# Enhanced Overexpression of an HIF-1/Hypoxia-Related Protein in Cancer Cells

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**Cap43 is a protein whose RNA is induced under conditions of severe hypoxia or prolonged elevations of intracellular calcium. Additionally, Ni and Co also induce Cap43 because they produce a state of hypoxia in cells. Cap43 protein is expressed at low levels in normal tissues; however, in a variety of cancers, including lung, brain, melanoma, liver, prostate, breast, and renal cancers, Cap43 protein is overexpressed in cancer cells. The low level of expression of Cap43 in some normal tissues compared with their cancerous counterparts, combined with the high stability of Cap43 protein and mRNA, makes the *Cap43* gene a new, important cancer marker. We hypothesize that the mechanism of *Cap43* overexpression in cancer cells involves a state of hypoxia characteristic of cancer cells where the Cap43 protein becomes a signature for this hypoxic state. *Key words:* antibody detection, $Ca^{2+}$, cancer-specific, HIF-1α, hypoxia signature. *Environ Health Perspect* 110(suppl 5):783–788 (2002).**
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Cap43 [Cap43 (GenBank access. no. AF006162) is our designation for this protein, but it has also been named Drg-1 (GenBank access. no. X92845) (*1*)] was originally cloned as a homocysteine-responsive gene in human endothelial cells (*2*). Subsequent studies in our laboratory showed that Cap43 mRNA was inducible in human lung cells exposed to nickel and cobalt (*3*). The reason for the induction by nickel and cobalt was because these metals mimicked a state of hypoxia in cells. Cap43 mRNA was shown to be induced by hypoxic stress in virtually every cell that has been studied (*4*). The dependence of *Cap43* on hypoxia was further investigated in HIF-1α knockout cells that had lost their ability to induce *Cap43* in response to nickel, cobalt, or hypoxia (*4*). Additionally, a computer analysis has found that there is one HIF-1 binding site in the promoter and two HIF-1 binding sites in the 3′ untranslated region of *Cap43,* further suggesting regulation of *Cap43* by HIF-1. An HIF-1 binding site in the 3′ region of the erythropoietin gene is known to regulate its transcription (*5*). Cap43 mRNA was also shown to be induced by a stressful elevation of intracellular calcium (*6*). However, the function of the Cap43 protein is currently unknown. The ability of *Cap43* to be induced by homocysteine in human endothelial cells suggested a possible role of *Cap43* in atherosclerotic disease (*6*). Recent studies have shown that *Cap43* is expressed at lower levels in colon cancer compared with normal tissue (*1*). In another study the expression of *Cap43* was found to be lower in breast and prostate cancer cells compared with normal cells (*7*). However, because expression of this gene is hormone dependent, the loss of expression in breast and prostate cancer cells is most likely due to the loss of hormone dependency in these cells. We have confirmed that there are lower levels of expression in prostatic cancer cells compared with normal prostate epithelium, but the induction by hypoxia was significantly higher in the more malignant prostate cancer (*8*). Here we discuss the relationship of *Cap43* induction by Ni and hypoxia to its overexpression in cancer cells.

## Materials and Methods

Tumor and normal tissue sections were obtained from the tumor registry of the Kaplan Comprehensive Cancer Center. Tumors and normal tissues were processed and embedded in paraffin wax. Sections 5 μm thick were cut, deparaffinized using xylol, and stained with hematoxylin–eosin (H&E) for histopathological diagnosis. For immunohistochemical detection, slides were heated in 1 mM EDTA buffer in a microwave oven, and endogenous peroxide was blocked with methanol containing 0.35% $H_2O_2$ for 30 min. A rabbit polyclonal antibody against Cap43 protein was incubated with the tissue sections for 2 hr and detected using routine avidin–biotin horseradish peroxidase complex and 3,3′-diaminobenzidine as the chromogen. Negative controls were performed with nonimmune sections instead of primary antibodies. The Cap43 antibody was made against a 30 amino acid segment near the C-terminal portion of the protein. The 30 amino acids consisted of three 10-amino-acid repeats. The antibody stains only one protein having the molecular weight of Cap43 (43,000 Da) in Western blots (see Figure 6).

