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The human *H19* gene is frequently overexpressed in myometrium and stroma during pathological endometrial proliferative events

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Abstract

We studied the patterns of *H19* expression in normal, hyperplastic and neoplastic human uterine tissues. *H19* RNAs were detected by an *in situ* hybridisation technique (ISH). In both normal and pathological conditions, *H19* was expressed in stromal and myometrial cells, but never in epithelial cells. 34/48 carcinomas overexpressed *H19* compared with the expression in normal tissues. This high expression was frequently observed in the vicinity of malignant epithelial cells. This suggests that the level of *H19* RNA synthesis could be the result of epithelium/stroma interactions. We also demonstrated that several cancerous or immortalised breast epithelial cells release factors into the culture medium, which in turn stimulate *H19* expression in stromal cells. The level of *H19* expression, estimated by ISH, was not significantly correlated with histological type when all types were considered together ($P = 0.108$), but was highly correlated to one type of cancer, i.e. carcinomas with an epidermoid component ($P = 0.0015$). The level of *H19* expression was also strongly correlated with tumour invasion of the reproductive organs ($P = 0.006$) and significantly correlated with neoplastic cell invasion of the myometrium ($P = 0.048$). In conclusion, our results indicate that *H19* overexpression is correlated with the progression of the disease and we propose that this frequent overexpression of the gene in the myometrium and in stroma is a reaction to pathological cell proliferation.

Keywords: Endometrial stroma; Endometrium cancers; *H19* gene; Hyperplastic endometrium; Myometrium; Oncogene

1. Introduction

H19 is a developmentally regulated gene [1,2]. The transcripts accumulate at high levels in tissues of various origins in early development and the gene is strongly repressed after birth in almost all organs. However, a basal but significant level of *H19* gene expression is maintained in a variety of adult organs, such as cardiac and skeletal muscles, the mammary gland and the uterus

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[2–8]. During both normal human and mouse development the gene is subjected to permanent genomic imprinting and only the maternal allele is expressed [9]. Studies of various tumours have demonstrated a re-expression or an overexpression of the *H19* gene in comparison to the healthy tissue. The abnormal expression status of the gene may or may not be accompanied by a loss of imprinting (LOI) or a loss of heterozygosity (LOH). LOH has been reported as being a primary event in several cancers: i.e: Wilms' tumours, liver, lung, ovarian, cervical and breast tumours. LOI of the gene has been shown in lung, oesophagus, bladder, ovarian, Mullerian and cervical carcinomas, rhabdomyosarcoma, hepatoblastoma, and testicular germ cell tumours. In contrast, normal imprinting was maintained in the pathogenesis of colorectal and breast carcinomas, neuroblastomas, leiomyomata and gliomas reviewed in [10].

The *H19* gene is transcribed by RNA polymerase II in a capped, spliced and polyadenylated RNA [1,2], but, to date, all attempts to characterise a H19 protein translated from endogenous *H19* RNA have failed [11,12]. The absence of translation of the native *H19* RNA led some authors to consider the *H19* gene product to be a 'ribomodulator' [11]. Indeed, the role of *H19* is not yet completely deciphered. A tumour suppressor role has been proposed by Hao and colleagues [13], who observed that the *H19* gene, stably transfected in cells lacking the homologous-endogenous sequence produced a growth retardation and a loss of tumourigenicity in a cancerous cell line (G401). However, Reid and colleagues [14], using the same cell lines, found that *H19* expression itself was not correlated with a suppression of malignancy. In agreement with the tumour suppressor status of the *H19* gene, Isfort and colleagues [15] demonstrated that the reintroduction of the gene into Syrian hamster embryo cells reduced their tumourigenicity, and, more recently, Ohana and colleagues [16] reported that human bladder cells with a high *H19* RNA level, when cultivated in low serum conditions, stopped proliferating before those lacking *H19* RNA.

