The product of the imprinted H19 gene is an oncofetal RNA

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Abstract

Aims/background—The H19 gene is an
imprinted, maternally expressed gene in
humans. It is tightly linked and co-
regulated with the imprinted, paternally
expressed gene of insulin-like growth fac-
tor 2. The H19 gene product is not
translated into protein and functions as an
RNA molecule. Although its role has been
investigated for more than a decade, its
biological function is still not understood
fully. H19 is abundantly expressed in many
tissues from early stages of embryogenesis
through fetal life, and is down regulated
postnatally. It is also expressed in certain
childhood and adult tumours. This study
was designed to screen the expression of
H19 in human cancer and its relation to
the expression of H19 in the fetus.

Methods—Using in situ hybridisation with
a [35S] labelled probe, H19 mRNA was
detected in paraffin wax sections of fetal
tissues from the first and second trimes-
ters of pregnancy and of a large array of
human adult and childhood tumours aris-
ing from these tissues.

Results—The H19 gene is expressed in
tumours arising from tissues which ex-
press this gene in fetal life. Its expression
in the fetus and in cancer is closely linked
with tissue differentiation.

Conclusions—Based on these and previ-
ous data, H19 is neither a tumour sup-
pressor gene nor an oncogene. Its product
is an oncofetal RNA. The potential use of
this RNA as a tumour marker should be
evaluated.

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Keywords: H19; tumour marker; oncofetal gene.

Genomic imprinting, the expression of only
one of two alleles according to the gamete of
origin, has been implicated in tumorigenesis.[1-3]
H19 is one of the small group of imprinted
genes in humans.[4-6] It is expressed from the
maternal allele, processed like other mRNAs,
but is not translated into a protein.[7] As such, it
belongs to a recently discovered distinctive
group of imprinted genes which transmit their
action directly through their RNA molecules.
This group also includes Xist[8] and IPW.[9] In
addition, the RNA from the 3' untranslated
region of α-tropomyosin functions as a ri-
boregulator and acts as a tumour suppressor.[10]

Like other imprinted genes, H19 is abun-
dantly transcribed from early stages of embryo-
genesis through fetal life and placental develop-
ment, and is down regulated postnatally.[11]
Despite extensive research for more than a
decade, its biological function is still not
understood fully. A clue to this enigma may be
its tight linkage with the insulin-like growth
factor 2 (IGF2) gene.[12] The latter plays an
important role in cell proliferation and differ-
entiation, and is well known as a growth factor
in many tumours, especially solid tumours of
childhood.[13] Both genes reside on an imprinted
domain on chromosome 11p11.5. They are
oppositely imprinted, IGF2 being expressed
from the paternal allele,[14] and are coexpressed
in many tissues.[15 16] It has been recently shown
(in mice) that these genes, together with
insulin-2, share a common cis-regulatory
sequence. Their regulation is probably modu-
lated through the methylation pattern of the
H19 promoter region.[17]

H19 was first discovered by Pachnis *et al*[11] as
a gene sharing trans-regulatory sequences with
α-fetoprotein (AFP), an oncofetal protein
widely used as a tumour marker. We have sug-
gested previously that H19, being abundantly
expressed in certain human fetal tissues and in
tumours arising from these tissues, also has
oncofetal characteristics.[18 19] We have extended
our study and examined the expression of H19
in the major types of human cancer as well as in
premalignant conditions. We suggest that H19
is an oncofetal RNA, and should be considered
for use as a tumour marker.

Methods

Representative samples of a large array of
tumours were selected from the files of the
Department of Pathology of the Hadassah
Medical Centre in Jerusalem. Over 50 samples
of testicular tumours were kindly supplied by
Dr LHJ Looijenga from the Laboratory for
Patho-Oncology at the Dr van der Hoed Can-
cer Centre in Rotterdam, and a few cases from
other medical centres were also included. In
many instances, samples of preneoplastic con-
ditions or tumours, or both, at different grades
of differentiation were investigated. The pat-
tern of H19 expression in these samples was
studied by in situ hybridisation (ISH) and was
compared with its expression in fetal tissues
from all three germ layers.

IN SITU HYBRIDISATION

Paraffin wax sections (5 μm) of formalin fixed
tissues were mounted on 3-aminopropyl-
triethoxylane (Tespa, Sigma) coated micro-
scope slides and dried at 37°C overnight. Sec-
tions were dewaxed with xylene, fixed with 4%

paraformaldehyde, and then treated with proteinase K (Sigma). Slides were acetylated to reduce non-specific binding of the probe and dehydrated through an ethanol series. [35S] labelled RNA probes (specific activity of 50 000 cpm/μl) were hybridised as described by Rangini *et al*[20] omitting the thio-AMP step. Slides were exposed for 10 days and counter stained with haematoxylin and eosin. The slides were examined and photographed using a Polyvar (Reichert-Jung) microscope under bright and dark field illumination.

Controls included hybridisation with sense RNA probe and RNase prehybridisation treatment. Negligible signal was observed in all the controls. A section of fetal kidney was used as a positive control and was included in each set of slides.

PREPARATION OF TRANSCRIPTION VECTOR

H19 cDNA corresponding to the 3' end of the mRNA (800 base pairs)[21] was subcloned at the EcoRI site into Bluescript II KS plasmid (Stratagene) behind the T7 and T3 RNA polymerase binding sites. In vitro RNA transcription with T7 RNA polymerase was used to produce antisense H19 RNA from linearised plasmid DNA. Sense H19 mRNA prepared with T3 polymerase was used as a control.

PREPARATION OF THE RIBOPROBE FOR IN SITU HYBRIDISATION

[35S] labelled in vitro RNA transcripts (10^7 cpm/μg) were produced using the Amersham

RPN 2006 kit and RNA polymerases from Boehringer Mannheim. Linearised plasmid was prepared by digestion with HindIII for the antisense (and EcoRI for the sense) strand. The fragments were separated from unincorporated nucleotides by precipitation in ethanol.

Results

Table 1 summarises the results. Some of these findings have been reported in part previously,[16 18 19 22 23] and have been included in this report for completeness.

TISSUES OF (EMBRYONIC AND EXTRAEMBRYONIC) ECTODERMAL ORIGIN

Nervous system

H19 was not expressed in the neural crest of an embryo of 35 days post conception, seven to nine, 13, and 14 weeks of gestation, nor in the cerebral cortex during the second trimester of pregnancy (17–22 weeks). Expression of H19 was also not detected in groups of neuroblasts migrating through the adrenal cortex towards the medulla during the second trimester of pregnancy, nor in the paravertebral ganglia at eight to nine weeks' gestation. A similar pattern was noted in gonadal immature teratoma, in that H19 was not expressed in primitive neural tissue present in these tumours. H19 was not expressed in two medulloblastomas; however, it was expressed in two of three astocytomas (one with prominent and diffuse expression, and one with weak expression) and in one ganglioneuroblastoma (fig 1). In the latter, the