Normal and cancerous tissues were also stained with HIF-1α antibody obtained from Neo Markers (Union City, CA, USA). Normal and cancerous tissues were also stained with the cytochrome P450 1B1 (CYP1B1) antibody obtained from GENTEST Corporation (Woburn, MA, USA).

### Cell Lines and Culture Conditions

A549 (human lung cancer), HCT116 (human colon tumor), and NCI-H69 (human lung small-cell carcinoma) cells were purchased from the American Type Culture Collection (Rockville, MD, USA). Human lung bronchoepithelial A549 cells were grown in Ham's F-12K medium; HCT116 and NCI-H69 were grown in RPMI 1640 supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. HIF-1$\alpha^{+/+}$ fibroblasts were obtained from C57 B mice with wild-type, normal, or knockout *HIF-1*α gene (*9*) and were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. All cells were incubated at 37°C in the presence of 5% of $CO_2$ in air. Cells were rendered hypoxic in a chamber with a gas mixture of 0.5% of $O_2$, 5% of $CO_2$ balanced with $N_2$ at 37°C. The level of oxygen in a chamber was verified using a gas monitor (SKC, Inc., Eighty Four, PA, USA).

### Northern Blot Analysis

Total RNA was extracted from cells immediately after exposure using an ULTRASPEC RNA isolation system (Biotecx, Houston, TX, USA), and the RNA was electrophoresed (15 μg of total RNA/lane) in 1.0% agarose/formaldehyde gels. Probes were labeled with [α-$^{32}$P]dcTP using the Random

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Primed DNA Labeling Kit (Boehringer Mannheim, Indianapolis, IN, USA).

### Western Blot Analysis

Cells were lysed in TNES buffer [50 mM Tris-HCl (pH 7.5), 2 mM EDTA, 100 mM NaCl, 1 mM sodium orthovanadate, and 1% NP40 containing protease inhibitors (20 µg/mL aprotinin, 20 µg/mL leupeptin, and 1 mM phenylmethylsulfonyl fluoride)]. The detection of HIF-1α was as described previously (*10*).

## Results

We have reported the cloning of a human gene, *Cap43,* that was significantly induced by nickel in A549, HTE, Calu-1, WI38, and HOS (human osteosarcoma) cells (*3*). The basal level of *Cap43* expression was very low in these cells, and it was induced by nickel within 4–6 hr. Exposure of cells to hypoxia (0.5% oxygen) induced Cap43 mRNA within 4 hr, and levels continued to increase up to 18 hr (Figure 1). The induction of this gene by hypoxia continued to increase up to 30 hr, and as with nickel, the induction remained high for at least 60 hr (not shown).

We had previously tested numerous stress conditions such as reducing or oxidative stress and heat shock; however, these conditions did not induce *Cap43* expression (*10*). The expression of *Cap43* in response to nickel or hypoxia in terms of the kinetics or *n*-fold of induction was very similar, suggesting that a common signaling pathway might be activated by both nickel and hypoxia (Figure 1A, B) (*3*). *GAPDH*, a gene known to be induced by hypoxia, was also induced by 1 mM nickel or 300 µM cobalt chloride in A549 cells (Figure 1B). The latter metal was also found to induce *Cap43*. It was conceivable that hypoxia represented a physiological inducer for the *Cap43* gene, and nickel stimulated *Cap43* expression by imitating hypoxic conditions in cells. The activation of HIF-1 transcription factor is an important part of the molecular response to hypoxia. To determine whether the HIF-1 transcription factor was involved in *Cap43* gene expression, we analyzed the time course of accumulation of HIF-1α in response to hypoxia in A549 cells. HIF-1α was an inducible subunit of HIF-1 transcription factor, and its accumulation correlated with the activation of HIF-1 transcriptional activity (*11*). A significant amount of HIF-1α protein was found in A549 cells after their exposure to hypoxia (Figure 1C). Similarly, accumulation of HIF-1α was observed in nickel-treated A549 cells (*10*). To confirm that the HIF-1 transcription factor was involved in the *Cap43* gene expression, we compared the *Cap43* gene expression in response to hypoxia, as well as nickel and cobalt, in mouse fibroblasts that originated from normal or HIF-1α knockout mice. An induction of *Cap43* by hypoxia was found in fibroblasts that originated from HIF-$1\alpha^{+/+}$ mice but not in those from HIF-$1\alpha^{-/-}$ mice (Figure 2A). Similarly, the induction of *Cap43* gene expression by nickel or cobalt was also observed in fibroblasts that originated from HIF-$1\alpha^{+/+}$ mice but not in those from HIF-$1\alpha^{-/-}$ mice (Figure 2A). The induction of another gene, *GAPDH*, by nickel, cobalt, or hypoxia was also found in fibroblasts that originated from HIF-$1\alpha^{+/+}$ mice and not in cells from HIF-$1\alpha^{-/-}$ mice (Figure 2B). These data suggest that the induction of gene expression in response to either hypoxia or metals requires the HIF-1 transcription factor.

