ORIGINAL ARTICLE

STRA13 expression and subcellular localisation in normal and tumour tissues: implications for use as a diagnostic and differentiation marker

A Ivanova*, S-Y Liao*, M I Lerman, S Ivanov, E J Stanbridge

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Correspondence to: Dr A V Ivanova, Laboratory of Immunobiology, Basic Research Program, SAIC-Frederick Inc, NCI-Frederick, Maryland 21702, USA; ivanova@mail.ncifcrf.gov

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*AI and SYL contributed equally to this study.

Background: STRA13 is a bHLH transcription factor that plays a crucial role in cell differentiation, proliferation, apoptosis, and response to hypoxia.
Objective: To assess STRA13 involvement in carcinogenesis and evaluate its diagnostic value.
Methods: A comprehensive analysis was undertaken of the endogenous protein expression in 389 normal and corresponding malignant specimens, using newly generated polyclonal antibodies.
Results: STRA13 was commonly expressed in epithelial cells of normal and neoplastic tissues where it was confined mostly to the nucleus. Intense cytoplasmic STRA13 immunoreactivity was characteristic of myoepithelial and differentiated squamous epithelial cells of all organ sites and their neoplastic counterparts, suggesting application of STRA13 as a myoepithelial cell marker. A distinctive apical granular cytoplasmic staining pattern observed in the pancreas and large intestine was retained in corresponding metastatic carcinomas, providing for identification of the primary sites of these disseminating tumours. In less differentiated tumours there was a tendency to lose the cytoplasmic staining or to switch to nuclear STRA13 staining. Analysis of STRA13, HIF-1α, and CAIX expression patterns in a large set of various tumours substantiated the association of STRA13 with HIF-1α expression and hypoxia in vivo. Investigation of the molecular mechanisms of STRA13 nucleo-cytoplasmic shuttling suggested that STRA13 employs nuclear import/export that utilises the NLS/NES motifs situated within the N-terminus and in the middle of the protein.
Conclusions: STRA13 may serve as a marker for myoepithelial cells, for the degree of tumour differentiation, and for identification of the primary site of certain metastatic tumours. In combination with CAIX and CAXII markers, it may lead to a more accurate classification of all renal carcinomas.

The bHLH-type transcriptional regulator STRA13, which is a capable of direct binding to the E-box (5′-CACGTG-3′),[1] is implicated in the pVHL/HIF, transforming growth factor β (TGFβ), and JAK/STAT pathways.[1–4] This transcription factor has been associated with many differentiation processes, such as neurogenesis,[5] adipogenesis,[6] chondrogenesis,[7] and also with the regulation of mammalian circadian rhythms.[8] Unexpectedly, a crucial role for STRA13 in the regulation of the early development of B and T cells, late B cell activation, and terminal differentiation emerged recently from studies on mouse knockout and transgenic models.[9 10] While experimental evidence supports an association of STRA13 with growth suppression[11] and the regulation of apoptosis,[4 10] the role of STRA13 in tumour progression remains unclear.[3]

Identification of STRA13 expressing cells is essential for the understanding of the role of STRA13 in human cancer and its prognostic value. We generated and affinity purified polyclonal antibodies against the 20 amino acid C-terminal peptide of the STRA13 protein and analysed STRA13 expression in 389 pathology specimens, including various tumours of breast, lung, kidney/urinary tract, the gastrointestinal tract, liver, pancreas, prostate, cervix, endometrium, ovary, skin, head and neck, secretory glands, and CNS. In this study, we analysed the expression patterns of STRA13 in normal tissues and corresponding neoplasms. We also compared STRA13, HIF-1α, and CAIX expression patterns in a large set of different tumours, and explored the possible molecular mechanism of STRA13 nucleo-cytoplasmic shuttling. We conclude that STRA13 antibodies can be used for identification of myoepithelial cells, and may help in the identification of the primary site of origin of certain metastatic tumours. Finally, we show that STRA13 antibodies in combination with CAIX and CAXII antibodies may advance the classification of renal tumours.

METHODS

Generation of anti-STRA13 polyclonal antibodies

A peptide corresponding to the C-terminal 20 amino acids of human STRA13 (NH_2-QALKPIPPLNLETKD-COOH) was synthesised and conjugated to keyhole limpet haemocyanin (KLH). The peptide was chosen on the basis of the antigenic analysis of the STRA13 protein. The peptide–KLH conjugate was injected into New Zealand rabbits; antiserum was obtained and affinity purified. All procedures described above were carried out by Washington Biotechnology Inc (Baltimore, Maryland, USA).

Western blot analysis

293T cells were transfected using Lipofectamine^{2000} (Invitrogen, Carlsbad, California, USA), according to the manufacturer's recommendations. Forty eight hours after transfection the cells were lysed in RIPA buffer (150 mM NaCl, 50 mM Hepes (pH 7.4), 10 mM NaF, 1 mM EDTA, 2 mM orthovanadate, 10% glycerol, 1% Triton, 1% deoxycholate, 0.5% SDS, protease inhibitors), after which the lysates were sheared by ultrasound and precleared by centrifugation at maximum speed for 15 minutes at 4°C. Cell lysates were further fractionated by 8–16% SDS-PAGE, transferred to a

Abbreviations: KLH, keyhole limpet haemocyanin; NES, nuclear export signal; NLS, nuclear localisation signal

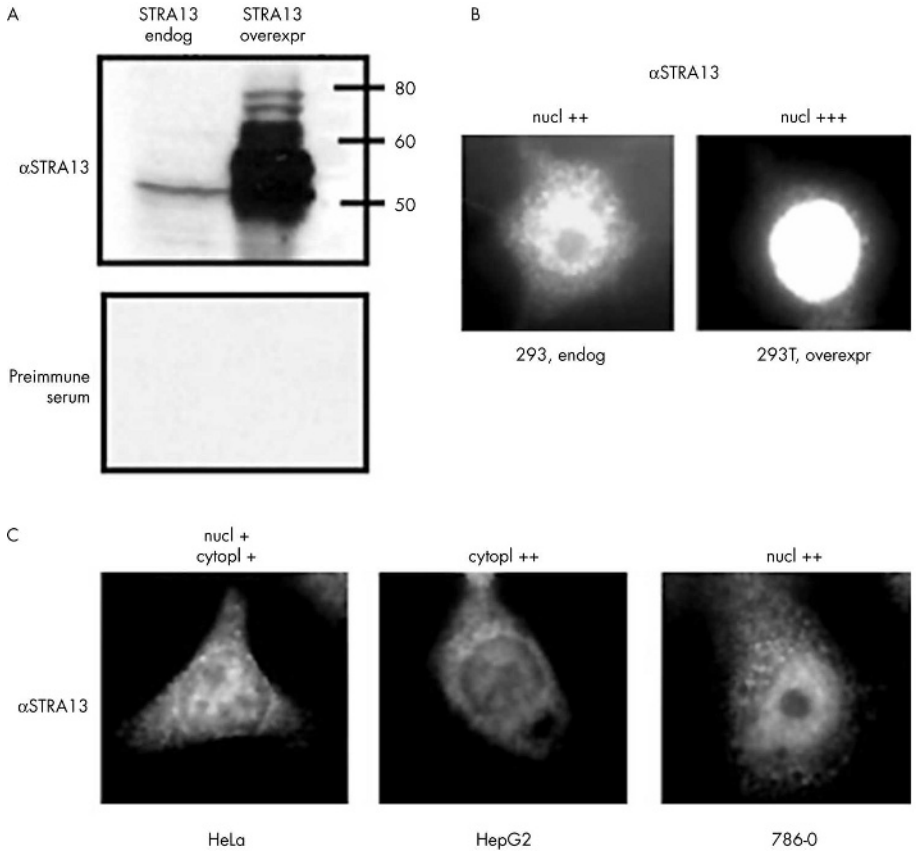

Figure 1 (A) Western blot analysis on the lysates of 293T cells transfected with pCMV2 and PCMV2/STRA13 plasmids. Upper panel: polyclonal STRA13 antibodies; lower panel: preimmune serum. (B) Immunostaining of non-transfected 293T cells (left) and transfected with FLAG/STRA13 plasmids (right) with STRA13 antibodies. (C) Cell specificity in STRA13 subcellular localisation. STRA13 polyclonal antibodies were used for immunostaining of different cell types. Cytopl+/++, predominantly cytoplasmic expression; nucl+/++, predominantly nuclear expression.

nitrocellulose membrane, and incubated with the STRA13 antibodies (1:1000) or preimmune serum (1:200). Proteins were visualised using the DURA ECL kit (Pierce, Rockford, Illinois, USA).