Table 1   Expression of the H19 gene in tissues from the three germ layers during embryogenesis and fetal development and in tumours arising from these tissues

| Tissue	Germ layer	Fetus	Hyperplasia/metaplasia Preneoplastic condition	Neoplasia
Neural crest derivatives	Ectoderm	−	In immature teratoma −	2 medulloblastomas − 2/3 astrocytomas + 1/1 ganglioblastoma + 4 meningiomas − 2 naevi −/1 melanoma −
Epidermis	Ectoderm	+	Squamous epithelium in dermoid cyst − Squamous epithelium in immature teratoma +	3 well differentiated squamous cell carcinomas −
Breast	Ectoderm	?	Fibrocystic disease − In situ lobular and intraduct carcinoma −	4 invasive duct and 3 invasive lobular carcinomas−
Trophoblast	Trophectoderm	+	Complete hydatidiform mole − With relaxation of imprinting +	1/1 placental site trophoblastic tumour + 4/4 choriocarcinomas +
Striated muscle	Mesoderm	+	In immature teratoma +	4/6 rhabdomyosarcomas +
Smooth muscle	Mesoderm	−	In immature teratoma −	
Cartilage	Mesoderm	+	In immature teratoma +	
Fibrous tissue	Mesoderm	+	In immature teratoma +	2/7 stroma in breast carcinomas +
Haemopoietic tissue	Mesoderm	−		5 B-cell lymphomas −
Adrenal	Mesoderm	+		
Prostate	Mesoderm	+	Benign prostatic hyperplasia −	
Kidney blastema tubules	Mesoderm	+ −	Blastema in nephrogenic rest +	Blastema in 4/7 Wilms' tumours + 4 renal cell carcinomas −
Germ cells in ovary and testis		−	Intratubular germ cell neoplasia −	21 seminomas − 21 embryonal carcinomas− 15/15 yolk sac tumours + 4/4 choriocarcinomas +
Gonadal interstitium & coelomic epithelium	Mesoderm	+	Leydig cell hyperplasia +	2/3 Leydig cell tumour + 1/1 Sertoli cell tumour + 2/3 borderline serous carcinomas + 1/2 serous carcinomas +
Urothelium	Mesoderm in pelvis & ureters endoderm in bladder	+	Carcinoma in situ +	4/24 grade I, 13/20 grade II, 14/24 grade III bladder carcinomas +
Small intestine (epithelium)	Endoderm	+		
Large intestine (epithelium)	Endoderm	+	Adenoma −	5 colonic carcinomas −
Liver	Endoderm	+	Regeneration +	14/22 hepatocellular carcinomas +
Pancreas ducts acini and islets	Endoderm	− +		4 ductal carcinomas −
Bronchus	Endoderm	+	Respiratory epithlium in immature teratoma +	3/7 adenocarcinomas and squamous carcinomas + 5 bronchioloalveaolar carcinomas − 1 small cell carcinoma − 3 carcinoid tumours −
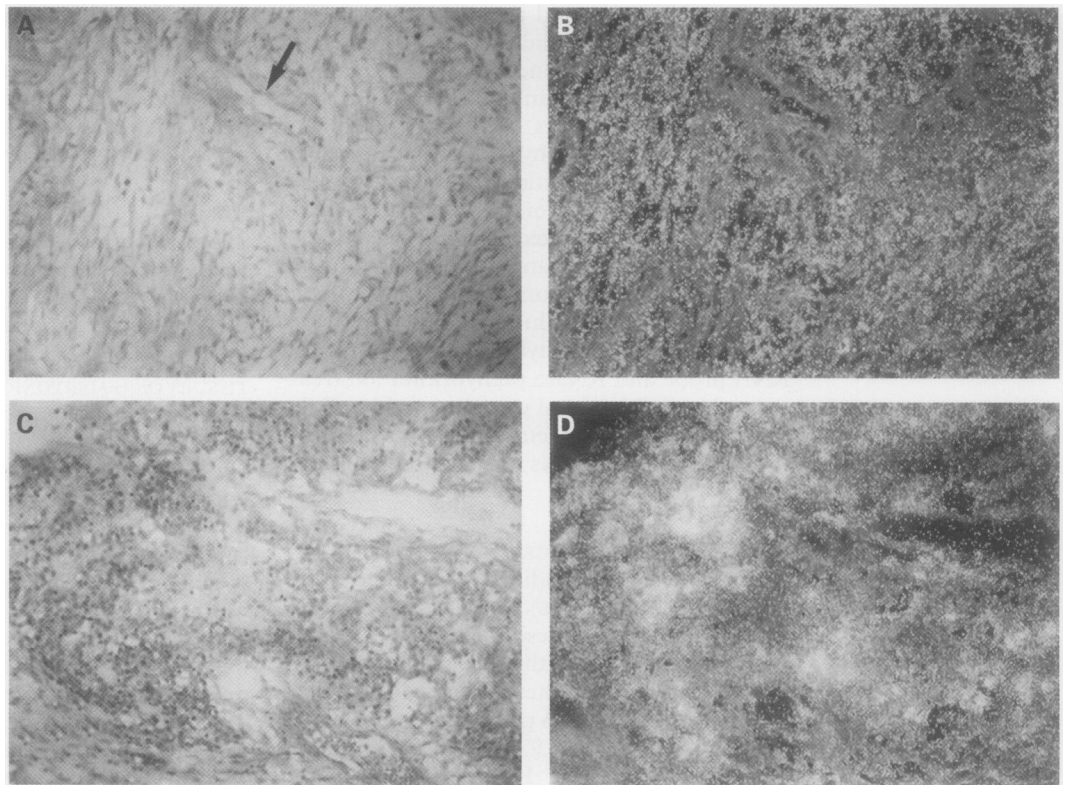
Figure 1   H19 expression in tumours arising in the central and peripheral nervous system. A and B, bright and dark field images, respectively. Diffuse expression of the gene is seen in astrocytoma, in contrast to the blood vessels in the tumour (arrow) (original magnification, ×125). C and D, bright and dark field images, respectively. Prominent expression of H19 is seen in clusters of neuroblasts in ganglioneuroblastoma (original magnification, ×125). (In situ hybridisation of [³⁵S] labelled antisense of H19 with haematoxylin and eosin as a counter stain.)

expression seemed to be present mostly in the zone of differentiating neuroblasts. No expression was evident in the four meningiomas examined.

H19 was not expressed in two intradermal naevi and one malignant melanoma, benign and malignant tumours, respectively, of melanocytes which migrate from the neural crest in fetal life.

Skin

Prominent expression of H19 was evident in the epidermis from the eighth week of gestation (fig 2) and in the epidermis and hair follicles in the second trimester. A similar pattern of expression was noted in an ovarian immature teratoma (see later). Expression of H19 was not detected in the epidermis lining the cyst of a mature ovarian teratoma (dermoid cyst)[18] and in intact skin postnatally.

In testicular germ cell tumours, which have been diagnosed as mature teratoma with immature elements, owing to the presence of primitive neural tissue, expression of H19 was noted in keratinising squamous epithelium that seemed to be mature on the basis of histological criteria alone (see later).

H19 was not expressed in three cases of well differentiated squamous cell carcinoma (SCC) of the skin. It should be mentioned here that SCC in several organs expressed H19. These include two of two SCCs of the uterine cervix (fig 2), one of three SCCs of the lung (fig 3), and a high grade SCC of the urinary bladder (fig 2).

Breast

Four sections of normal breast tissue and breast with mild fibrocystic changes, two sections of ductal carcinoma in situ, four invasive ductal carcinomas, and three invasive lobular carcinomas, but not fetal breast tissue, were studied. H19 was not expressed in any of these cases. It is, however, remarkable that moderate expression was noted in the fibroblasts in the stroma of one case of fibrocystic disease, one invasive ductal carcinoma and one invasive lobular carcinoma (fig 4).