We examined, by immunohistochemical staining, the levels of Cap43 protein in a variety of human normal and cancer tissues. An antibody was raised against a 30-amino acid sequence at the C-terminal end of the Cap43 protein, which includes three 10-amino-acid repeats. Figure 3 shows a variety of normal and cancer cells stained with this Cap43 polyclonal antibody. To understand whether elevations of Cap43 correlated with a state of hypoxia, we also stained tissues with an antibody to HIF-1α (Figure 4). Figure 3A shows lung cancer tumor cells, as well as surrounding normal tissue. Cap43 antibody preferentially stains malignant lung cancer cells, including both non–small-cell and small-cell types. Figure 4D and E shows normal lung tissue such as the bronchus or the alveolus have very low expression of Cap43 compared with the cancer cells (Figure 3). As shown in Table 1, all 10 of the lung cancer tissues from 10 different patients had elevations of Cap43 in the cancer cells, whereas surrounding normal cells did not. In contrast, HIF-1α was present in both normal and lung cancer cells (Figure 5A, Table 1). As shown in Figure 5A, HIF-1α was present at higher levels in some cancer cells but not to the extent that was found with Cap43. Cap43 stains the nucleus as well as the cytoplasm of the neoplastic cells but nonneoplastic lung cells such as the bronchus and alveolus stain very poorly with Cap43 antibody (Figure 4D, E). Cap43 protein is generally found at low levels in most normal tissues, with the exception of

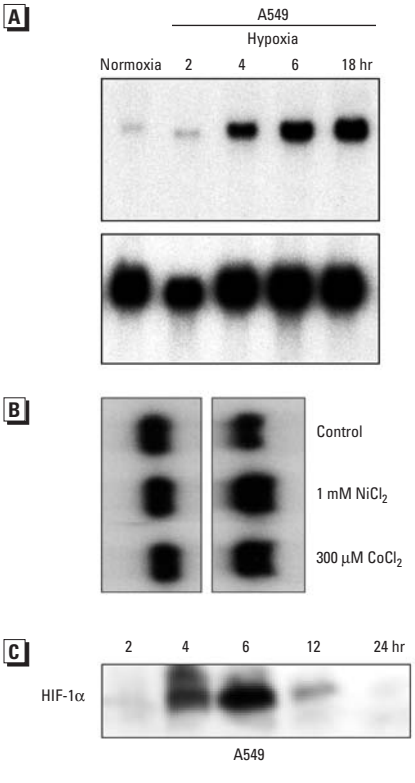


Figure 1. Induction of *Cap43* and *GAPDH* gene expression in A549 cells by metals or hypoxia. (*A*) Induction of *Cap43* gene expression by hypoxia. A549 cells were exposed to hypoxia (0.5% $O_2$) for the time periods indicated. Fifteen micrograms of total RNA were isolated and subjected to Northern blot analysis. The blot was first hybridized with the *Cap43* probe (top); then the membrane was stripped and rehybridized with β-actin (bottom). (*B*) Induction of *GAPDH* gene expression in A549 cells by metals. A549 cells were exposed to 1 mM $NiCl_2$ or 300 µM $CoCl_2$ for 20 hr. Fifteen micrograms of total RNA were isolated and subjected to a Northern blot analysis. The blot was first hybridized with the *GAPDH* probe (right); then the membrane was stripped and rehybridized with β-actin (left). (*C*) Time course of HIF-1α induction by hypoxia in A549 cells. A549 cells were exposed to hypoxic conditions (0.5% $O_2$) for the time periods indicated. Forty micrograms of nuclear extracts were subjected to Western blot analysis.

