. 3*J*).

A panel of fresh and paraffin-embedded tumor tissues was

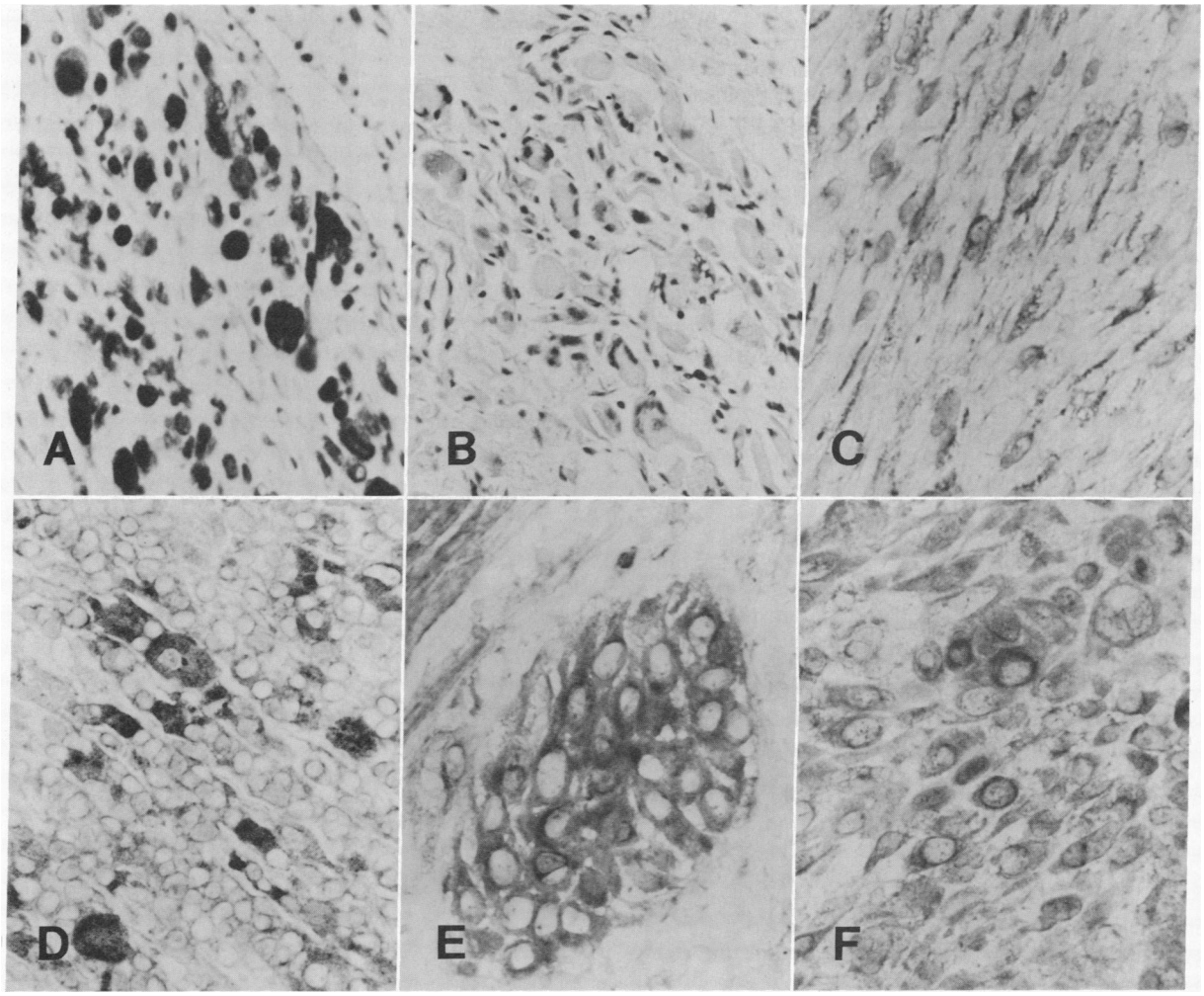

FIG. 4. Reactivity of monoclonal antibody 142-24E05 with human tumor tissues tested by the avidin-biotin immunoperoxidase method. Tumor tissues were tested with antibody 142-24E05 (*A*, *C–F*) or with unrelated negative control antibody (*B*). (*A*) Germ cell tumor with strongly labeled areas of muscle differentiation. (*B*) Same germ cell tumor as in *A*, tested with negative control monoclonal antibody. (*C*) Fibrosarcoma, strong reactivity. (*D*) Malignant melanoma, strong reactivity of a small subpopulation of tumor cells. (*E*) Epidermoid carcinoma of the urinary bladder, strong reactivity of tumor cells (center) and normal smooth muscle (upper left). (*F*) Epidermoid carcinoma of the lung, strong reactivity of tumor cells. (Hematoxylin counterstain; *A* and *B*, ×200; *C–F*, ×400.)

tested with antibody 142-24E05 and the results are summarized in Fig. 4 and Table 2. It is apparent that expression of $p21^{ras}$ is not restricted to any specific type of tumor. Instead, a majority of carcinomas, sarcomas, lymphomas, endocrine tumors, and neuroectodermal neoplasms were found to express $p21^{ras}$, although at times with marked heterogeneity (Fig. 4 *A* and *D*). Furthermore, within tumors that show recognizable subsets of tumor cells with various degrees of cellular differentiation, stronger immunoreactivity was commonly associated with a more mature cellular phenotype. This pattern was observed for well-differentiated squamous carcinomas of lung, bladder, and esophagus as well as for teratocarcinomas; in fact, in germ cell tumors areas of seminoma and embryonal carcinoma were unreactive or weakly reactive with antibody to $p21^{ras}$ (echoing the low reactivity seen with germ cells in normal testis), whereas tumors showing mature somatic differentiation were generally much more strongly reactive.