Trophectoderm

Prominent expression of H19 was found in the placenta from the early stages of gestation and continued until term (nine cases) and also in one case of missed abortion with hydropic degeneration of the villi. H19 expression was most prominent in the intermediate trophoblasts and to a lesser extent in cytotrophoblasts. A similar pattern of expression was present in two partial hydatidiform moles. In six complete hydatidiform moles, the human counterpart of an androgenetic conceptus, expression of H19 was absent from the villous stroma and surrounding trophoblast, but prominent expression was seen in groups of proliferating mononuclear trophoblastic cells. This expression of H19 from the paternal allele represents a relaxation of the imprinting phenomenon.[22 24] H19 was abundantly expressed in one placental site trophoblastic tumour studied by us and in four choriocarcinomas.[22]
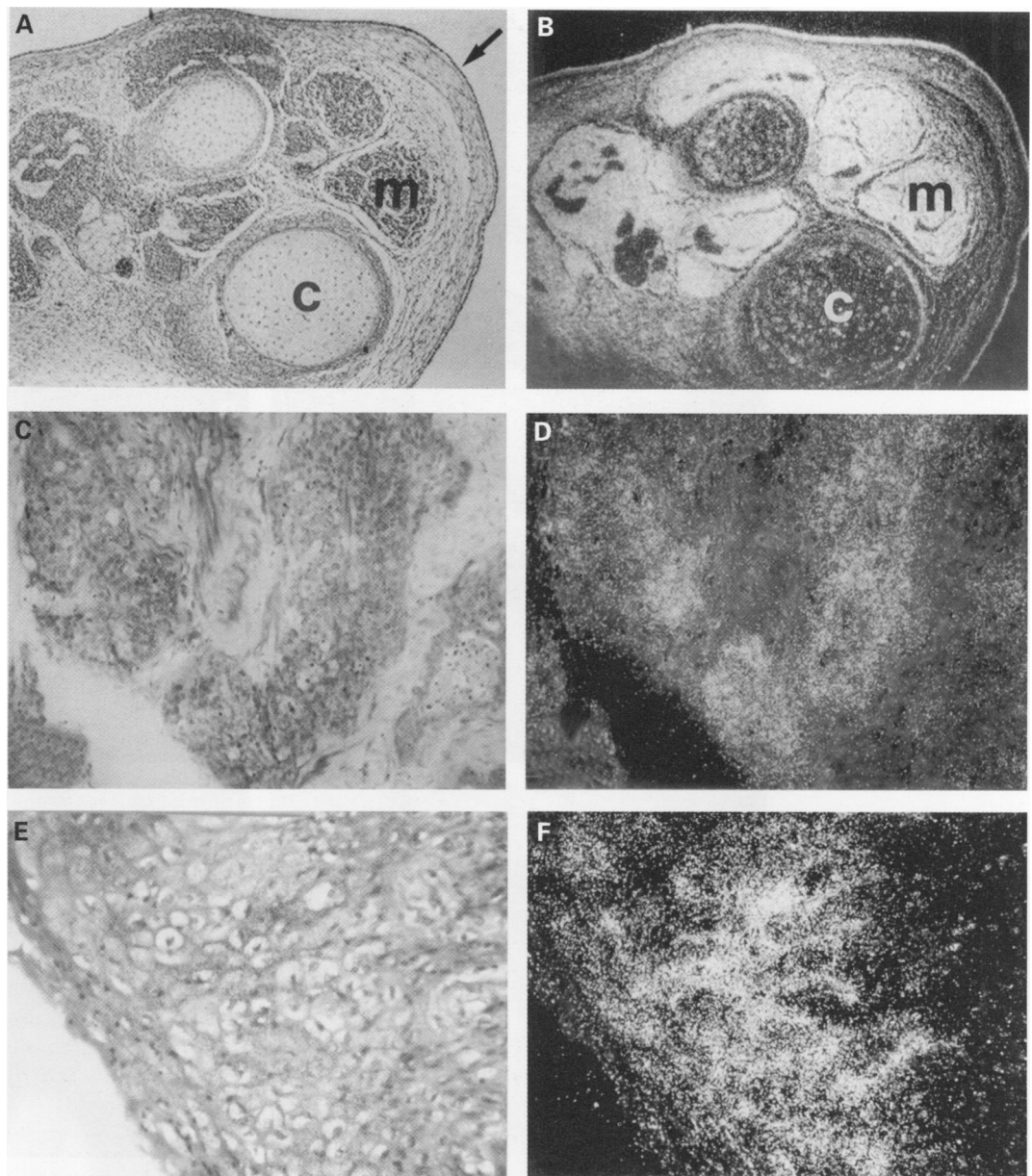
Figure 2   H19 expression in tissues with epidermoid differentiation. A and B, bright and dark field images, respectively. An upper limb of an embryo at eight weeks' gestation. Prominent expression of H19 is evident in the primitive epidermis (ectoderm) (arrow), as well as in the mesenchymal tissues (mesoderm) including skeletal muscle (m), fibrous tissue and cartilage (c) (original magnification, ×20). C and D, bright and dark field images, respectively. Prominent expression of H19 is seen in invasive squamous cell carcinoma of the uterine cervix (original magnification, ×125). E and F, bright and dark field images, respectively. Expression of H19 in high grade invasive squamous cell carcinoma of the urinary bladder (original magnification, ×200). (In situ hybridisation of [^{35}S] labelled antisense of H19 with haematoxylin and eosin as a counter stain.)

TISSUES OF MESODERMAL ORIGIN

Kidney

In the fetus from the eighth week through the second trimester of pregnancy, prominent expression of H19 was evident in the metanephric blastema, and was reduced dramatically upon differentiation to tubular structures (fig 5). No expression was found in the adult kidney.

The pattern of H19 expression in intralobar nephrogenic rests in WAGR syndrome (Wilms' tumour-aniridia-genital anomalies-mental retardation) associated with 11p13 deletion was similar to that of the fetal kidney: it was concentrated in the metanephric blastema but not in primitive tubules. No expression was seen in the adjacent mature renal tissue (fig 5).[25] A similar pattern of expression was noted in nephrogenic rests in a patient with Perlman

syndrome, another syndrome with predisposition for developing Wilms' tumour.

In two of seven Wilms' tumours examined by us, H19 expression was similar to that in the fetal kidney—that is, H19 was expressed in the metanephric blastema but not in the tubular epithelial elements (fig 5). In two other cases, H19 expression was noted mainly in the stroma and only focally in the blastema.

In four cases of renal cell carcinoma, arising histogenetically from the proximal tubular epithelium, no expression of H19 was observed.

Female reproductive organs

Expression of H19 was observed in the endometrium and ovaries. There was a cyclic change in the level of expression in the endometrial stroma and in the ovarian
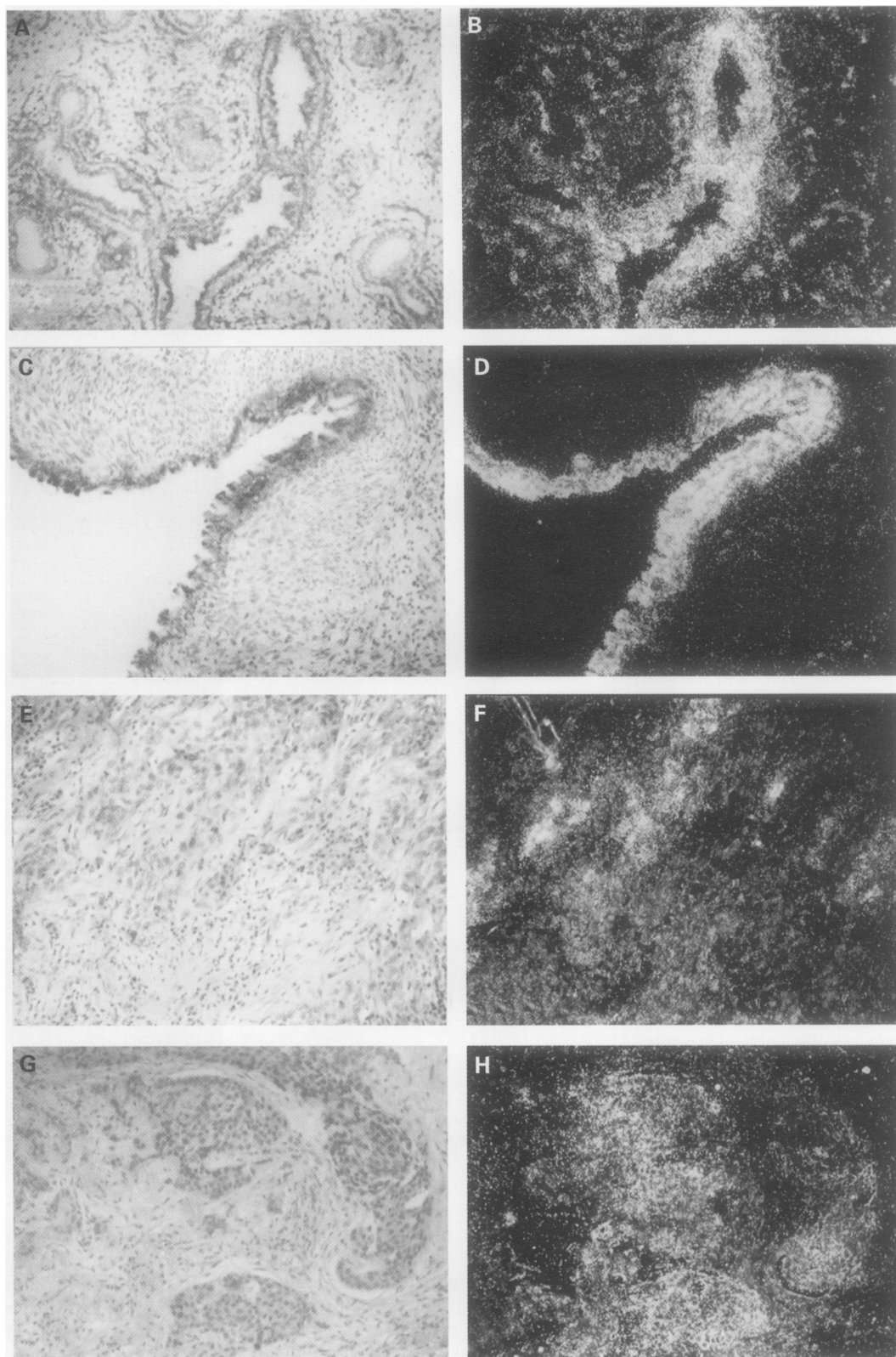
Figure 3   H19 expression in the lung. A and B, bright and dark field images, respectively. Expression is confined to the mucosa of the large airways in the fetus at 14 weeks' gestation (original magnification, ×125). C and D, bright and dark field images, respectively. In testicular immature teratoma, prominent H19 expression can be seen in respiratory-like epithelium (original magnification, ×125). E and F, bright and dark field images, respectively. H19 expression in squamous cell bronchogenic carcinoma (original magnification, ×125). G and H, bright and dark field images, respectively. H19 expression in primary pulmonary adenocarcinoma (original magnification, ×125). (In situ hybridisation of [³⁵S] labelled antisense of H19 with haematoxylin and eosin as a counter stain.)