By contrast, the *H19* gene expression pattern in foetal tissues and in carcinomas led other authors to consider this gene to be an 'oncofetal' RNA, with the status of a tumour marker [17]. This latter statement was based on the demonstration that *H19* activity is correlated with tumour grade and progression in bladder and urothelial carcinomas [18]. These discrepancies emphasised the complex nature of the *H19* gene. Interestingly, Li and colleagues [19] re-examined the putative *H19* 'ribomodulator' function and demonstrated that *H19* RNA forms a complex with polysomes translating insulin-like growth factor *IGF2* RNA and appears to be an antagonist of *IGF2* expression in *trans*. More recently, our group demonstrated that *H19* overexpression of ectopic origin conferred a proliferative advantage for breast epithelial cells in a soft agar assay and in severe combined immunodeficient (SCID) mice [20]. Furthermore, this overexpression increases thioredoxin levels in cancerous cells [21]. These phenotypical characteristics are in agreement with an oncogenic role for the *H19* gene.

Few studies have reported *H19* gene expression in the uterus. Lustig and colleagues [22] indicated that *H19* RNA synthesis in the human foetus, occurred in the endometrial stroma, but not in the glandular epithelium. Ariel and colleagues [6] examined *H19* expression in human endometrium during the menstrual cycle by *in situ* hybridisation; *H19* RNA was confined to the stroma and fluctuated with the endometrial dating and reached a peak in the late secretory stage.

We have previously studied *H19* gene expression in the uterus. We demonstrated that *H19* RNA synthesis is under steroid hormone control at various normal stages of the physiological functions of this organ [8]. These findings prompted us to examine *H19* expression in cells recovered from human endometrial tissues exhibiting abnormal overproliferation, especially neoplastic cell growth. To analyse endometrial adenocarcinomas, we undertook a statistical approach to *H19* expression in relation to basic clinicopathological factors usually requested in the diagnosis, prognosis and therapy of these common cancers of the female reproductive system.

2. Patients and methods

2.1. Tissue samples and clinicopathological information

Our study examined 48 specimens of adenocarcinomas from hysterectomy patients who were treated in 1993 at the Oscar Lambret Centre (Lille, France). Survival five years after hysterectomy was ascertained for all patients. Only cases characterised by a primary adenocarcinoma were selected. For each tissue sample, the following clinicopathological factors (*CPF*) were considered (*CPF 3–9* in the World Health Organization (WHO) classification):

CPF 1: Age: number of patients who were younger than 40 years, between 41 and 50 years, 51 and 60 years, 61 and 70 years and older than 71 years was: 1, 5, 14, 20 and 8 cases, respectively;
CPF 2: Menopausal status of patients when surgery occurred: post-menopausal, 36 cases; pre-menopausal, 10 cases; undetermined (UD), 2 cases;
CPF 3: Histological classification of malignant neoplasms of the uterine corpus: endometrioid, 23 cases; adenocarcinoma with epidermoid component, 11 cases; endometrioid and mucinous, 11 cases; undifferentiated, 2 cases; UD, 1 case;

CPF 4: Histological grading of endometrial carcinomas: grade I, well differentiated; grade II, moderately differentiated; grade III, undifferentiated or poorly differentiated 16, 26 and 4 cases, respectively; UD, 2 cases;
CPF 5: Tumour characterised by the *International Federation of Gynecology and Obstetrics* (FIGO) classification (1976, modified in 1988): stage I, classes Ia and Ib, tumour was contained within the uterine body, but was smaller (Ia) or greater (Ib) than 80 mm: 11 and 32 cases, respectively; stage II, tumour was extended to the cervix, but limited to the uterus: 4 cases; stage III, extension outside of the uterus, but limited to the pelvis, IIIb, vaginal metastases: 1 case;
CPF 6: Tumour invasion within the genital organs: Fallopian tubes not invaded, 8 cases; one or two Fallopian tubes invaded: 21 cases; Fallopian tubes and isthmus invaded: 12 cases; Fallopian tubes, isthmus and/or the cervix and ovaries invaded: 7 cases;
CPF 7: Myometrial invasion: less than half of the myometrium invaded: 23 cases; from half to the whole of the myometrium invaded: 24; UD: 1 case;
CPF 8: Pelvic lymph node metastases: invaded: 3 cases; not-invaded: 33 cases; UD: 12 cases;
CPF 9: Occurrence of metastases, other than in the pelvic lymph nodes: positive: 2 cases; negative: 46 cases.