Immunofluorescent staining of cultured cells

Cells were cultured on coverslips overnight. In the morning, cells were fixed with 3% paraformaldehyde for 30 minutes and permeabilised with 0.1% Triton X-100 for five minutes. After preincubation with 1% fetal bovine serum, cells were incubated with primary antibodies against STRA13 (1:200) or with preimmune serum (1:100) at room temperature for one hour. This was followed by incubation with goat anti-rabbit Alexa Fluor conjugated secondary antibodies (Molecular probes, Eugene, Oregon, USA). Cells were mounted in mounting medium containing DAPI (Vector Laboratories, Burlingame, California, USA).

Tissue specimens

The normal adult tissues from all organ sites and the corresponding benign or malignant neoplastic tissues were obtained from 389 routine pathology specimens at St Joseph Hospital (Orange, California, USA). The tissues examined included brain, nose, throat, upper and lower respiratory system, upper and lower gastrointestinal system (oesophagus, stomach, small and large intestine), pancreas, liver, bile ducts and gallbladder, female and male urogenital system (kidney, ureter, bladder, testicle, prostate, cervix, uterus, fallopian tube, ovary), breast, adrenal gland, thyroid, parathyroid, salivary gland, spleen, lymph node, bone, cartilage, muscle, skin, and the body cavities. All tissue samples were processed within six hours of surgical resection and fixed in 10% neutral buffered formalin. The formalin fixed tissues were paraffin embedded, sectioned, and stained with haematoxylin and eosin (H&E) for light microscopy examination.

Immunohistochemical studies

The mouse monoclonal antibody (MN75) used to detected the MN/CAIX protein has been described previously.[12] The peroxidase method and the primary antibody dilution for CAIX has also been described before.[12 13] For STRA13 immunostaining we used the avidin–biotin enzyme complex kit (LSAB2, Dako Corporation, Carpinteria, California, USA). Briefly, 5 micron sections of paraffin embedded tissues were deparaffinised and pretreated in citrate buffer (pH 6.0) for 10 minutes in a pressure cooker. Endogenous peroxidase was blocked with methanol and 3% H_2O_2 for 10 minutes. All sections were incubated with primary antibody at a 1:250 dilution for 30 minutes, with biotinylated secondary linking antibody for 30 minutes, with the streptavidin–enzyme complex for 30 minutes, with diaminobenzidine as chromogen for 10 minutes, and with haematoxylin as a counterstain for two minutes. Positive and negative controls were included in each run. Selected cases ($n = 95$) were also immunostained with an anti-HIF-1α mouse monoclonal antibody (NeoMarkers Inc, Fremont, California, USA) at a 1:100 dilution. The sections were exposed to pressure cooking pretreatment in citrate buffer (pH 6.0) for 15 minutes and the Dako catalysed signal amplification system (CSA) was used according to the manufacturer's instructions.

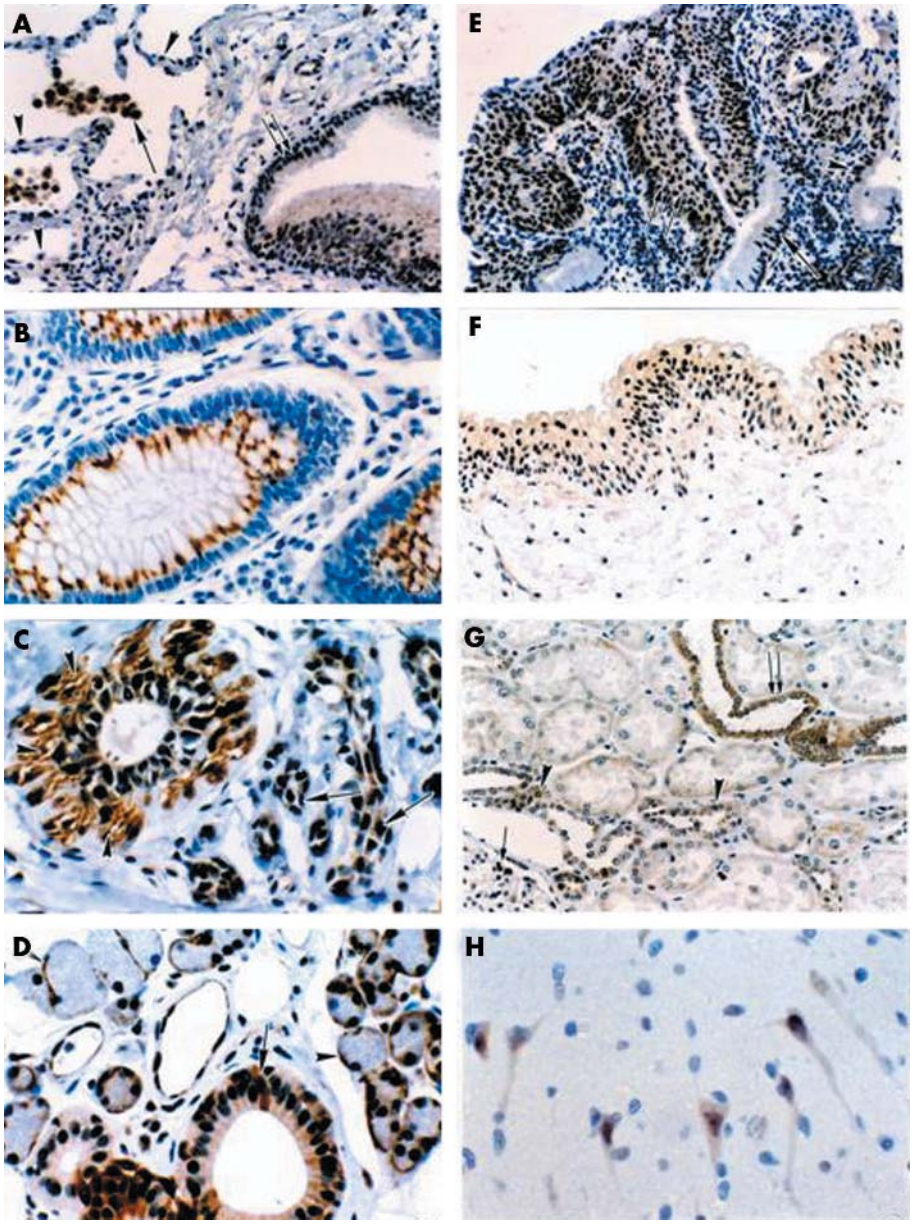

Figure 2 Stra13 nuclear staining, with a varying degree of cytoplasmic reactivity is seen in many organ sites. Nuclear/cytoplasmic staining in (A) bronchial epithelium (double arrows), type II alveolar cells (arrowhead) and alveolar macrophages (single arrow) and blood vessels; (C and D) acinar/ductal cells (arrow) of the breast and salivary gland; (E) the reserve cells (arrowhead), metaplastic squamous cells (double arrows) and rare columnar cells (single arrow) of the cervix; (F) the urothelial cells of the bladder; and (H) the neurones of the brain. In the kidney (G), cytoplasmic (double arrows) or nuclear (arrowhead) positivity, or both, is seen in the distal convoluted ducts and Henley's loop. Nuclear reactivity is also seen in the endothelial cells of the glomeruli (single arrow). In the colon (B), Stra13 expression is cytoplasmic and restricted to the apical region. Diffuse cytoplasmic staining is seen in all myoepithelial cells of the breast (C) and salivary gland (D). Original magnification, ×200 (B, C, D, H) and ×100 (A, E, F, and G).