follicles.[26] However, these physiological changes are beyond the scope of this article.

Mature ovarian teratoma (dermoid cyst) which is the result of parthenogenesis and is endowed with two sets of maternal chromosomes[27] consists only of mature tissue.

It is, therefore, not surprising that expression of H19 was not seen in this tumour.[18] In one immature ovarian teratoma, the pattern of H19 expression was similar to that in the fetus.[18] We have not studied any other ovarian germ cell tumours so far.
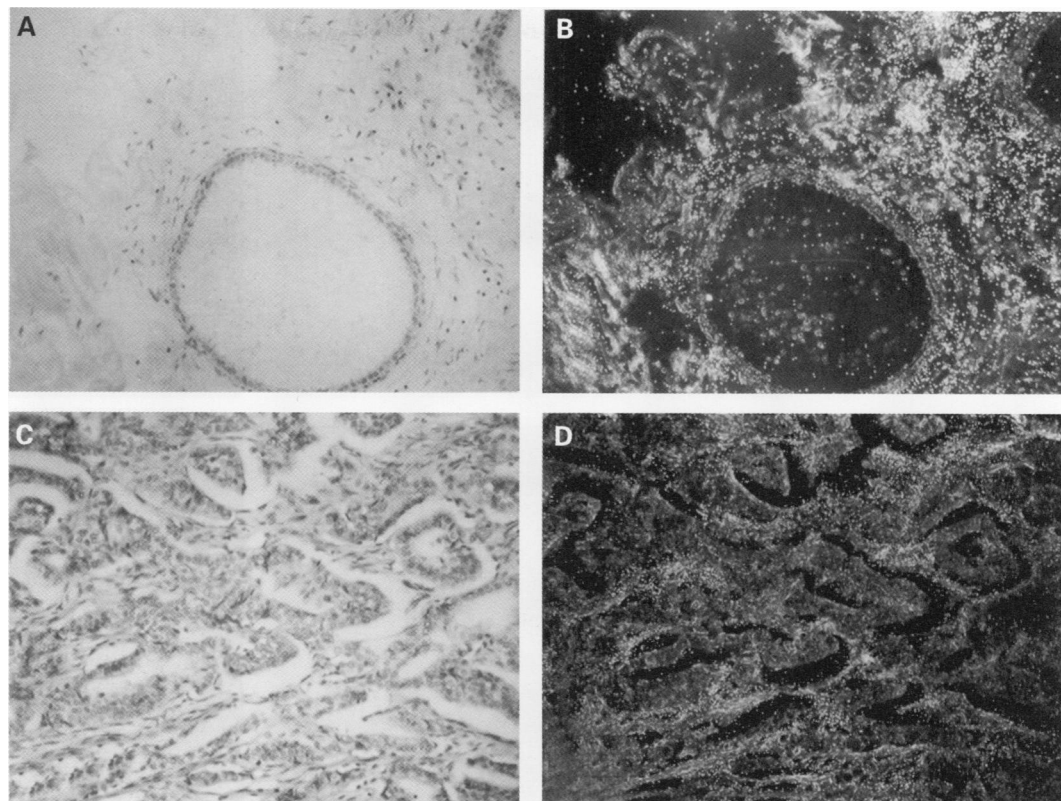
Figure 4   H19 expression in breast disease. A and B, bright and dark field images, respectively. Fibrocystic disease of the breast. Labelling for H19 RNA can be seen in the fibrous tissue and not in the ductal epithelium (original magnification, ×125). C and D, bright and dark field images, respectively. Invasive ductal carcinoma. H19 expression is again noted in the fibrous stroma and not in the epithelial cells (original magnification, ×125). (In situ hybridisation of [^{35}S] labelled antisense of H19 with haematoxylin and eosin as a counter stain.)

In the embryo, expression of H19 was most prominent in the ovarian stroma and was also noted to a lesser extent in the coelomic epithelium. During the second trimester of pregnancy, H19 expression was weak to moderate, and was detected only in the stroma. In one steroid producing tumour, diffuse moderate expression was present. In a small series of serous tumours, abundant expression of H19 was observed in one of two papillary serous carcinomas and in two of three serous tumours of borderline malignancy. In the latter, labelling was most prominent in the groups of proliferating cells shedding from the papillary structures.

Prominent expression was also noted in one of two SCCs of the uterine cervix (fig 2), and focal rather weak expression in another such tumour.

Male reproductive organs
During the first trimester, at eight weeks of gestation, prominent expression of H19 was observed in the interstitium, whereas no expression was seen in the sex cords. In the second trimester, prominent expression of H19 was noted in interstitial Leydig cells, but not in the seminiferous tubules. H19 is not usually expressed in adult testes. We have noted, however, moderate expression of H19 in Leydig cells in instances of Leydig cell hyperplasia, and a low level of expression in Sertoli cells in seminiferous tubules with aspermatogenesis adjacent to a testicular germ cell tumour.

Moderate expression of H19 was found in one Sertoli and low to moderate expression in two of three Leydig cell tumours.