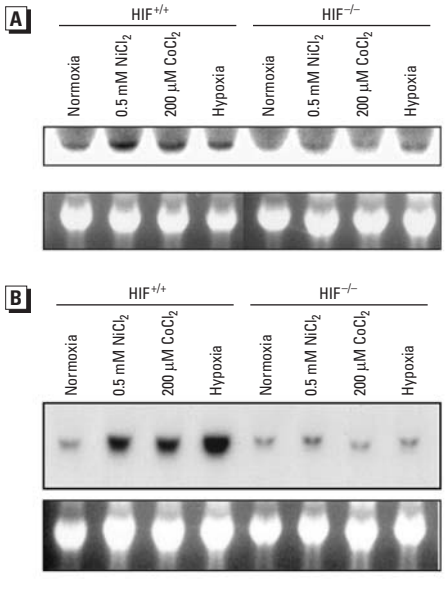


Figure 2. HIF-1 transcription factor was involved in nickel-induced gene expression. (*A*) Induction of *Cap43* gene expression in mouse HIF-$1\alpha^{+/+}$ or HIF-$1\alpha^{-/-}$ fibroblasts by nickel or hypoxic conditions. Cells originating from HIF-$1\alpha^{+/+}$ or HIF-$1\alpha^{-/-}$ mice were exposed to hypoxia (0.5% $O_2$), $NiCl_2$, or $CoCl_2$ for 20 hr. Fifteen micrograms of total RNA were isolated and subjected to a Northern blot analysis. (*B*) Induction of *GAPDH* gene expression in HIF-$1\alpha^{+/+}$ or HIF-$1\alpha^{-/-}$ mouse fibroblasts by nickel or hypoxic conditions. *GAPDH* gene expression was assessed by Northern blot analysis. Bottom panels show loading control.