Other proliferative diseases of the uterine endometrium from patients treated in 1999 and 2000 were studied: i.e. adenomyosis, endometrial polyps, simple hyperplasia with or without atypia and complex hyperplasia with or without atypia.

2.2. Fixation, embedding and histological staining

Immediately after resection, human endometrial tumours and adjacent normal tissues were fixed with formalin (10% v/v) for 24 h, dehydrated through increasing ethanol concentrations and embedded in Paraplast plus. Five micron sections were transferred to Esco Superfrost Plus (Polylabo) slides for immunohistochemical staining (IH) or *in situ* hybridisation (ISH). For IH, tumour sections were fixed on slides using a glycerinated albumin (10% v/v) solution.

Hemalun–phloxine–safran (HPS) staining was performed on one section of each tumour. This section indicated histological structures of samples and HPS allowed us to localise precisely the most interesting areas for observation after the various IH procedures and the ISH. As a control, we analysed normal tissues located near the tumour and *H19* gene expression in these areas was compared with that observed within the tumour.

2.3. Immunohistochemical staining

Before immunostaining, slides were treated as previously described by Balaton and colleagues in [23] and summarised by Adriaenssens and colleagues in [7]. In order to establish, at the cellular level, any relationships between *H19* gene expression and the biological activity of cells, we considered, in parallel to the ISH, four immunostaining reactions: (1) Mib-1 antibody directed against a proliferation-related antigen (prediluted, Immunotech, France); (2) anti-p53 raised against the amino-terminal amino-acid sequence of both the wild-type and mutant versions of this molecule (DO-1, prediluted, Immunotech); (3) anti-oestrogen receptor (anti-ER) and anti-progesterone receptor (anti-PR) named 1D5 and 1A6, respectively, from Dako (Denmark) and diluted to 1/20.

2.4. In situ hybridisation

2.4.1. Riboprobes

A 1.3 kb *Stu*I fragment of *H19* cDNA was recombined with pSP65 plasmids at a *Sma*I site in both orientations (sense and antisense). Plasmids were linearised by *Hind*III digestion. Sense and antisense probes were transcribed in the presence of [^{35}S] cytosine triphosphate (CTP) by SP6 polymerase and reduced to a 150 bp average length before use.

2.4.2. ISH protocol

The basic experiments were previously described by Quéva and colleagues in [24]. After hybridisation, slides were dipped in NTB2 nuclear track emulsion (Kodak) and exposed for three weeks. Autoradiographic development (D19 developer) and fixation (Unifix, Kodak) were performed at 12 °C. A fluorescent post-staining of the nuclei was performed (Hoechst, 33258 Bisbenzimidine, Serva, $\lambda = 340$ nm). Coverslips were fixed with Dako-glycergel (Sebia). Observations were performed through an Olympus BH2 microscope.

2.5. Statistical methods

Labelling levels were evaluated from a series of 10–15 sections on each slide. Several microscopic fields were considered for each section on a given slide. Low power magnification was used allowing an easier evaluation of the *H19* labelling. The intensity of the *H19* labelling was classified as follows: weak, + (similar to normal tissues); intermediate, ++; high, +++. Intermediate or high labelling was established independently by the authors *versus* the weak *H19* expression that is observed in normal tissues. A general statistical analysis was performed using the Fisher's exact test. The latter is used to test for row and column independence in a single contingency table (StatXact, 1992) indicating the level and distribution of *H19* labelling. For one subclass of adenocarcinomas (adenocarcinomas with an epidermoid component), we used the S-Plus 4 Guide to statistics, 1997; Data

Analysis Products Division, MathSoft, Inc., Seattle. The threshold P value of 0.05 was chosen.

2.5.1. Cell culture and northern-blot analysis

Normal breast epithelial and fibroblast cells were established from mammoplasty material (20- to 40-year-old women) obtained from the Department of Plastic Surgery (Pr. Pellerin) of the Medical University of Lille (France). MDA-MB-231 and MCF-7 are two human cancer mammary epithelial cell lines (ATCC). HBL-100 is an immortalised human mammary epithelial cell line generously provided by Pr. Crespin (Medical Faculty, Bobigny, France). All mammary epithelial cell lines were cultured as previously described in [25]. Medium conditioned by mammary epithelial cells was collected after 48 h of cell culturing, centrifuged and frozen prior to use.