Assessment of STRA13, CAIX, and HIF-1α protein expression

Slides stained with immunoperoxidase and haematoxylin–eosin were reviewed by one of the authors (SYL), and the immunohistochemical results were scored semiquantitatively on the basis of the percentage of positive cells present in a single section's total field. The intensity of staining was scored as very weak (+/−), weak (+), or strong (++). The percentage of cells with nuclear or cytoplasmic positivity, or both, was recorded as follows: diffuse (high expression) when more than 25% of cells were positive; focal (low expression) when equal or less than 25% of cells were positive; negative when there was no brown colour reaction or very weak (+/−) positivity.

RESULTS

Specificity of STRA13 antibodies and assessment of nuclear/cytoplasmic localisation of the STRA13 protein

Affinity purified STRA13 rabbit polyclonal antibodies specifically recognised the overexpressed STRA13 protein in 293T cells transfected with the STRA13/FLAG construct, as well as endogenous STRA13 (fig 1). In both cases bands showed the same electrophoretic mobility as the band produced with α-FLAG antibodies (data not shown). Western blot with preimmune serum did not reveal any signal (fig 1A, bottom panel) implying that the band shown in fig 1A (upper panel) was specific. Immunofluorescent staining of 293T cells transfected with the STRA13/FLAG construct using STRA13

antibodies showed predominantly nuclear localisation of the overexpressed protein, and nuclear/cytoplasmic localisation of the endogenous STRA13 protein in 293T cells (fig 1B). Immunostaining of HeLa, HepG2, and 786-0 cells with the anti-STRA13 antibodies showed cell specificity in the intracellular distribution of the STRA13 protein: in HeLa cells, STRA13 was equally present in both the nucleus and cytoplasm; in HepG2 cells, localisation of STRA13 was mostly cytoplasmic; and in the 786-0 cell line, nuclear expression of STRA13 was predominant (fig 1C). Preimmune serum that was used as a control did not produce any specific staining (data not shown).

STRA13 protein expression in normal adult human tissues

Nuclear localisation of STRA13 protein expression was observed in mesothelial cells and coelomic epithelium of the body cavities and in the respiratory epithelial cells of the larynx and trachea. In the lung, persistent nuclear expression with a varying degree of cytoplasmic reactivity was found in the bronchial epithelium, type II alveolar cells, and alveolar macrophages (fig 2A). Thus STRA13 expression in the lung was similar to that described previously.[14] In the gastrointestinal system, both nuclear and cytoplasmic staining patterns were identified. The nuclear staining was seen in the epithelial cells of the gastric and duodenal mucosa, gallbladder, bile ducts, and pancreatic ducts/acinar cells. The hepatocytes showed both nuclear and cytoplasmic staining. Interestingly, epithelial cells of the large intestine, including the rectum and appendix, showed a very characteristic diffuse apical cytoplasmic granular expression but no nuclear staining (fig 2B). In the breast, salivary glands, and sweat glands, nuclear staining was primarily seen in luminal cells of the secretory units. Intensive cytoplasmic or plasma membrane staining, or both, was also identified but was restricted to myoepithelial cells and a limited number of ductular/ductal cells (fig 2C and 2D). In the genital organs, positive nuclei were randomly distributed in the secretary endometrium, glandular cells of fallopian tubes and endocervix, reserve cells/metaplastic squamous cells of the cervix (fig 2E), the ductular unit, and the epididymis of the testis. A varying degree of nuclear staining was also observed throughout the urothelial epithelium (pelvis, ureter, and bladder) (fig 2F). In the kidney, STRA13 protein expression varied from case to case. In the majority of renal tissues nuclear staining was primarily seen in the endothelial cells of the glomeruli and distal convoluted ducts, though in some cases positive nuclei were seen throughout the ductal system. Granular cytoplasmic immunoreactivity was consistently noted in the distal convoluted ducts, Henle's loop and, to a lesser degree, in the collecting ducts (fig 2G). In the central nervous system, both nuclear and cytoplasmic positive staining was limited to the neurones and a few glial cells and the choroid plexus (fig 2H). A very weak nuclear staining was occasionally found in the germ cells of the testis and the sex cord cells of the ovary. In the mesenchymal tissues, a varying degree of positive nuclear immunoreactivity with or without cytoplasmic staining was present in the chondrocytes, endothelial cells, fibroblasts, and muscle, but the numbers of cells staining positively and the degree of staining intensity varied significantly depending on the organ sites tested. No immunoreactivity was observed in the lymph nodes, spleen, or prostate gland, with the exception of myoepithelial cells and macrophages.

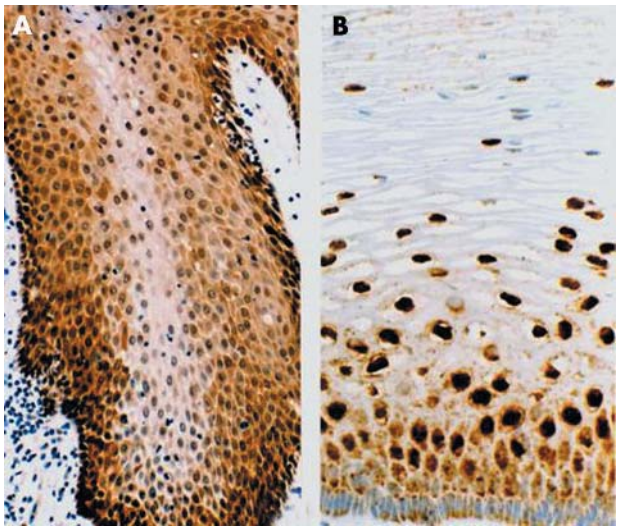

Figure 3 STRA13 expression in the normal squamous epithelium. (A) STRA13 immunoreactivity in most of the squamous epithelium is primarily cytoplasmic, with a varying degree of nuclear positivity. (B) STRA13 expression in the cervical squamous epithelium at the transformation zone is localised to the nucleus. Original magnification ×200 (A and B).

We persistently found STRA13 diffuse cytoplasmic staining in certain cell types. These cell types were myoepithelial cells, thymocytes, sebaceous glands of the skin, and terminally differentiated squamous cells. The STRA13 protein was consistently expressed in the cytoplasm of the squamous epithelium in all organ sites, such as oral cavity, tongue, oesophagus, anus, and vagina (fig 3A), and in the lower half of the epidermis and hair follicles. Interestingly, metaplastic squamous cells derived from the glandular type epithelium and the squamous epithelium of the cervix at the transformation zone retained the STRA13 nuclear localisation (fig 2E and 3B).

In this study, we also made a comparative evaluation of STRA13 and CAIX protein expression in normal adult tissues. While expression of the STRA13 protein, either nuclear or cytoplasmic or both, was widely distributed throughout the organ systems, the CAIX protein expression, in contrast, was limited to a few organ sites. Those cells that co-expressed STRA13 and CAIX were restricted to mesothelial cells and coelomic epithelium, the surface glands of stomach and duodenum, and the biliary tract system. Table 1 summarises the data on the distribution of the STRA13 and CAIX protein expression in normal tissues. The pattern of the CAIX protein expression was very similar to that reported previously.[15]

STRA13 expression in different tumours

We surveyed a large number of benign and malignant human tissues by immunostaining with STRA13 antibodies. The results are summarised in table 2 and illustrated in figs 4 to 6. A wide variety of examined tumours showed three major patterns of STRA13 staining:

- positive nuclei accompanied by a varying degree of weak cytoplasmic immunoreactivity;
- diffuse/intensive cytoplasmic reactivity with or without positive nuclei;
- granular cytoplasmic reactivity, either diffuse or limited to the apical region of cells.