Expression of H19 in testicular germ cell tumours was consistently observed in certain components and absent in others, in a pattern of expression similar to that seen during embryogenesis and in fetal life (fig 3). No expression was evident either in intratubular germ cell neoplasia or in 19 classic and two spermatocytic seminomas. No expression was seen in elements mimicking the earliest stages of embryonic differentiation—that is, the epiblast. This was clearly demonstrated in 21 cases, including pure embryonal carcinoma (epiblastoma) and embryonal carcinoma component in mixed germ cell tumours as well as in embryoid bodies. In teratoma, expression seemed to be directly related to differentiation in tissues expressing the gene in fetal life. In six cases of mixed mature/immature teratomas and residual mature teratomas, tissue elements which seemed to be mature by histological criteria—for example, keratinising squamous epithelium, expressed the embryonal gene H19. Expression was also observed in two infantile mature testicular teratomas, but not in three mature adult teratomas. H19 expression was also evident in germ cell tumours with extraembryonic differentiation. In all four cases with a component of choriocarcinoma, prominent H19 expression was evident in trophoblastic cells and was similar to that seen in gestational choriocarcinoma. In seven cases of pure yolk sac tumour, four of which occurred
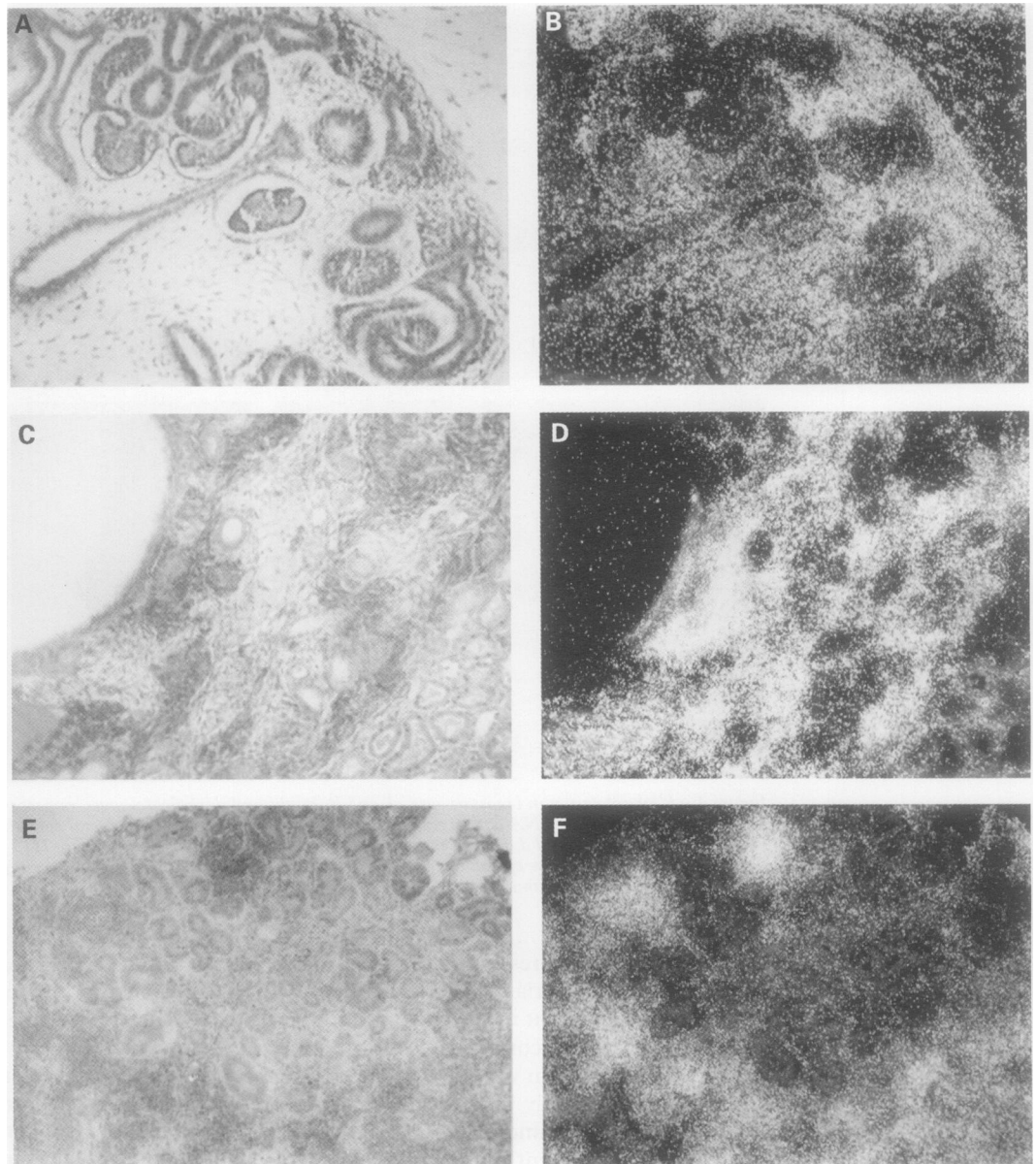
Figure 5 H19 expression in the kidney. A and B, bright and dark field images, respectively. In the embryonal kidney (eight weeks' gestation), H19 expression is most prominent in the metanephric blastema and is reduced dramatically upon differentiation to tubular structures (original magnification, ×125). C and D, bright and dark field images, respectively. H19 expression is evident in nephrogenic rests in a kidney removed from a patient with WAGR syndrome. No expression is seen in the adjacent mature renal tissue (original magnification, ×125). E and F, bright and dark field images, respectively. The pattern of H19 expression in Wilms' tumour is similar to the embryonal kidney. H19 is expressed in the blastema but not in the tubular epithelial component (original magnification, ×125). (In situ hybridisation of [³⁵S] labelled antisense of H19 with haematoxylin and eosin as a counter stain.)

in infants and young children, H19 expression was consistently abundant in the mesotheloid-reticular areas and in loose stroma, and absent in other typical elements, including the Schiller-Duval bodies and vitelline differentiation. A similar pattern of expression was observed in elements of yolk sac tumour present in eight mixed germ cell tumours in adolescents and young adults.[23]

H19 was not expressed in fetal prostatic tissue. No expression was found in sections of adult prostatic tissue with benign prostatic hyperplasia, examined while studying the cases of bladder carcinoma.

Soft tissue

Primitive mesenchymal tissue in the fetus and placenta expressed H19 and similar prominent expression was seen accordingly in immature teratomas. This was also the case for fetal striated muscle and cartilage (fig 2). Conversely, H19 was not expressed in smooth muscle of various organs during fetal life, or in bundles of smooth muscle present in immature teratoma.

H19 was expressed in four of six embryonal rhabdomyosarcomas. The pattern of expression was diffuse in two and focal in the other two (fig 6).

Expression of H19 was observed in the fibrous stroma in carcinoma of the breast (fig 4) and also in the stroma of a metastatic colonic carcinoma (fig 7). This may represent gene expression induced by paracrine factors produced by the tumour cells.

Haemopoietic tissue

Haemopoietic tissue, abundantly present in the liver in the second trimester of pregnancy, did
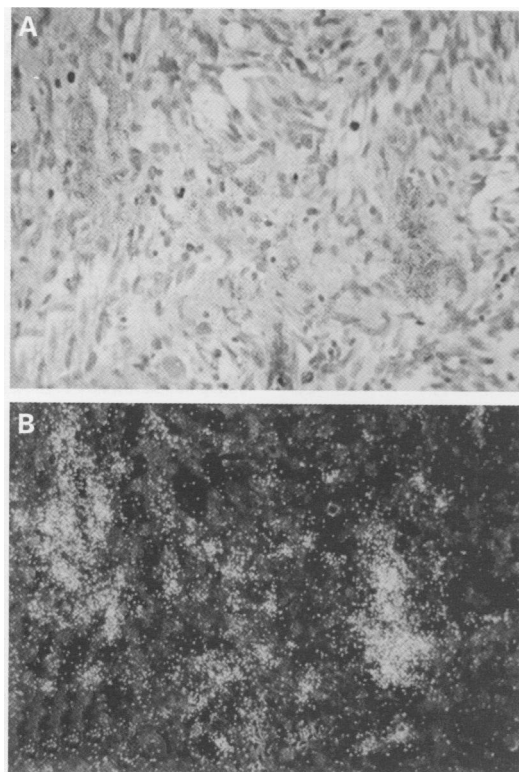
Figure 6 *H19 expression in embryonal rhabdomyosarcoma. A and B, bright and dark field images, respectively. Prominent expression is noted in single tumour cells and in small groups (original magnification, ×200). (In situ hybridisation of [^{35}S] labelled antisense of H19 with haematoxylin and eosin as a counter stain.)*

Figure 7 *H19 expression in the fibrous stroma of adenocarcinoma of the colon. A and B, bright and dark field images, respectively. Expression of H19 is evident in the stroma between glandular structures of adenocarcinoma cells, which do not express the gene (original magnification, ×200). Also see figs 4C and 4D for gene expression in the stroma of a tumour. (In situ hybridisation of [^{35}S] labelled antisense of H19 with haematoxylin and eosin as a counter stain.)*

not express H19.[16] This was also the case in adult lymph nodes and in five B-cell lymphomas of the testis.