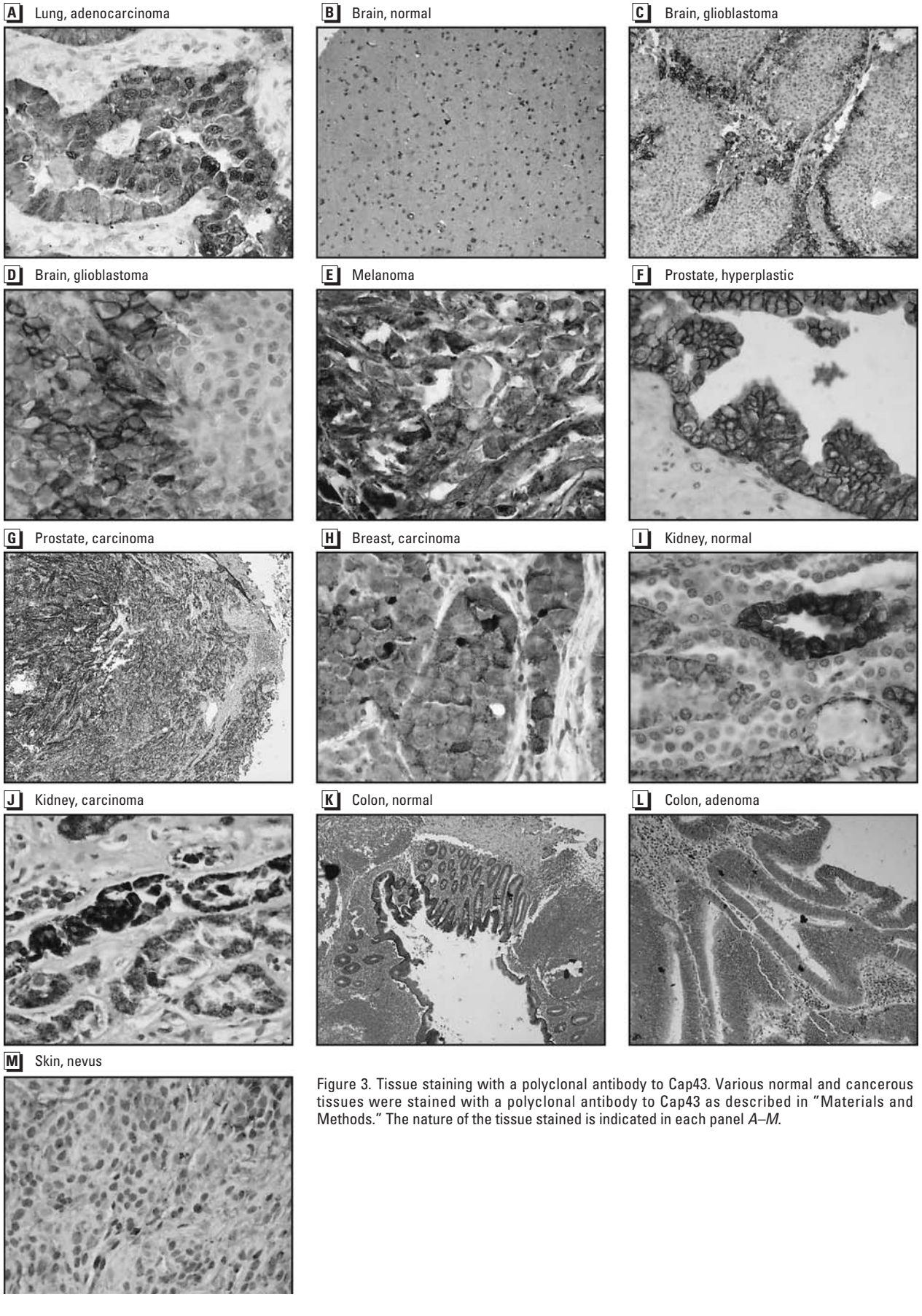


Figure 3. Tissue staining with a polyclonal antibody to Cap43. Various normal and cancerous tissues were stained with a polyclonal antibody to Cap43 as described in "Materials and Methods." The nature of the tissue stained is indicated in each panel *A–M.*

some higher expression in the distal and proximal convoluted tubules of the kidney (Figure 3I). The distal and proximal convoluted tubules of the kidney also express HIF-1α (Figure 5C). There is also expression of Cap43 protein in colon mucosa, colon smooth muscle, and normal prostate, as well as some expression in normal breast cells and in normal lung tissue (Figures 3, 4). However, the expression of Cap43 in the cancer cells of these tissues is considerably higher or very different, as shown in Figure 3. Figure 3B shows a low level of Cap43 protein expression in normal human brain. Cells in the brain slices that we examined express low levels of Cap43 protein (Table 1). Figure 3C is a low-magnification view of a malignant human glioblastoma multiform; Figure 3D shows a higher magnification of this brain tumor and surrounding normal tissue. Cap43 antibody preferential stains the tumor cells adjacent to the necrotic areas. The staining of Cap43 in these cells is localized to the membrane. In this particular case, HIF-1α antibody also similarly stained the same areas as did Cap43 antibody (Figure 5G), suggesting that the induction of HIF-1α by hypoxia may be responsible for the elevation of Cap43 in the malignant brain cells (Figures 3C, D, 5G). Table 1 shows that both for astrocytomas and for hemangioblastoma tumors, there is intense staining for both Cap43 and HIF-1α in several different patients.

Figure 3E shows a malignant melanoma with intense Cap43 staining in the cancer cells, whereas normal skin has little Cap43 staining (Figure 3M). In contrast, melanoma has little staining for HIF-1α (Figure 5B). Figure 3F shows hyperplastic prostatic epithelial cells that stained positively for Cap43. Figure 3G shows a low magnification of Cap43 staining of a high-histological-grade prostatic adenocarcinoma. Cap43 staining is intense in the tumor cells and very different from the normal cells. Figure 3H as well as Figure 4A–C shows that normal breast cells stain poorly for Cap43 but that the cells of invasive ductal carcinoma *in situ* stain intensely for Cap43. Table 1 shows the total number of patients examined for normal breast (0 of 6) and breast cancer. In contrast to Cap43 (4 of 4), HIF-1α (2 of 4) is not as high in breast cancer cells as is Cap43. The distribution of Cap43 in breast cancer cells seems localized to the membrane predominantly and focally to the cytoplasm. The staining is consistent for either histological grade of breast cancer.