With a few exceptions, the pattern of STRA13 expression in a given tumour was similar to the corresponding normal tissues.

Tumours with STRA13 nuclear localisation

The majority of the tumours studied showed predominantly nuclear localisation of STRA13. High levels of nuclear staining were commonly observed in adenocarcinomas

Table 1 The distribution of STRA13 and CA IX protein expression in normal adult human tissues

	STRA13		CA IX
	Nuclear staining	Cytoplasmic staining	Membrane/cytoplasm
Lining cells of body cavity			
Mesothelial cells (serous membrane)	Focal	Negative	Diffuse
Coelomic epithelium (surface of the ovary)	Focal	Negative	Diffuse
Squamous mucosa of all organs	Variable	Diffuse	Negative
Oral mucosa, tonsil, larynx	Rare	Diffuse	Negative
Tongue, oesophagus	Focal	Diffuse	Negative
Anus/vagina	Rare	Diffuse	Negative
Ectocervix	Diffuse	Focal	Negative
Myoepithelial cells of all organs			
Salivary glands, breast, prostate gland	Negative	Diffuse	Negative
Skin			
Epidermis, rare	Diffuse	Focal	
Sweat/sebaceous glands	Rare	Diffuse	Negative
Hair follicle	Rare	Diffuse	Focal
Upper/lower respiratory system			
Respiratory epithelial cells	Focal	Negative	Negative
Reactive reserve cells	Focal	Negative	Rare
Type II alveolar cells	Diffuse	Weak	Negative
Alveolar macrophges	Rare	Diffuse	Negative
Gastrointestinal system			
Gastric/duodenal surface glands	Diffuse	Negative	Diffuse
Crypt cells of duodenum/small intestine, appendix	Negative	Negative	Diffuse/focal
Crypt cells of large intestine	Negative	Negative	Rare
Surface glandular cells of large intestine	Negative	Diffuse	Negative
Pancreas			
Ductal cells	Focal	Negative	Negative
Acinar cells	Focal	Negative	Negative
Gallbladder/biliary tract	Diffuse	Negative	Diffuse
Genitourinary system			
Kidney			
Proximal convoluted ducts	Variable	Negative	Negative
Distal convoluted ducts	Variable	Diffuse	Negative
Collecting duct	Negative	Variable	Negative
Renal pelvis/ureter/bladder			
Transitional cells	Focal	Negative	Negative
Prostate gland	Negative	Negative	Negative
Testis			
Ductular unites/epididymis	Focal	Negative	Negative
Germ cells	Rare	Negative	Negative
Uterine cervix			
Mature squamous cells	Diffuse	Weak/negative	Negative
Basal cells of squamous mucosa	Negative	Diffuse	Negative
Reactive reserve cells of the glands	Diffuse	Negative	Rare
Metaplastic squamous cells	Diffuse	Negative	Rare
Uterine endometrial glands			
Proliferative phase	Negative	Negative	Negative
Secretory phase	Variable	Negative	Negative
Ovary			
Surface coelomic epithelium	Focal	Negative	Diffuse
Granulosa/theca cells	Focal	Negative	Negative
Breast			
Myoepithelial cells	Negative	Diffuse	Negative
Ductal units	Focal	focal	Negative
Lobular units	Diffuse	Negative	Negative
Lymph node/spleen			
Lymphocytes	Negative	Negative	Negative
Macrophages	Negative	weak	Negative
Thymus gland			
Lymphocytes	Negative	Negative	Negative
Thymocytes	Negative	Diffuse	Negative
Central nervous system			
Glial cells	Rare	Rare	Negative
Neurone	Diffuse	Diffuse	Negative
Choroid plexus	Focal	Focal	Diffuse

(62%) and squamous cell carcinomas (75%) of the lung, cervical intraepithelial neoplasia (50%), and in situ adenocarcinomas (75%) of the cervix, lobular/tubular carcinomas of the breast (100%), transitional cell carcinomas of the urothelial epithelium (72%), and clear cell carcinomas of the kidney (56%). Typical patterns of STRA13 staining in

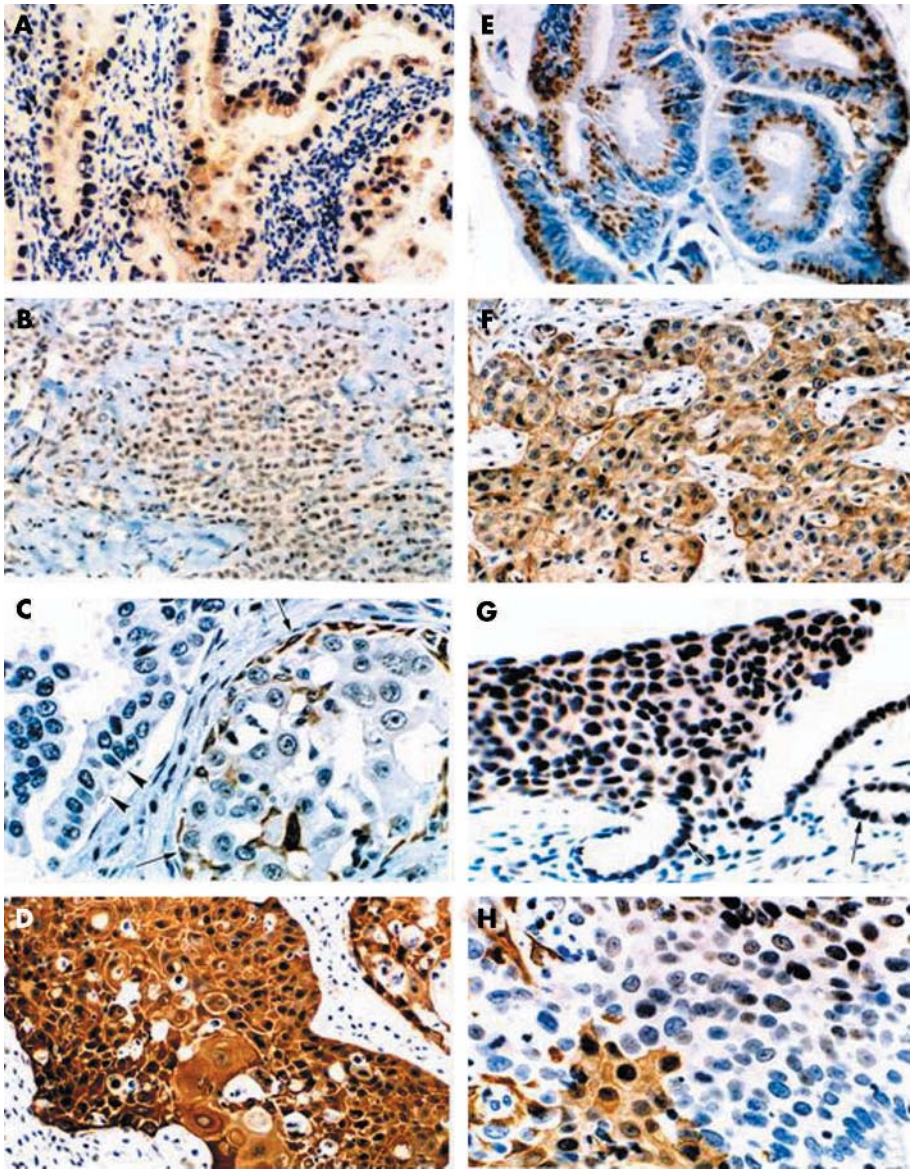

Figure 4 STRA13 expression in the tumour tissues recapitulates the normal tissue distribution. (A) STRA13 high nuclear expression in the adenocarcinomas of the lung, (B) lobular carcinomas of the breast, and (G) immature dysplastic cervical epithelium; note the nuclear reactivity in the normal reserve/columnar cells (arrow). (C) STRA13 expression in the myoepithelial cells is persistent in the in situ (arrow) but not in the invasive (arrowhead) ductal carcinoma. (D) STRA13 diffuse cytoplasmic reactivity in squamous cell carcinomas of the larynx and (E) apical cytoplasmic reactivity in the colonic adenocarcinomas. (F) STRA13 aberrant cytoplasmic expression in the high grade ductal carcinomas of the breast. (H) In cervical squamous cell carcinomas, STRA13 nuclear expression in the less differentiated cells and cytoplasmic expression in the highly differentiated squamous cells. Original magnification ×200.

tumours of lung and breast are shown in figs 4A and 4B. A high level of nuclear expression was observed in primitive neuroectodermal tumours, high grade gliomas (including glioblastoma multiforme), and mesotheliomas. However, owing to the very limited number of these tumour samples, these observations should be considered preliminary. In the benign lesions, diffuse nuclear positivity was seen in all cases of inverted papillomas of the larynx, bile duct adenomas, and in 92% of ovarian mucinous tumours.