Urinary tract
The transitional epithelium of the pelvicalyceal system and the ureter is continuous with the transitional epithelium of the bladder, which arises from the hindgut, and will be described together.

TISSUES OF ENDODERMAL ORIGIN
Urinary tract
The transitional mucosa of the urinary tract in the fetus abundantly expressed H19. No expression was found in the adult bladder mucosa. Samples of bladder carcinoma were selected at random from the files of the Department of Pathology and were examined for expression of H19, which was correlated with the histological grade of the tumour.[19] H19 was expressed in four (16%) of 24 grade I, in 13 (65%) of 20 grade II, and in 14 (58%) of 24 grade III papillary transitional cell carcinomas of the urinary bladder (p = 0.0027, Pearson χ^2).

H19 was also expressed in 10 (71%) of 14 samples of carcinoma in situ of the urinary bladder mucosa, most of which were taken from sites adjacent to invasive cancer.

Selected recurrent biopsy specimens of seven patients with grades I–II bladder carcinoma were examined for H19 expression. Two to seven specimens from each patient (average 4.4) were studied. Three patients had multiple recurrences, but progression and invasion did
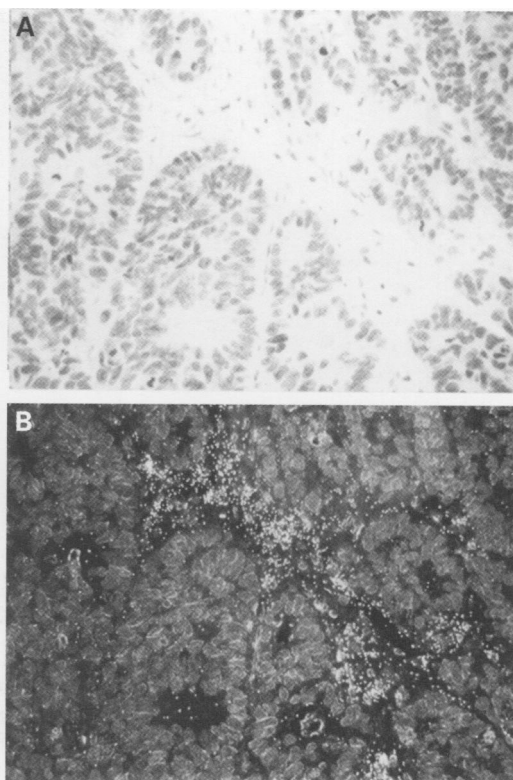
not occur during the course of the disease. H19 was not expressed in any of the biopsy specimens of these patients. In four patients, progression to invasive tumour occurred during the course of disease. In two of these patients, H19 expression was evident in tumour cells, either from the first biopsy specimen (one patient), or with progression to invasive cancer.

Using ISH, expression of H19 was not detected in any of the seven biopsy specimens of patients with chronic cystitis (interstitial or eosinophilic).

Intestine
In the fetus, H19 expression was observed in small intestinal mucosa, but was absent in the mucosa of the large intestine. As carcinomas arising in the small intestinal mucosa are very rare, and when they occur they have similar differentiation to colonic carcinomas, only tumours arising in the large intestine have been investigated. These ranged from colonic adenomas with different grades of dysplasia to overt carcinoma. H19 was not expressed in a single hyperplastic polyp and three tubulovillous adenomas with dysplasia ranging from mild to severe, in three adenocarcinomas and in metastatic adenocarcinoma of the liver (one case) (fig 7) and lung (one case).

Lung
H19 was not expressed above background level in the bronchial mucosa of fetuses from the
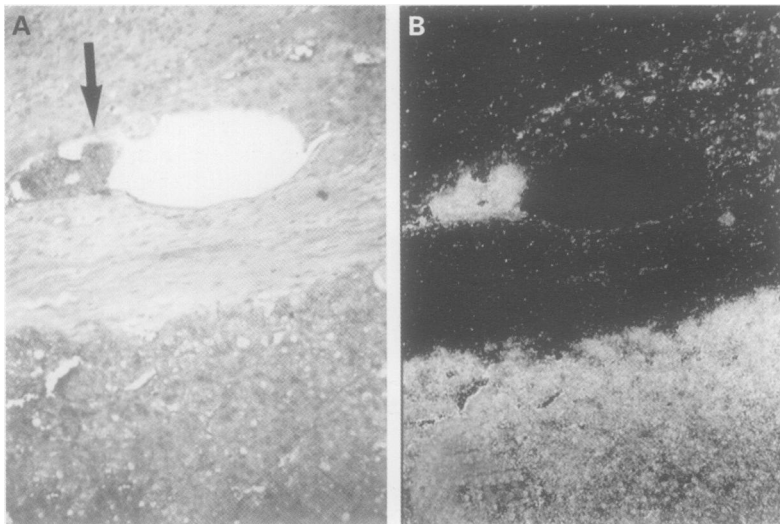
Figure 8   H19 expression in hepatocellular carcinoma. A and B, bright and dark field images, respectively. Prominent expression is evident in hepatocellular carcinoma in the lower half of the picture and also in a group of tumour cells in the lumen of a vascular space (arrow) (original magnification, ×125). (In situ hybridisation of [^{35}S] labelled antisense of H19 with haematoxylin and eosin as a counter stain.)

first trimester (eight weeks) and mid-second trimester (17–22 weeks). Prominent expression of the gene was, however, noted in the large airways at week 14, towards the end of the glandular phase in pulmonary differentiation (fig 3), and also in the tracheal mucosa in the second trimester. In immature teratoma, expression of H19 was seen in epithelium resembling respiratory epithelium (fig 3). Our study of lung carcinoma included 16 primary lung tumours. Moderate expression of H19 was evident in groups of malignant cells in one of three SCCs, one of two adenosquamous carcinomas and one of two adenocarcinomas (fig 3). No expression was detected in the five bronchioloalveolar carcinomas or in tumours with neuroendocrine differentiation (three carcinoid tumours and one small cell carcinoma).

Pancreas
In the fetus, prominent expression of H19 was evident in the acini and islets (not clearly separated at this stage of differentiation), but no expression was seen in the ducts. H19 was not expressed in the four ductal pancreatic carcinomas.

Liver
In the embryo at 35 days post conception, prominent expression of H19 was evident in the liver. The expression was also noted later in gestation during the second trimester of pregnancy.[16]

Prominent expression of H19 was observed in eight of 22 hepatocellular carcinomas (fig 8), moderate expression in four, and weak expression in two cases. Focal expression of the gene was also noted in regenerative nodules in liver cirrhosis and in hepatocytes adjacent to a metastasis of an adenocarcinoma and periportal hepatocytes in the same liver.

Discussion
The H19 gene is one of the few genes imprinted in humans.[28] Since its discovery in mice in 1984,[11] the biological function of this

abundantly transcribed fetal gene has not been elucidated. Activity as a tumour suppressor gene has been suggested on the basis of reduced tumorigenicity of certain cell lines derived from embryonal tumours following the stable induction of a H19 construct.[29] This reduced tumorigenicity was linked with morphological changes suggestive of cellular differentiation. Some of these results could not be repeated by other investigators.[30] The discovery of another imprinted, maternally expressed gene at 11p15.5—that is, p57^{kip2}, can explain some of the data with regard to the tumour suppressor activity of this region.[30 31] This second imprinted gene produces a cyclin dependent kinase inhibitor which may function as a tumour suppressor by inhibiting replication and inducing differentiation.[32]

The role of H19 in neoplasia was studied at first in tumours which resemble morphologically the embryonal tissues from which they arose, primarily Wilms' tumour of the kidney,[6 33 34] and later hepatoblastoma.[35] Trophoblastic tumours[22] and testicular germ cell tumours[36] which differentiate along lines of development of embryonic and extraembryonic tissues also belong to this group of tumours. Later, it became apparent that H19 is also expressed in tumours arising in adulthood, and data regarding carcinoma of the bladder,[19 37 38] lung,[39 40] oesophagus,[41] uterine cervix,[42] and breast[39 43] have accumulated rapidly in the past three years.