Figure 3I shows the staining of the proximal and distal convoluted tubules of the normal kidney. There is no staining for HIF-1α and Cap43 in the glomerulus; however, there was differential staining of the tubular system for both Cap43 (Figure 3I) and HIF-1α (Figure 5C). The proximal tubules appear to have polarized membrane staining, whereas the distal convoluted tubules have homogeneous cytoplasmic staining for Cap43 (Figure 3I). Figure 3J shows a renal cancer (renal cell carcinoma) demonstrating diffused cytoplasmic staining for Cap43. Figure 3K shows a normal colon, and Figure 3L shows an adenoma of the colon. There is less staining of Cap43 in the colon cancer cells compared with the normal cells. The lower levels of Cap43 protein in colon cancer have been reported by other groups of investigators (*1*). Similarly, HIF-1α was higher in normal colon (Figure 5F) compared with colon cancer (Figure 5E). We have also stained many of the same tumor tissues with CYP1B1 that were stained with Cap43 and HIF-1α. However, as reported by another group (*12*), CYP1B1 is widely expressed at higher levels in normal cells and thus is not a good cancer marker. It should be noted, as shown in Figure 6, that the antibodies used to stain tissues for Cap43 and HIF-1α stain only one protein with Western blot analysis.

## Discussion

There have been very few proteins that exhibit cancer-specific overexpression. One promising cancer-specific marker has been CYP1B1,

Table 1. Presence of Cap43 and HIF-α proteins in various normal and malignant tissues.

| | Cap43 | HIF-α |
|---|---|---|
| Normal lung | 0/10 | 10/10 |
| Lung cancer | 10/10 | 10/10 |
| Normal liver | 0/3 | 0/3 |
| Liver cancer | 3/3 | n.d. |
| Normal breast | 0/6 | 0/6 |
| Breast cancer | 4/4 | 2/4 |
| Lymphocytes (tonsils) | 0/3 | 0/3 |
| Smooth muscle | 0/30 | 10/30 |
| Smooth muscle tumor | 4/6 | n.d. |
| Normal brain | 0/3 | 0/3 |
| Brain cancer astrocytoma | 7/7 | 7/7 |
| Brain cancer hemangioblastoma | 3/3 | 3/3 |
| Renal cancer | 22/22 | 0/3 |

Normal and cancerous tissues were stained with either Cap43 or HIF-1α antibody as described in "Materials and Methods." Data are from single tissue samples from individual patients. Low expression levels or background expression of normal breast and lung cells (as shown in Figure 4) was counted as 0.