Tumours with cytoplasmic expression

All tumours composed of myoepithelial cells or differentiated squamous cells showed a high level of cytoplasmic positivity. Thus the cytoplasmic STRA13 immunoreactivity may serve in a given tumour as an indicator of myoepithelial cells or terminally differentiated squamous cells. For example, STRA13 protein expression was seen in the myoepithelial component of all mixed tumours of the salivary glands (n = 6), fibroadenomas (n = 3), and in situ ductal carcinoma of the breast (n = 6) (fig 4C). High levels of STRA13 expression were also observed in all squamous cell carcinomas derived from squamous epithelium such as skin (n = 5), oral mucosa/tongue, and larynx (n = 12) (fig 4D).

Tumours with granular cytoplasmic immunoreactivity

The granular cytoplasmic positivity was found mainly in the pancreas, large intestine, and kidney (fig 2B). This pattern was reproduced in 70% of primary adenocarcinomas of the pancreas and large intestine (fig 4E), and in 100% of colonic adenomas. Interestingly, even in metastatic carcinomas the pattern of expression was retained as compared to the tissue of origin. Thus apical granular cytoplasmic STRA13 positivity

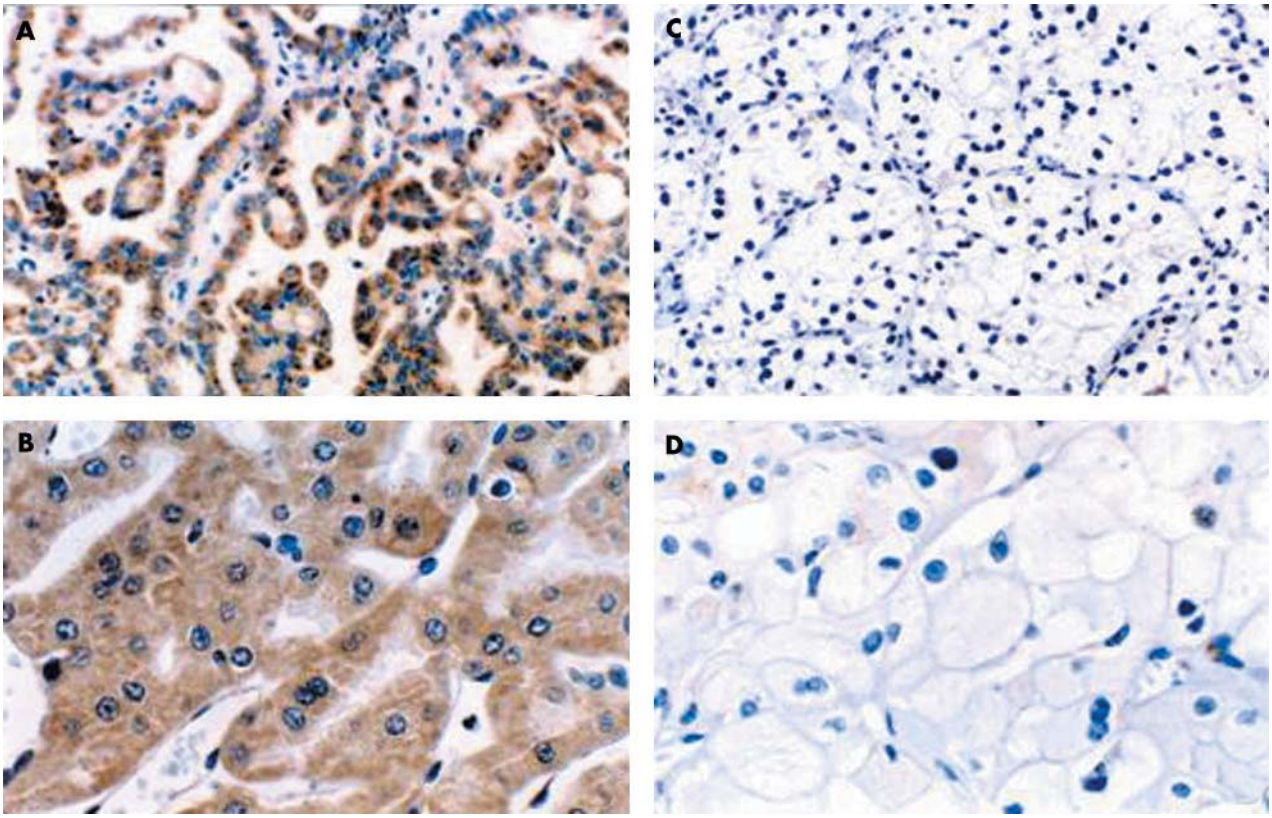

Figure 5 Examples of STRA13 expression in the renal cell neoplasms. Diffuse cytoplasmic staining is consistently present in the papillary carcinomas (A) and oncocytomas (B). A varying degree of nuclear positivity is seen in the majority of clear cell carcinomas (C), but no immunoreactivity is observed in the chromophobe cell carcinomas (D). Original magnification ×200.

in the metastatic disease may point to the pancreas or the large intestine as sites of tumour origin.

In the kidney, the diffuse granular cytoplasmic STRA13 staining was seen in all papillary renal cell carcinomas (n = 3), collecting duct carcinomas (n = 3), tubular adenomas (n = 2), and oncocytomas (n = 7), but not in clear cell carcinomas (n = 25) or chromophobe cell carcinomas (n = 8). While clear cell carcinomas showed STRA13 nuclear staining, chromophobe cell carcinomas appeared to be STRA13 negative (fig 5A to 5D). Thus STRA13 staining may aid the classification of renal cell tumours.

Association of STRA13 protein localisation with the tumour type and the degree of differentiation

The most fascinating observation made in this study was the switch from cytoplasmic to nuclear STRA13 localisation or vice versa within a given tumour, depending on the degree of differentiation. We consistently observed this phenomenon in the tumours of the breast, cervix, transitional mucosa, large intestine, and the squamous mucosa of all organs. Although STRA13 staining in tumours appeared to recapitulate the normal pattern of expression, there was a tendency to lose or show an aberrant STRA13 expression in the high