Total RNAs from cells were isolated using the guanidium isothiocyanate–CsCl gradient method. Denatured RNA samples (20 μg/well) were fractionated on formaldehyde 1.2% w/v agarose gel, transferred to a Hybond-C-Extra filter and analysed by Northern-blot hybridisation. RNA amounts were quantified by gel staining with ethidium bromide allowing the visualisation of ribosomal RNA (28S and 18S). Hybridisations were performed in stringent conditions with a 1.2 kbp *H19* cDNA fragment, labelled by random priming using deoxycytidine 5′-[α^{32}P] triphosphate.

3. Results

3.1. Expression pattern of the H19 gene in normal and pathological uterine tissues

We used ISH to study *H19* gene transcription in normal, hyperplastic and neoplastic uterine tissues. Hybridisations with a sense probe were used as controls and provided no significant labelling (data not shown). In normal tissues, this gene was weakly expressed in mesenchymal cells (Fig. 1.1, asterisks), but not in luminal or glandular epithelium (Fig. 1.1, arrow-heads). In hyperplastic endometrium, the *H19* labelling was very high in the stroma surrounding epithelial structures and absent from epithelial cells; this feature is illustrated, for instance, in a simple hyperplasia without atypia, (Fig. 1.2, luminal and glandular epithelial cells, arrow-head and arrow, respectively; (lu) indicates the uterine luminal cavity). This *H19* expression pattern was also observed in other proliferative events occurring in endometrial diseases: i.e.: adenomyosis, endometrial polyps, simple hyperplasia with atypia, as well as complex hyperplasia with or without atypia (data not shown). In cancerous endometrium, *H19* was exclusively expressed in the endometrial stroma surrounding large areas of neoplastic cells (Fig. 1.3 (s)), but never in epithelial structures (Fig. 1.3 (c, arrow-head)). Overexpression of the *H19* gene in cancerous endometrium was observed in 34/48 cases (compare Fig. 1.1 with Fig. 1.3). In 28/34 specimens, we observed an increasing *H19* RNA accumulation gradient from distal to proximal endometrial stroma. This quite frequently gave a high labelling of the area surrounding the neoplastic cells (Fig. 2). Similarly, several myometrial cells