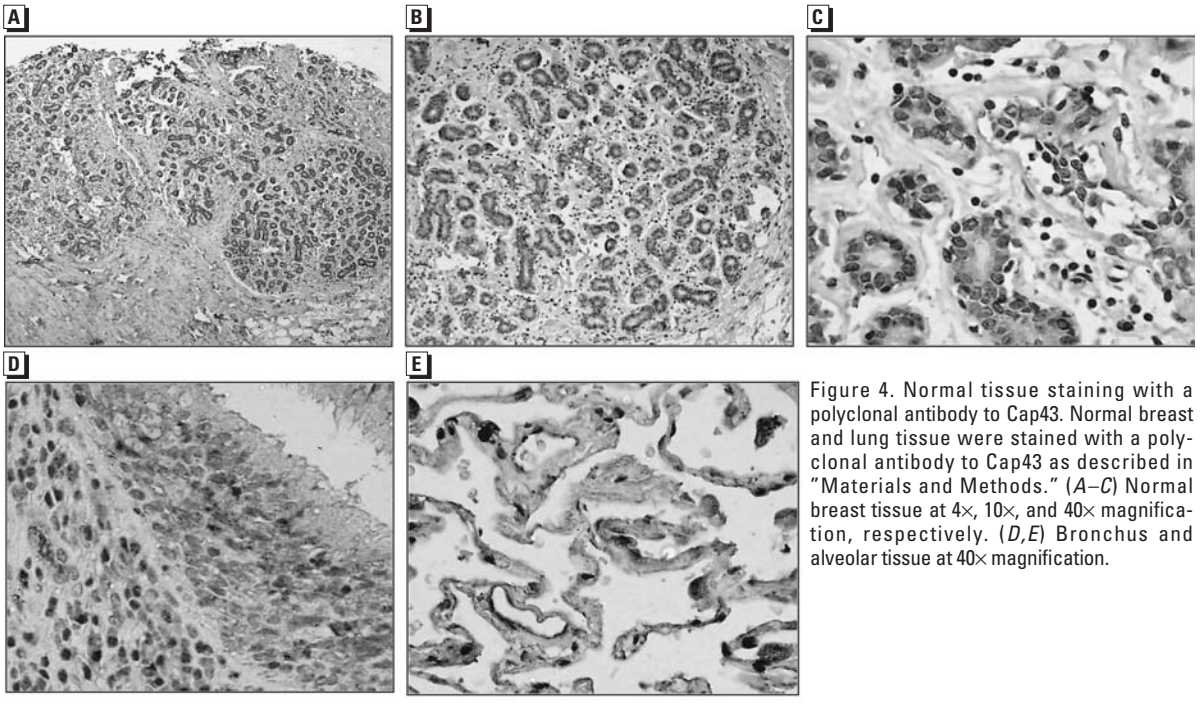


Figure 4. Normal tissue staining with a polyclonal antibody to Cap43. Normal breast and lung tissue were stained with a polyclonal antibody to Cap43 as described in "Materials and Methods." (*A–C*) Normal breast tissue at 4×, 10×, and 40× magnification, respectively. (*D,E*) Bronchus and alveolar tissue at 40× magnification.

which was found by a group of investigators to be overexpressed in a variety of cancer cells (*12*). However, another group of investigators was unable to repeat this finding and found CYP1B1 to also be highly expressed in normal tissue (*13*). Our results are in agreement with this latter group. *p53* mutations greatly increased the half-life of the protein in cancer cells, which may be a cancer-specific marker for populations of cancer cells that have *p53* mutations (*14,15*). Although *p53* mutations are common in many kinds of cancer, they are not sufficiently high in incidence for p53 protein overexpression to be used as a cancer-specific marker. With the exception of colon cancer, which tends to have decreased levels of Cap43 for reasons that are unknown, most of the other major cancers overexpress Cap43 protein. In some of these cancers, the reason for the overexpression of Cap43 protein may be the hypoxic conditions of the tumor cells or adjacent tissues that overexpress HIF-1α, which induces Cap43. In fact, in some instances HIF-1α was also elevated in the same cancer cells in which Cap43 was overexpressed. However, in other instances, HIF-1α does not stain all the tumor cells that overexpress Cap43, although consistently the antibody to Cap43 protein stains the tumor cells. These differences may be due to Cap43 protein being considerably more stable than HIF-1α, and thus Cap43 may be a better signature for hypoxic cells than HIF-1α. Another possible explanation is that some of the Cap43 induction may be HIF-1 independent, as has recently been shown for p27 induction by hypoxia (*16*).

The remarkable overexpression of Cap43 protein makes it an extremely valuable marker for cancer cells, and in fact, staining with Cap43 antibody allows one in many instances to identify cancer cells from normal cells. There is also the possibility of Cap43 protein being present in serum, and it might be elevated in patients who have cancer. Thus, the elevation of Cap43 protein in human serum may be used as a test for early cancer detection. However, these studies have not been conducted at the present time, and future studies will address this problem. Additionally, it may be possible to direct therapy toward Cap43 protein with drugs

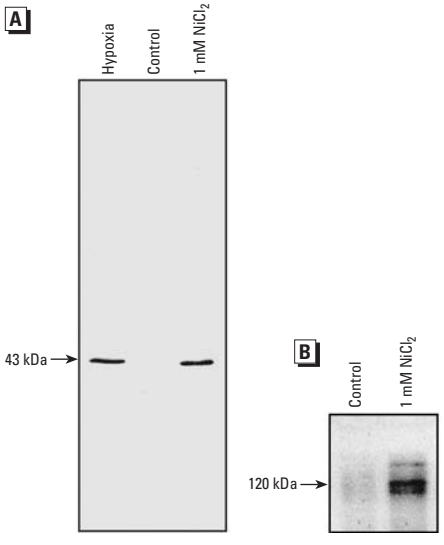


Figure 6. Western blot of human cellular extract stained with Cap43 and HIF-1α antibody. Cap43 and HIF-1α were induced in human cells with either hypoxia or NFi, and protein was separated with sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Antibody staining was accomplished by Western blot transfer using a Cap43 antibody (*A*) or an HIF-1α antibody (*B*).