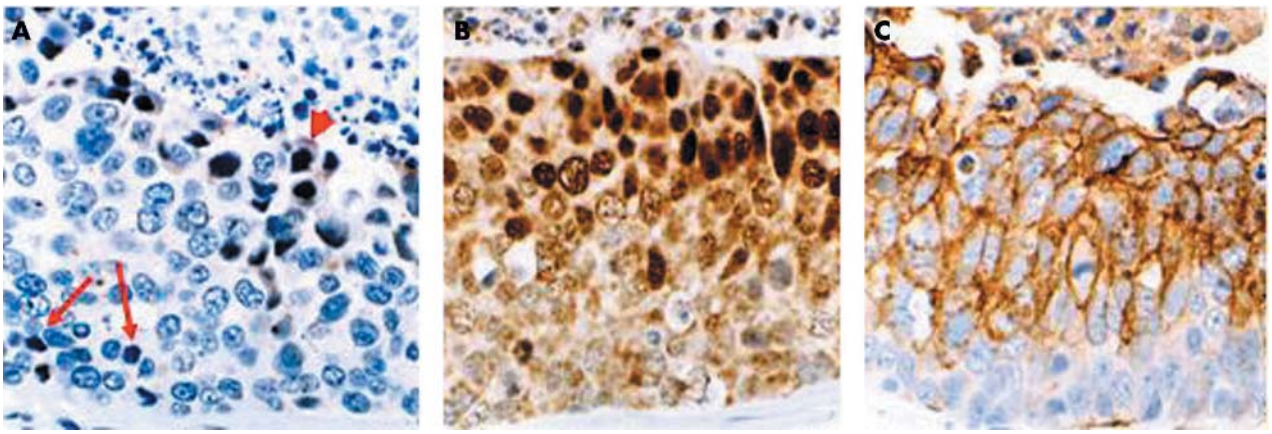

Figure 6 Co-expression of STRA13 (A), HIF-1α (B), and CA IX (C) in cervical carcinomas. The STRA13 positive cells are not restricted to the region adjacent to the necrosis (arrowhead) but are also seen in the areas away from necrosis (arrow). The HIF-1 α and CA IX expression is restricted to the necrotic/hypoxic regions. Original magnification ×400.

Table 2 Expression of STRA13 and CA IX in benign and malignant human tumours

Tumour category	Cases studied (n)	STRA13: Positive cytoplasmic (C+) and/or nuclear (N+) staining (n (%))				CA IX: Positive staining (n (%))
		(C+) and (N+)	C+ only	N+ only	High N+ expression (>25%)	Membrane/cytoplasm
Cervix						
Carcinomas	28	23 (82)	2 (7)	21 (75)	10 (36)	27 (96)
Squamous cell Ca	15	15 (100)	2 (7)	13 (87)	6 (40)	14 (92)
Adenoca	9	7 (78)	0	7 (78)	4 (44)	9 (100)
Adenosquamous Ca	4	1 (33)	0	1 (33)	0	4 (100)
CIN (I–III)	14	13 (93)	6 (40)	7 (50)	7 (50)	14 (100)
In situ adenoca	4	4(100)	0	4 (100)	3 (75)	4 (100)
Uterine corpus						
Endometrial Ca	9	9 (100)	2 (22)	9 (100)	4 (44)	9 (100)
Ovary						
Epithelial Ca of all types	25	18 (72)	0	18 (72)	9 (36)	20 (80)
Cystadenoma benign or LMP	13	13 (100)	1 (8)	12 (92)	12 (92)	13 (100)
Sex cord tumour	6	4 (67)	4 (67)	0	0	3 (50)
Metastatic Ca of colon primary	4	4 (100)	4 (100)	0	0	4 (100)
Germ cell tumour	5	3 (60)	0	3 (60)	0	2 (40)
Breast						
Ductal Ca	40	26 (70)	8 (25)	22 (55)	12 (30)	14 (35)
high grade	15	9 (60)	8 (53)	3 (20)	1 (6)	11 (73)
intermediate/low grade	25	19 (76)	2 (8)	19 (76)	11 (44)	3 (12)
Lobular Ca	11	11 (100)	1 (9)	11(100)	11 (100)	0
Kidney/urinary tract						
Transitional cell Ca	11	10 (91)	0	10 (91)	8 (73)	11 (100)
Squamous cell Ca	3	3 (100)	3 (100)	0	0	3 (100)
Renal cell Ca	42	36 (86)	22 (52)	19 (45)	15 (36)	34 (81)
Subtype:						
Clear cell	25	19 (76)	5 (20)	19 (76)	14(56)	25 (100)
Papillary	6	6 (100)	6 (100)	0	0	6 (100)
Chromophobe cell	8	8 (0)	8 (0)	0	0	0
Collecting duct	3	3 (100)	3 (100)	0	0	3 (100)
Oncocytoma	7	7 (100)	7 (100)	0	0	0
Tubular adenoma	2	2 (100)	2 (100)	0	0	0
Wilm's tumour	3	3 (100)	1 (33)	2 (67)	0	2 (67)
Prostate						
Adenocarcinoma	2	0	0	0	0	0
Gastrointestinal tract						
Stomach/duodenum adenoca	3	3 (100)	3 (100)	1 (33)	1 (33)	3 (100)
Colon/pancreas adenoca	40	34 (85)	32 (80)	13 (33)	0	37 (93)
Colon adenoma	3	3 (100)	3 (100)	0	0	3 (100)
Liver						
Hepatoma	6	5 (83)	5 (83)	0	0	3 (50)
Bile duct adenoma	2	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)
Lung						
Squamous cell Ca	12	12 (100)	12 (100)	9 (75)	9 (75)	12 (100)
AdenoCa	13	12 (92)	4 (31)	11 (85)	8 (73)	10 (77)
Head and Neck						
Squamous cell Ca	10	10 (100)	8 (80)	6 (60)	4 (67)	10 (100)
Inverted papilloma	2	2 (100)	0	2 (100)	2 (100)	2 (100)
Salivary glands						
Pleomorphic adenoma	4	4 (100)	4 (100)	0	0	2 (50)
Mucoepidermoid Ca	3	3 (100)	3 (100)	0	0	3 (100)
Acinic cell Ca	4	3 (75)	0	3 (75)	3 (75)	0
Thyroid gland						
Papillary/follicular Ca	3	3 (100)	3 (100)	0	0	0
Tumours with secretory gramules						
PNET	2	1 (50)	0	1 (50)	1 (100)	0
Neuroblastoma	10	7 (70)	7 (70)	0	0	0
Paraganglioma	2	2 (100)	2 (100)	0	0	0
Phaeochromocytoma	2	0	0	0	0	0
Neuroendocrine Ca	4	1 (25)	0	1(25)	1 (100)	4 (100)
Carcinoid tumour	5	4 (80)	2 (40)	5 (80)	2 (40)	0
Central nervous system						
Glioma						
Low grade (grade I–II)	7	7 (71)	1 (14)	7 (71)	1 (14)	0
High grade/glioblastoma	8	7 (88)	7 (100)	7 (88)	7 (88)	8 (100)
Oligodendroglioma	2	0	0	0	0	0
Ependymoma	4	0	0	0	0	4 (100)
Meningioma	2	2 (100)	2 (100)	0	0	2 (100)
Choroid plexus tumour	3	3 (100)	3 (100)	2(67)	1(33)	3 (100)
Body cavity						
Mesothelioma	2	1 (50)	0	1 (50)	1 (100)	2 (100)
Skin						
Squamous/basal cell Ca	5	5 (100)	5 (100)	0	0	4 (80)
Melanoma	2	0	0	0	0	0

Adenoca, adenocarcinoma; Ca, carcinoma; CIN, cervical intraepithelial neoplasia; LMP, low malignancy potential; PNET, primitive neurectodermal tumour.

grade or poorly differentiated carcinomas. For example, in the tumours of the breast high levels of STRA13 nuclear staining were seen in 100% of lobular and tubular carcinomas (fig 4B), in 44% of ductal carcinomas of low or intermediate grade, and only in 6% of high grade ductal carcinomas. In contrast, diffuse cytoplasmic positivity was seen in 53% of high grade ductal carcinomas, as opposed to only 8% of low or intermediate grade ductal carcinomas (fig 4F). Other examples were squamous cell carcinomas of all organs. Well differentiated squamous carcinomas derived from the squamous mucosa always showed diffuse cytoplasmic immunoreactivity (fig 4D). However, there was a tendency to lose cytoplasmic expression or to switch from cytoplasmic to nuclear localisation when the tumours became less differentiated. In particular, in the cervix immature type CIN (cervical intraepithelial neoplasia) recapitulated the normal pattern of STRA13 expression (fig 4G) but the differentiated type CIN showed diffuse cytoplasmic positivity (fig 4H). STRA13 nuclear immunoreactivity was usually reduced in the invasive carcinomas as compare to the CIN lesions (fig 4H).