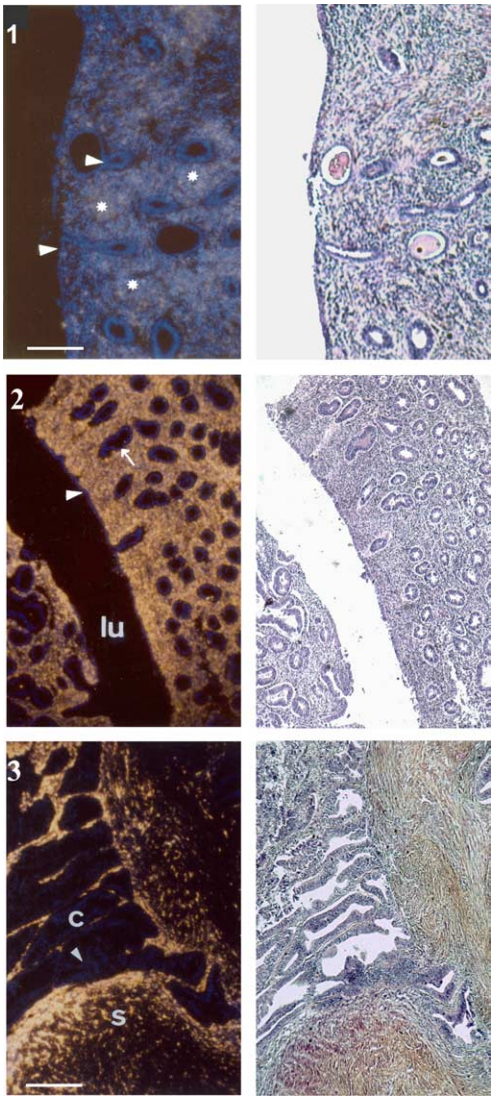

Fig. 1. *H19* gene expression in normal, hyperplastic and cancerous endometrium after *in situ* hybridisation (ISH) using an antisense sequence probe (left pictures). Photographs on the right illustrate counterparts of the tissue sections stained by hemalun–phloxine–safran (HPS). Scale marker: 10 mm = 625 μm. 1.1: A healthy area. A very weak labelling is observed in stromal cells (asterisks), but not in luminal or glandular epithelium (arrow-heads). 1.2: Hyperplastic endometrium. *H19* is obviously overexpressed in the stroma of simple hyperplasia without atypia. The glandular (arrow) and luminal (arrow-head) epithelial cells are not labelled. (lu) indicates the luminal cavity of the uterus. 1.3: Adenocarcinomas quite frequently show high *H19* labelling of the stroma (s) surrounding the cancerous area (c). Epithelial cells are negative for *H19* gene expression (arrow-head).

expressed the gene at a higher level in the internal part of this tissue than in the external one (Fig. 2).

3.2. Comparisons between H19 gene expression in adenocarcinomas and several biological cell markers

Cell proliferation was evaluated by immunohistochemical staining with a Mib-1 monoclonal antibody, which detects an epitope of the Ki-67 protein in formalin-fixed tissues. In all of the endometrial tumours examined, some epithelial cells were Ki-67/Mib-1-positive and we distinguished three classes of specimens: (a) no or only a very few labelled cells (28 cases), (b) less than 10% of labelled cells (15 cases), (c) 10–50% of labelled cells (5 cases). However, stromal cells never expressed this antigen. Therefore, no obvious co-localisation could be established at the cellular level between *H19* expression and the presence of the Mib-1 marker (data not shown).

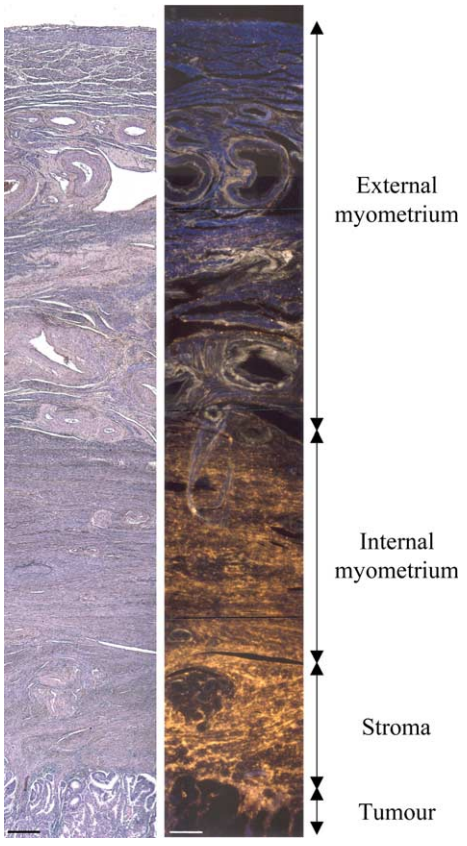

Fig. 2. Section of an uterus showing tissues from the tumour to the external myometrium. The tumour area comprises an external stroma, which is highly labelled by the *H19* antisense probe (right picture). In contrast, the internal part of the tumour is essentially composed of epithelial cells where the *H19* gene is not expressed, and thin ribs of labelled stroma. There is frequently (in 28/34 cases) an obvious gradient where labelling increases from the external part of the organ to the area of cancerous cells. Photograph on the left illustrates the counterpart of the tissue section stained by HPS. Scale marker: 10 mm = 625 μm.

Using an anti-p53 antibody, we found that when a tumour exhibited a positive signal, the latter labelled the nucleus of epithelial, but not stromal cells. Tumours have been classified as follows: negative reaction (23 cases), less than 10% of labelled cells (15 cases), 10–50% of labelled cells (7 cases), 51–100% of positive cells (3 cases). There was no overlap between the presence of *H19* RNA, determined by ISH and p53 accumulation in the tumours studied (compare Fig. 3.1 with Fig. 3.2).