The expression of imprinted genes in cancer involves parameters such as allelic status and methylation pattern, on the one hand, and level of gene expression, on the other. It should be emphasised here that biallelic expression of an imprinted gene is not necessarily accompanied by overexpression, and the two phenomena probably represent two different and independent events in the abnormal expression of imprinted genes in neoplasia.[41 44 45] It has been shown that at the very beginning of embryogenesis, H19 is expressed from both alleles, and silencing of the paternal allele occurs at a later step.[46] We propose that biallelic expression of an imprinted gene is an oncofetal feature which is not always linked to reversal to the high embryonic level of gene expression. It is our impression that whereas H19 expression may occur in cancer and sometimes in regenerative and hyperplastic states, the reversal to biallelic expression is probably resticted to neoplasia alone, and may be linked to tumour progression.[41–43]

This study concentrated solely on the comparison of H19 expression in fetal tissues and in tumours arising from these tissues, using ISH. Although the allelic status cannot be studied using ISH, the advantage of using this method is that gene expression can be correlated with the type of cell expressing it.

The necessity to support data regarding gene expression in tumours by ISH is clearly demonstrated in breast cancer. Although H19 expression has been documented in breast carcinoma using northern blotting and PCR,[39 45] we found in a small number of cases that expression was limited to the mesenchymal

stromal cells of the tumour and to the fibrous stroma in fibrocystic disease of the breast. Similar findings were recently documented by Dugimont *et al*[43] who observed H19 expression in the stroma of fibroadenoma and carcinoma of the breast in most of their cases, and only rarely in the neoplastic cells.

H19 was first described as a gene transcribed in endodermal and mesodermal tissues.[47] For convenience, we have classified the tissues and organs according to the original embryonic germ layers. One should remember, however, that tissues originating in different germ layers may mature along the same line of differentiation. We conclude that with regard to H19 expression the dominant factor is probably differentiation. For example, the transitional epithelium of the urinary tract is derived from the endoderm (the hindgut) in the bladder and from the mesoderm (mesonephric duct) in the ureters and pelvicalyceal system. We have shown that H19 is abundantly transcribed in the transitional epithelium of all parts of the urinary tract in fetal life and in transitional cell carcinomas arising from it.

Tissues of different embryological origin which undergo squamous differentiation, whether benign or malignant, have the potential to express H19. The gene is expressed in the epidermis (ectoderm) from the early stages of embryogenesis in the first trimester of pregnancy, and in the squamous epithelial mucosa of the oesophagus (endoderm) in the second trimester of pregnancy. H19 is also expressed in benign squamous metaplasia of the urinary bladder mucosa (endoderm), in morphologically mature squamous epithelium in testicular and sacrococcygeal teratomas, and in SCCs arising in the lung (endoderm) (fig 3), urinary bladder (endoderm) (fig 2) and uterine cervix (mesoderm) (fig 2).

Embryonal and neoplastic tissues share many characteristics—for example, rapid proliferation rate and the ability of cells to migrate. These processes are strictly controlled and intricately coordinated during fetal life, wheras neoplastic tissue is characterised by the loss of equilibrium between determinants of cell proliferation and tissue organisation. The resemblance between embryonal and neoplastic tissue also includes expression of embryonal genes in cancer. The oncofetal genes include carcinoembryonic antigen (CEA), AFP and the beta subunit of choriogonadotropin (βCG). It is of note that H19 was first discovered as a gene which shares trans-regulatory factors with AFP in the liver.[11]

Expression of oncofetal genes is tightly linked to stage of differentiation, both during the fetal period and in neoplasia. We have shown that the expression of H19 is also linked to differentiation which may differ in various tissues. There are fetal tissues—for example, liver, which express H19 from early stages of embryogenesis through at least most of the second trimester of pregnancy. In other tissues, H19 is expressed in a very narrow window of differentiation—for example, in the fetal lung, where H19 is expressed neither in the eighth week nor during 17–22 weeks' gestation but in the large airways during the 14th week, towards the end of the glandular phase of pulmonary development.

One of the most intriguing findings in our study is the expression of H19 in tumours arising from tissues of the central and peripheral nervous system—that is, astrocytoma and ganglioneuroblastoma. The lack of expression of H19 in neural tissue is one of the established characteristics of this gene.[15 16 47] We have examined H19 expression in the nervous system from the embryo at 35 days post conception[16] at two to three week intervals to the 22nd week of gestation and have never detected labelled H19 mRNA using ISH. Moreover, no expression was detected in neural tissue in gonadal and sacrococcygeal immature teratomas. It is possible that H19 is expressed during a very narrow window of differentiation at an earlier stage of embryonal differentiation. Another possibility is ectopic RNA transcription by tumour cells, as occurs in certain types of human cancer and is best demonstrated by ectopic hormone production by tumours.

H19 is expressed during tumorigenesis in an array of human tissues, suggesting that it is probably not a candidate tumour suppressor gene.[38 40-43] A possible role as an oncogene has been ruled out by diverse approaches of investigation.[1 3] It seems from this study that H19 RNA is re-expressed in tumours arising in tissues which express it in fetal life. This expression is linked to the stage of differentiation. The H19 product is, therefore, neither a tumour suppressor gene nor an oncogene, but an oncofetal RNA. We propose to investigate further its potential for use as a tumour marker in certain types of human neoplasia.

1 Feinberg AP. Genomic imprinting and gene activation in cancer. *Nature Genet* 1993;4:110–13.
2 Hochberg A, Gonik B, Goshen R, de Groot N. A growing relationship between genomic imprinting and tumorigenesis. *Cancer Genet Cytogenet* 1994;73:82–3.
3 Tycko B. Genomic imprinting: Mechanism and role in human pathology. *Am J Pathol* 1994;144:431–43.
4 Rachmilewitz J, Goshen R, Ariel I, Schneider T, de Groot N, Hochberg A. Parental imprinting of the human H19 gene. *FEBS Lett* 1992;309:25–8.
5 Zhang Y, Tycko B. Monoallelic expression of the human H19 gene. *Nature Genet* 1992;1:40–4.
6 Rainier S, Johnson LA, Dobry CJ, Ping AJ, Grundy PE, Feinberg AP. Relaxation of imprinted genes in human cancer. *Nature* 1993;362:747–9.
7 Brannan CI, Dees EC, Ingram RS, Tilghman SM. The product of the H19 gene may function as an RNA. *Mol Cell Biol* 1990;10:28–36.
8 Brockdorff N, Ashworth A, Kay GF, McCabe VM, Norris DP, Cooper PJ, et al. The product of the mouse Xist gene is a 15kb inactive Xist specific transcript containing no conserved ORF and located in the nucleus. *Cell* 1992;71:515–26.
9 Wevrick R, Kerns J, Francke U. Identification of a novel paternally expressed gene in Prader-Willi syndrome region. *Hum Mol Genet* 1994;3:1877–82.
10 Rastinejad F, Conboy MJ, Rando TA, Blau HM. Tumor suppression by RNA from the 3' untranslated region of α-tropomyosin. *Cell* 1993;75:1107–17.
11 Pachnis V, Belayew A, Tilghman SM. Locus unlinked to a-fetoprotein under the control of the murine raf and Rif genes. *Proc Natl Acad Sci USA* 1984;8:5523–7.
12 Zemel S, Bartolomei MS, Tilghman SM. Physical linkage of the mammalian imprinted genes H19 and insulin-like growth factor 2. *Nature Genet* 1992;2:61–5.

13 Garvin AJ, Gansler T, Gerald W, Sens DA. Insulin-like growth factor production by childhood solid tumors. *Persp Pediatr Pathol* 1991;**15**:106–16.