The correlation between STRA13, CAIX, and HIF-1α protein expression in the tumours

The transcription factor STRA13 has been identified as one of a plethora of pVHL/HIF targets regulated at the mRNA level.[3 16] However, the role of STRA13 in tumour response to hypoxia remains to be elucidated.[3] Giatromanolaki and colleagues reported that STRA13 expression was associated with the increase in HIF-1α and CAIX expression.[14] CAIX is a pVHL/HIF target expressed on the cell surface of many epithelial tumours under hypoxic conditions.[17–21] We surveyed 95 cases of various tumour tissues for the co-expression of STRA13, CAIX, and HIF-1α. In this particular survey, only nuclear staining of STRA13 and HIF-1α protein was considered as positive immunoreactivity. The results of this study are summarised in tables 2 and 3. With a few exceptions, the majority of these tumours showed co-expression of STRA13 and CAIX protein, although CAIX had much higher staining intensity. However, studies on parallel tumour sections showed that co-expression of both proteins in the same cells was not very striking. This was particularly true in the areas of necrosis. Cells expressing high level of CAIX were found adjacent to the necrotic areas (fig 6C), while the STRA13 positive cells were not only seen adjacent to necrosis but were also present in the areas more distant from the necrotic region. Overall, STRA13 nuclear staining in a given tumour was more similar to that of HIF-1α than CAIX (fig 6A and B).

The STRA13 protein possesses functional NLS and NES motifs

Nucleocytoplasmic shuttling of STRA13 observed at various differentiation stages stimulated us to investigate possible mechanisms responsible for the STRA13 subcellular localisation. Analysis of the STRA13 amino acid sequence (NetNes 1.1 server, http://www.cbs.dtu.dk/services/NetNES/) revealed two potential nuclear localisation signal (NLS) and one nuclear export signal (NES) motifs (fig 7). A series of STRA13 deletion mutants fused to the FLAG tag was used to assess their functional significance. While the entire FLAG/STRA13 protein overexpressed in 293T cells showed nuclear localisations, deletion of all three NLS/NES motifs (mutant 293–412) prevented STRA13 nuclear transport, supporting the functional importance of the two NLSs (fig 7). In experiments with mutants 123–412, 259–412, and 123–299 we further demonstrated that the centrally located NLS (position 261–268) is sufficient for nuclear transport. Interestingly, while two of the truncated variants (123–412 and 123–299 mutants) showed nuclear localisation only, mutant 259–412 was equally distributed between nucleus and cytoplasm. These results suggest that the position of the NLS within the protein terminus may affect the efficiency of NLS usage. Indeed, protein localisation of the mutant 1–299 was changed to ~70%/20%/5% nuclear/nucleo-cytoplasmic/cytoplasmic when compared with 100% nuclear for the wild type protein, thus providing yet another example of the NLS 261–268 terminal location effect. In this case, however, protein distribution differed from that of mutant 123–412, suggesting that NLS 62–65 and NES 85–101 are also involved in the dynamics of STRA13 exchange between the nucleus and cytoplasm.

Table 3 Expression of STRA13, CA IX, and HIF-α1 in human tumours

	No of positive cases/total No of cases studied			No of positive cases (%) associated with hypoxia (necrosis)		
	STRA13* (nuclear)	CAIX	HIF-α1* (nuclear)	STRA13* (nuclear)	CAIX	HIF-α1* (nuclear)
Cervical carcinomas						
Squamous cell Ca	4/12	10/12	10/12	1 (8)	10 (83)	10 (83)
Adenocarcinoma	4/4	4/4	0/4	0	0	0
Endometrial adenocarcinoma	3/3	2/3	3/3	0	0	0
Ovary						
Adenocarcinoma	10/14	9/14	9/14	0	4 (29)	4 (29)
Mucinous tumours of LMP	4/4	4/4	1/4	0	0	0
Breast						
Ductal Ca	5/5	3/5	3/5	0	3 (60)	3 (60)
Lobular Ca	4/4	0/4	0/4	0	0	0
Colon						
Adenocarcinoma	3/17	17/17	3/17	0	5 (29)	0
Adenoma	0/3	3/3	0/3	0	0	0
Lung						
Squamous cell Ca	1/3	3/3	3/3	1 (33)	3 (100)	3 (100)
Adenocarcinoma	5/6	3/6	4/6	0	0	0
Kidney/urinary tract						
Renal cell Ca (clear cell)	6/10	9/10	6/10	0	0	0
Transitional cell Ca	4/4	4/4	4/4	0	0	0
Glioblastoma multiforme	4/4	4/4	1/4	0	4 (100)	1 (25)
Mesothelioma	1/2	2/2	0/2	0	0	0

*The positive staining is limited to nuclear reactivity.
Ca, carcinoma; LMP, low malignancy potential.

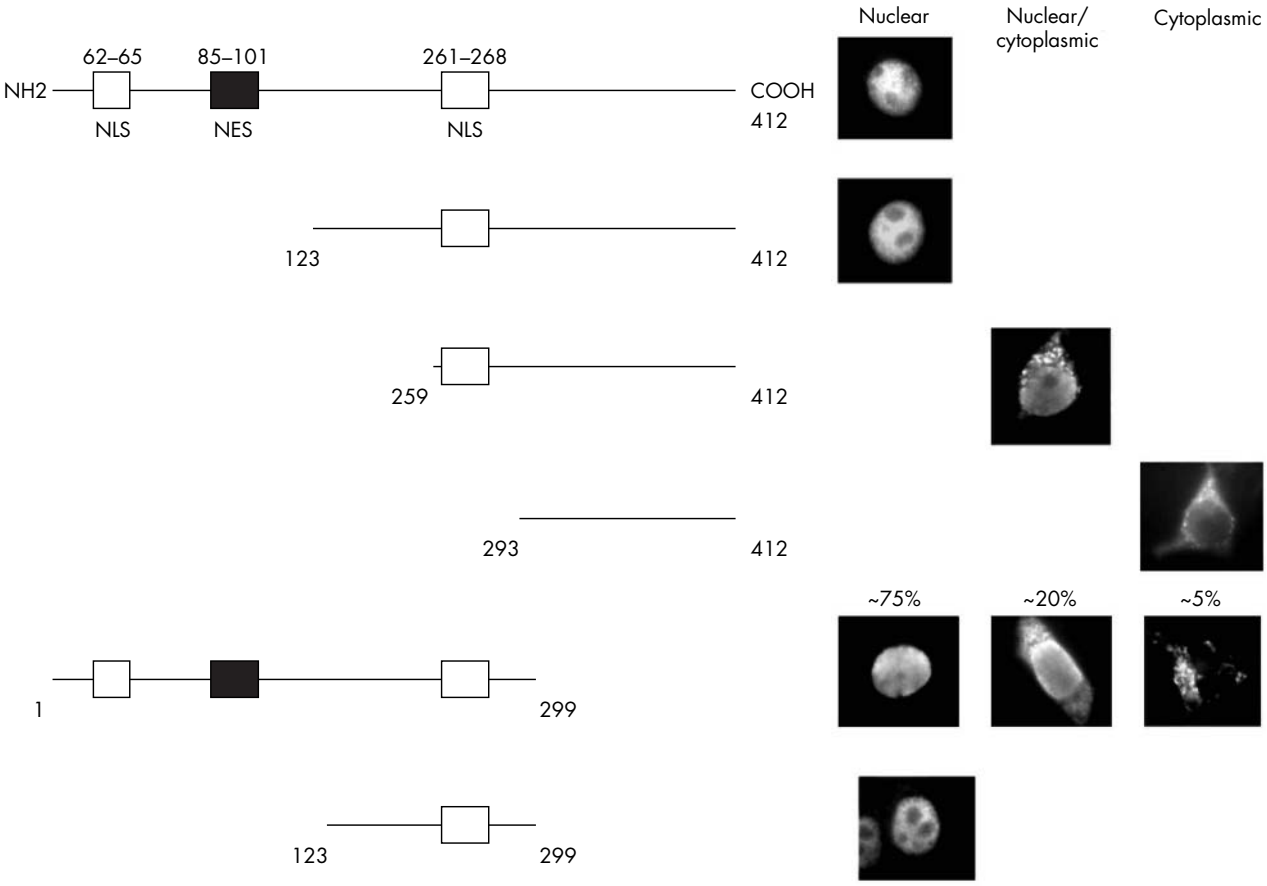

Figure 7 Identification of nuclear localisation signals (NLS) and nuclear export signals (NES) in the primary structure of the STRA13 protein. Left panel shows FLAG/STRA13 deletion constructs with predicted NLS and NES. Right panel illustrates immunofluorescent staining of 293T cells transfected with the indicated constructs. Cells were immunostained with anti-FLAG M2 monoclonal antibodies (Sigma) and visualised with anti-mouse Alexa Fluor 488 (green) antibodies.