The expression of both the ER and PR was examined. It is known that ER-α is abundant in stromal and epithelial cells of the endometrium, while only a very low ER-β protein expression is detected in these cells. Well-differentiated tumours are more likely to be ER-positive (ER^+) and PR-positive (PR^+) than poorly differentiated neoplasms. Positive reactions were observed in some stromal or epithelial cells, but, no co-localisation with *H19* RNA was observed (compare Fig. 3.1 with Fig. 3.3 and Fig. 3.4).

3.3. Statistical analysis

We examined the relationship between *H19* expression level in the endometrial stroma and myometrium and the clinicopathological factors indicated in Section 2.

In 14 adenocarcinomas, expression of the gene in the endometrial stroma and myometrium was not different to the pattern exhibited in normal tissues, but in 34 adenocarcinomas, it was clearly higher.

The statistical methods described in Section 2 were applied to the series of clinicopathogical factors: *CPF 1–9*.

Different classifications of patient age (*CPF 1*) were used for the establishment of the statistical test. First, we applied the distribution shown in Section 2, and we obtained no significant correlation between this classification and the level of *H19* expression ($P = 0.311$, Table 1). Secondly, we calculated the arithmetical mean and median ages, which are 62.42 and 63.00 years, respectively and we applied the statistical test to the *H19* gene expression level in the two classes of patients determined as being less than or equal to 63 years and more than 63 years. The test did not demonstrate any correlation between the patient's age and *H19* RNA expression.

There was also no correlation between the *H19* expression level and each of the following factors: menopausal status (*CPF 2*), histological tumour type (*CPF 3*), histological grading (*CPF 4*) and FIGO classification (*CPF 5*) (Table 1).

Histological classification of adenocarcinomas was analysed (*CPF 3*). When *H19* labelling levels are considered for the subclass of "adenocarcinomas with epidermoid component" only, it appeared that tumours were not randomly distributed between the three classes (1 case +, 1 case ++, 9 cases +++), $P = 0.0015$, indicating that high *H19* labelling could be considered a marker of this