14 DeChiara T, Robertson E, Efstratiadis A. Parental imprinting of the mouse insulin-like growth factor II gene. *Cell* 1991;**64**:849–59.

15 Goshen R, Rachmilewitz J, Schneider T, de Groot N, Ariel I, Palti Z, *et al.* The expression of the H19 and IGF2 genes during human embryogenesis and placental development. *Mol Reprod Dev* 1993;**34**:374–9.

16 Lustig O, Ariel I, Ilan J, Lev-Lehman E, de Groot N, Hochberg A. Expression of the imprinted gene H19 in the human fetus. *Mol Rerod Dev* 1994;**38**:239–46.

17 Leighton PA, Ingram RS, Eggenschwiler J, Efstratiadis A, Tilghman SM. Disruption of imprinting caused by deletion of the H19 gene region in mice. *Nature* 1995;**375**:34–9.

18 Biran H, Ariel I, de Groot N, Shani A, Hochberg A. Human imprinted genes as oncodevelopmental markers. *Tumor Biol* 1994;**15**:123–34.

19 Ariel I, Lustig O, Schneider T, Pizov G, Sappir M, de Groot N, Hochberg A. The imprinted H19 gene as a tumor marker in bladder carcinoma. *Urology* 1995;**45**:335–8.

20 Rangini Z, Ben Yehuda A, Shapira E, Gruenbaum Y, Fainsod A. Chox E, a chicken homogene of the H2.0 type exhibits dorsal-ventral restriction in proliferating regions of the spinal cord. *Mech Dev* 1991;**35**:13–24.

21 Rachmilewitz J, Gileadi O, Eldar-Geva T, Schneider T, de Groot N, Hochberg A. Transcription of the H19 gene in differentiating cytotrophoblasts from human placenta. *Mol Reprod Dev* 1992;**32**:196–202.

22 Ariel I, Lustig O, Oyer CE, Elkin M, Gonik B, Rachmilewitz J, *et al.* Relaxation of imprinting in trophoblastic disease. *Gynecol Oncol* 1994;**53**:212–19.

23 Verkerk AJMH, Ariel I, Dekker MC, Schneider T, van Gurp RJHLM, de Groot N, *et al.* Unique expression patterns of H19 in human testicular cancers of different etiology. *Oncogene* 1997 (in press).

24 Mutter GL, Stewart CL, Chaponot ML, Pomponio RJ. Oppositely imprinted genes H19 and insulin-like growth factor 2 are coexpressed in human androgenetic trophoblast. *Am J Hum Genet* 1993;**53**:1096–102.

25 Ariel I, Abeliovich D, Bar-Ziv J, Hochberg A. Renal pathology in WAGR syndrome. *Pediatr Pathol* 1996;**16**:1015–22.

26 Ariel I, Weinstein D, Voutilainen R, Schneider T, Lustig-Yariv O, de Groot N, *et al.* Genomic imprinting and the endometrial cycle. the expression of the imprinted gene H19 in the human female reproductive organs. *Diagn Mol Pathol* 1997;**6**:17–25.

27 Linder D, Kaiser McCaw BK, Hecht F. Parthenogenetic origin of benign ovarian teratomas. *N Engl J Med* 1975;**292**:63–6.

28 Hurst LD, Moore T. Imprinted genes have few and small introns. *Nature Genet* 1996;**12**:234–7.

29 Hao Y, Crenshaw M, Moulton T, Newcomb E, Tycko B. Tumour-suppressor activity of H19 RNA. *Nature* 1993;**365**:764–7.

30 Reid LH, West A, Gioeli DG, Phillips KK, Kelleher KF, Araujo D, *et al.* Localization of a tumor suppressor gene in 11p15.5 using the G401 Wilms' tumor assay. *Hum Mol Genet* 1996;**5**:239–47.

31 Koi M, Johnson LA, Kalikin LM, Little PFR, Nakamura Y, Feinberg AP. Tumor cell growth arrest caused by subchromosomal transferable DNA fragments from chromosome 11. *Science* 1993;**260**:361–4.

32 Matsuoka S, Edwards MC, Bai C, Parker S, Zhang P, Baldini A, *et al.* p57[kip2], a structurally distinct member of the p21[CIP1] Cdk inhibitor family, is a candidate tumor suppressor gene. *Genes Dev* 1995;**9**:650–62.

33 Moulton T, Crenshaw T, Hao Y, Moosikasuwan J, Lin N, Dembitzer F, *et al.* Epigenetic lesions at the H19 locus in Wilms' tumour patients. *Nature Genet* 1994;**7**:440–7.

34 Taniguchi T, Sullivan MJ, Ogawa O, Reeve A. Epigenetic changes encompassing the IGF2/H19 locus associated with relaxation of IGF2 imprinting and silencing of H19 in Wilms' tumor. *Proc Natl Acad Sci USA* 1994;**92**:2159–63.

35 Rainier S, Dobry CJ, Feinberg AP. Loss of imprinting in hepatoblastoma. *Cancer Res* 1995;**55**:1836–8.

36 Van Gurp RJLM, Oosterhuis JW, Kalscheuer V, Mariman ECM, Looijenga LHJ. Human testicular germ cell tumors show biallelic expression of the H19 and IGF2 gene. *J Natl Cancer Inst* 1994;**86**:1070–5.

37 Cooper MJ, Fischer NF, Komitowski D, Shevelev A, Schulze E, Ariel I, *et al.* Developmentally imprinted genes as markers for bladder tumor progression. *J Urol* 1995;**155**:2120–7.

38 Elkin M, Shevelev A, Schulze E, Tykocinski ML, Cooper MJ, Ariel I, *et al.* The expression of the imprinted H19 and IGF-2 genes in human bladder carcinoma. *FEBS Lett* 1995;**374**:57–61.

39 Douc-Rasy S, Coll J, Barrois, M, Joubel A, Frost S, Dozier C, *et al.* Expression of the human fetal BAC/H19 gene in invasive cancers. *Int J Oncol* 1993;**2**:753–8.

40 Kondo M, Suzuki H, Ueda R, Osada H, Takagi K, Takahashi T, *et al.* Frequent loss of imprinting of the H19 gene is often associated with its overexpression in human lung cancer. *Oncogene* 1995;**10**:1193–8.

41 Hibi K, Nakamura H, Hirai A, Fujikake Y, Kasai Y, Akiyama S, *et al.* Loss of H19 imprinting in esophageal cancer. *Cancer Res* 1996;**56**:480–2.

42 Douc-Rasy S, Barrois M, Fogel S, Ahomadegbe JC, Stehelin D, Coll J, *et al.* High incidence of loss of heterozygosity and abnormal imprinting of H19 and IGF2 genes in invasive cervical carcinomas. Uncoupling of H19 and IGF2 expression and biallelic hypomethylation of H19. *Oncogene* 1996;**12**:423–30.

43 Dugimont T, Curgy JJ, Wernert N, Delobelle A, Raes MB, Joubel A, *et al.* The H19 gene is expressed within both epithelial and stromal components of human invasive adenocarcinomas. *Biol Cell* 1995;**85**:117–24.

44 Glassman ML, de Groot N, Hochberg A. Relaxation of imprinting in carcinogenesis. *Cancer Genet Cytogenet* 1996;**89**:69–73.

45 Yballe CM, Vu TH, Hoffman AR. Imprinting and expression of insulin-like growth factor-II and H19 in normal breast tissue and breast carcinoma. *J Clin Endocrinol Metab* 1996;**81**:1607–12.

46 Jinno Y, Ikeda Y, Yun K, Maw M, Masuzaki H, Fukuda H, *et al.* Establishment of functional imprinting of the H19 gene in human developing placentae. *Nature Genet* 1995;**10**:318–24.

47 Poirier F, Chan CTJ, Timmons TM, Robertson EJ, Evans MJ, Rigby PWJ. The murine H19 gene is activated during embryonic stem cell differentiation in vitro and at the time of implantation in the developing embryo. *Development* 1991;**113**:1105–14.