DISCUSSION

In this study, we used newly generated polyclonal antibodies raised against the STRA13 C-terminus for identification of STRA13 positive cells in normal adult tissues and in neoplasias. We showed that STRA13 is commonly expressed in epithelial cells of both normal and neoplastic origin and confined mostly to the nucleus. Nuclear localisation of STRA13 observed in multiple cell types is in agreement with its function as a transcriptional regulator.[2–5 11 14 22 23]

Our study indicates that the STRA13 protein expression in normoxic regions of solid neoplasms represents, in the majority of the cases, recapitulation of its expression pattern in normal tissues. Thus the high level of STRA13 mRNA found in certain tumours and cell lines[3] may be explained by tumour tissue origin rather than by a cancer associated increase in STRA13 expression.

The consistent STRA13 immunoreactivity in the terminally differentiated squamous cells of normal tissues or carcinomas reinforces the crucial role of STRA13 in cell differentiation suggested previously from studies of cell lines.[5–7] Thus overexpression of Stra13 in P19 cells resulted in neuronal differentiation[5]; forced expression of STRA13 promoted chondrogenic differentiation, hypertrophy, and/or mineralisation in the cultures of embryonic cells, mesenchymal stem cells, and chondrocytes.[7] Overexpression of STRA13 in the mesenchymal cells induced mRNA expression of type II collagen, Indian hedgehog, and Runx2, as well as cartilage matrix accumulation.[7]

The observed switch of STRA13 localisation from the nucleus to cytoplasm depending on the cellular differentiation stage is another indication of STRA13 participation in the process of differentiation. Nuclear export of proteins regulating transcription is a very efficient mechanism to downregulate an induced transcriptional response when the differentiation signal is turned off.[24] Thus, while in undifferentiated keratinocytes the transcription factor E2F-5 is scarce and largely cytoplasmic, induction of differentiation by Ca^{2+} treatment resulted in E2F-5 accumulation in the nucleus.[25] Likewise, histone deacetylase HDAC4 also shuttled between nuclear and cytoplasmic compartments during C2C12 myoblast differentiation.[26] The most common mechanism of nucleocytosolic transport employs stretches of basic amino acids, such as nuclear localisation signals (NLS) and leucine-rich regions as nuclear export signals (NES) in the cargo proteins, and the protein transport receptors of the importin/karyopherin β superfamily.[24 27] In this study, we showed that STRA13 contains two NLS and one NES responsible for the subcellular localisation of the protein. The nature of the signals promoting STRA13 nucleocytoplasmic shuttling remains to be established.

Based on our data a scenario can be proposed in which STRA13 is maintained in the nuclei of proliferating and differentiating cells to serve as a transcriptional repressor and (or) an activator of various sets of genes, depending on the external stimuli. Upon completion of the differentiation, STRA13 is sequestered from its transcriptional activity to the

cytoplasm employing its NES peptides. However, this sequestration may also serve for switching the functional activity of the protein if STRA13 plays a special role in the cytoplasm.

Functional links between HIF-1α, CAIX, and STRA13 have been established previously.[2 3 14] Our comparison of STRA13, HIF-1α, and CAIX expression patterns in a large set of various tumour tissues further substantiates the association of STRA13 with HIF-1α expression and hypoxia in vivo. Nevertheless, unlike CAIX,[14] the pattern of STRA13 staining was more diffuse and was not always restricted to the areas immediately adjacent to necrosis. It appeared that the pattern of STRA13 expression was more related to HIF-1α expression and independent of necrosis. These data are in line with other studies[14] and suggest that STRA13 may play a special role in the cells in the preapoptotic stage. Given that STRA13 was recently associated with apoptosis[4 10] it is tempting to speculate that this transcriptional factor may also mediate hypoxia associated apoptosis in tumours.

Implications for tumour diagnosis

STRA13 is a candidate biomarker for distinguishing in situ from invasive breast carcinomas

Consistent expression of STRA13 in myoepithelial cells, both in normal tissues and neoplasms, makes STRA13 a valuable potential diagnostic marker for breast tumours. In the mammary gland the myoepithelial cell layer is the basal cell layer. During breast carcinogenesis, the myoepithelial cells rarely undergo malignant transformation, but remain as a continuous basal cell layer of the carcinoma in situ lesion. Loss of the outer myoepithelial layer is considered a hallmark of invasive carcinomas. Thus STRA13 antibodies may help in solving important problems in breast pathology: distinguishing in situ versus infiltrating carcinoma, discriminating sclerosing adenosis and other benign sclerosing lesions from infiltrating carcinoma, and differentiating benign papillomas from papillary carcinoma.

STRA13 expression specific for myoepithelial cells may provide a clue to its possible role in cancer. The cumulative data suggest that myoepithelial cells are natural paracrine suppressors of invasion and metastasis and may specifically inhibit the progression of precancerous disease states to invasive cancer in vivo.[28] Thus STRA13 might play an important role in the process of natural suppression of tumour invasion as it is expressed specifically in myoepithelial cells, which provide a cellular barrier for the spreading cancer cells.

STRA13 is a candidate marker for the identification of the primary site of certain metastatic tumours

The apical granular cytoplasmic positivity in normal mucosa and adenocarcinomas of the colon and pancreas appears to be a unique pattern of STRA13 expression. As this pattern is retained in metastatic carcinomas, detection of the STRA13 apical cytoplasmic immunoreactivity in metastatic tumour cells may be helpful in localisation of the primary site of the colon or pancreas tumours.

STRA13 is a potential classification marker for renal cell carcinomas

Previously we have described the patterns of CAIX and CAXII expression in renal cell tumours and its diagnostic value.[15] In this study, we identify STRA13 as yet another potentially important biomarker for renal carcinomas. We found that the diffuse granular cytoplasmic STRA13 expression was present in specific types of renal cell neoplasms, namely papillary carcinomas, oncocytomas, and collecting duct carcinomas; however, all renal clear cell carcinomas examined showed STRA13 nuclear staining, while none of the chromophobe cell carcinomas examined showed STRA13 immunoreactivity. Thus the combination of three biomarkers (STRA13, CAIX, and CAXII) may serve as a powerful tool in the classification of all forms of renal cell tumours. To determine the value of STRA13 as a diagnostic biomarker in these various malignancies it will be necessary to increase the relevant sample sizes in order to provide the necessary statistical power. These studies are in progress.

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Authors' affiliations

A Ivanova, S Ivanov, Laboratory of Immunobiology, Basic Research Program, SAIC-Frederick Inc, NCI-Frederick, Maryland, USA
E J Stanbridge, S-Y Liao, Department of Microbiology and Molecular Genetics, School of Medicine, University of California, Irvine, California, USA
M I Lerman, Laboratory of Immunobiology, NCI-Frederick

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