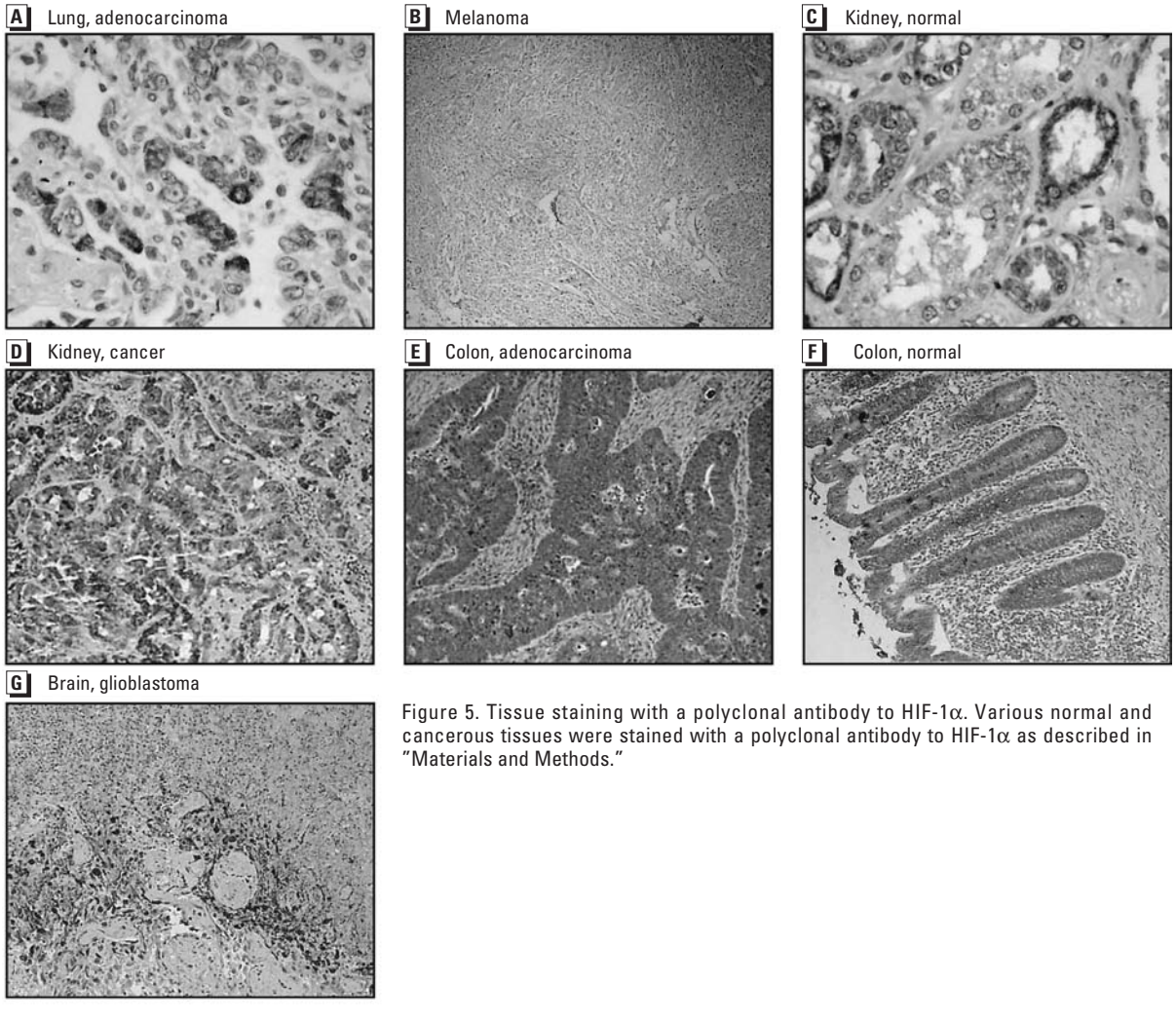


Figure 5. Tissue staining with a polyclonal antibody to HIF-1α. Various normal and cancerous tissues were stained with a polyclonal antibody to HIF-1α as described in "Materials and Methods."

that specifically destroy Cap43 protein. Although the function of Cap43 protein is unknown at the present time, its induction by hypoxia and $Ca^{2+}$ suggests that it could play a pivotal function for cancer cell survival.

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