Table 2. Summary of immunohistochemical typing results of 74 malignant human tumors for $p21^{ras}$ expression

Tumor type	Number of cases	$p21^{ras}$ expression			
		+++	++	+	−
Carcinomas	33	20	5	8	—
Lymphomas	6	3	2	1	—
Melanoma	4	1	—	2	1
Neuroblastoma	4	1	3	—	—
Astrocytoma	2	—	2	—	—
Sarcomas	25	16	5	2	2

Sections of paraffin-embedded and fresh frozen tumor tissues were tested with monoclonal antibody 142-24E05 by the avidin-biotin immunoperoxidase procedure. Reactivity is symbolized as follows: +++, very strong; ++, strong; +, weak; −, negative.

DISCUSSION

Characterization of retroviral oncogenes and the identification of related cellular protooncogenes (25) have rapidly expanded our understanding of viral carcinogenesis in a number of animal tumor systems. The implications of these findings for spontaneous tumors of humans are still unclear. However, a model has been proposed for control of normal cell proliferation by the interaction of several classes of protooncogenes (26), and changes in the expression of controlling elements of normal proliferation are thought to result in the uncontrolled cellular growth commonly associated with human malignant tumors. In the present study, we have examined the relationship between cellular proliferation and $p21^{ras}$ expression in normal human tissues. We were surprised to find that in those tissues that show a distinct spatial separation of proliferation and maturation

compartments, $p21^{ras}$ expression is associated with a mature cellular phenotype and *not* with high proliferative activity. Thus, in the rapidly self-renewing epithelia of stomach and small and large intestine no $p21^{ras}$ is detected in the proliferative compartments, whereas several mature cell types, most prominently the parietal cells of the stomach, show strong $p21^{ras}$ expression. Similarly, in the testis, skin, esophagus, and uterine cervix, $p21^{ras}$ reactivity is much weaker in stem cells and rapidly proliferating cells than in the fully differentiated cells of these organs. Finally, terminally differentiated neurons, muscle, and endocrine cells also show strong $p21^{ras}$ expression.

Another characteristic of $p21^{ras}$ expression in normal tissues is its wide distribution among derivatives of all three germ layers. These observations are in striking contrast to the results of several previous immunohistochemical studies, which had failed to detect $p21^{ras}$ expression in normal urothelium, colonic mucosa, breast and prostate epithelium, and muscle (13–21). The fact that we and others have used one common antibody, Y13-259, which we now show to react with these normal tissues, suggests that the more limited $p21^{ras}$ distribution described previously was most likely due to technical problems of tissue preservation or immunohistochemical technique. Furthermore, our findings with a large number of malignant tumors indicate that most epithelial, mesenchymal, neuroectodermal, and hematopoietic neoplasms express detectable levels of $p21^{ras}$ (Fig. 4, Table 2). We propose that in the majority of human tumors $p21^{ras}$ expression reflects cellular differentiation rather than the transformed phenotype or abnormal growth characteristics. There are numerous precedents for expression of specific differentiated phenotypes in tumors; for example, we have shown that the pattern of keratin expression in colon carcinomas closely resembles the pattern seen in cells of the surface epithelium of the colonic mucosa rather than the pattern seen in the crypts of Lieberkühn, which include the proliferative compartment of the colonic mucosa (24). In analogy to this keratin distribution we now find that $p21^{ras}$ expression is also characteristic for cells of the normal surface epithelium (but not of the glands of Lieberkühn) and is also expressed in most colon carcinomas. Thus, both $p21^{ras}$ and keratin expression in the tumors may merely indicate that the specific differentiation phenotype is preserved or reexpressed after transformation. This hypothesis clearly contrasts with the suggestion (13–16) that high levels of $p21^{ras}$ expression correlate with advanced stage of disease or poor clinical prognosis. In a parallel study, we have extended our analysis of $p21^{ras}$ expression in human malignant tumors to a series of over 50 germ cell tumors and, consistent with the results reported here, $p21^{ras}$ expression was found to correlate with cellular differentiation rather than clinical prognosis (P.G.C., unpublished data).

While the observed heterogeneity of $p21^{ras}$ expression in germ cell tumors (Fig. 4*A*) seems to correlate with mature somatic differentiation, other mechanisms may account for the phenotypic heterogeneity seen in additional tumors. Thus, Fig. 4*D* shows a dramatic example of $p21^{ras}$ heterogeneity in a malignant melanoma, reminiscent of the selective *ras* activation in cultured tumor cells derived from only some metastases of a melanoma patient described by Albino *et al.* (27). A more general question concerning *ras* oncogenes is whether expression of these genes, which are functionally related to malignant transformation in certain experimental tumors, may serve quite different cell-specific functions in normal differentiated cell types and a majority of human malignant tumors. The latter may continue to express $p21^{ras}$ as part of their differentiated phenotype, retained after transformation. A similar paradox has been encountered for another protooncogene, *src* (28, 29): the normal tissue distribution of $pp60^{c\text{-}src}$ is also not restricted to cells with high proliferative activity but, instead, this protein is particularly abundant in neurons and other nondividing cell types.

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