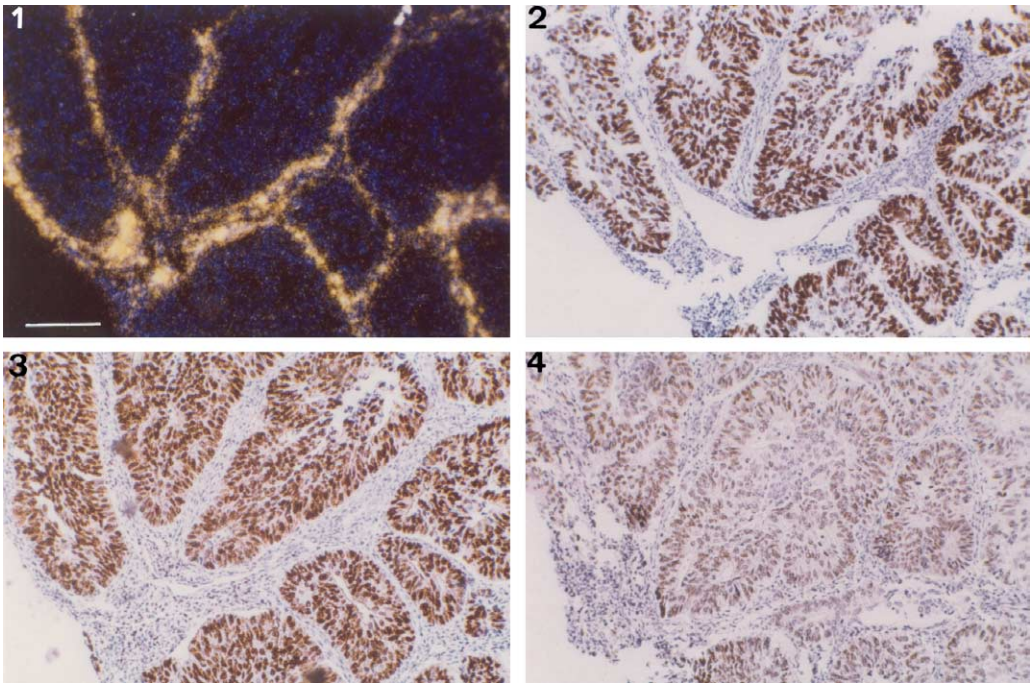

Fig. 3. Comparisons between *H19* gene expression pattern and p53 or steroid receptor patterns in an endometrial adenocarcinoma. Scale marker: 10 mm = 625 μm. 3.1: ISH performed with an antisense *H19* riboprobe. 3.2: Anti-p53 labelling pattern. 3.3: Anti-progesterone receptor (PR) labelling pattern. 3.4: Anti-oestrogen α-receptor (ER) labelling pattern. No overlap between anti-p53, anti-PR or anti-ER and *H19* RNA labellings was observed.

histological subclass of adenocarcinomas (probability test in S-Plus 4). However, for the other two subclasses, namely endometrioid adenocarcinomas (8 cases +, 5 cases ++, 10 cases +++) and endometrioid and mucinous adenocarcinomas (3 cases +, 4 cases ++, 4 cases +++), *H19* expression could not be considered a neoplastic marker (probability test in S-Plus 4; $P = 0.99$).

The level of *H19* expression was significantly correlated with tumour invasion of the female reproductive system (*CPF 6*): $P = 0.006$. Finally, *H19* expression intensity correlated with myometrium invasion (*CPF 7*): $P = 0.048$.

Because of the low number of cases in one class of each factor *CPF 8* and *CPF 9* (i.e: the pelvic lymph node metastases, the occurrence of other metastases), no statistical analysis of *H19* expression compared with the status of these factors was possible.

The statistical test failed to establish any significant correlation between the different classes of epithelial cells labelled by Ki-67/Mib-1, p53 protein or hormone receptors immunohistochemical staining and the level of *H19* expression in the stromal cells.

3.4. Epithelial cells regulate H19 expression in mesenchymal cells

The frequent observation that the level of *H19* expression was high in stromal cells in the vicinity of epithelial cells led us to question whether there was the cross-talk between epithelial and mesenchymal cells. Interactions between these two types of cells can be observed using cell line models. We tested the possibility that epithelial cells release factors into the culture medium which are able to modify their environment. Experiments were performed using several breast epithelial cell types; normal breast epithelial cells (NBEC) or tumour cells (HBL-100, MCF-7, MDA-MB-231) and normal breast human fibroblasts (primary culture). The breast model was chosen because normal fibroblasts and normal epithelial cells (NBEC) from reductive surgery are routinely used in our laboratory, and, as in tumour endometrium, we frequently observed an overexpression of *H19* near the tumour cells in breast benign or malignant tumours [5,7]. Northern-blot analysis showed that soluble factors produced by various breast epithelial cells differentially regulated *H19* gene expression in fibroblasts and that this expression was dependent on the cell line. All cancer cells tested induced a higher rate of *H19* gene transcription in fibroblasts than NBEC did (Fig. 4). It is worth noting that MDA-MB-231, considered to be the most aggressive cells, displayed the greatest effect (Fig. 4).

4. Discussion

Endometrial carcinoma is a common invasive malignancy of the reproductive system in industrial countries.

Table 1
Statistical analysis performed using the Fisher's exact probability test considering the *H19* labelling level in the stromal area for each clinicopathogical factor (*CPF*)

Clinicopathological factors (CPF)	*H19* expression			
	+	++	+++	
CPF 1 ($n = 48$)				
Age				
⩽40	0	0	1	$P = 0.311$
41–50	0	3	2	
51–60	6	1	7	
61–70	7	4	9	
⩾71	1	2	5	
CPF 2 ($n = 46$)				
Menopausal status				
Yes	12	6	18	$P = 0.350$
No	1	3	6	
CPF 3 ($n = 47$)				
Histological tumour type				
Endometrioid carcinomas	8	5	10	$P = 0.108$
Carcinomas with epidermoid component	1	1	9	
Endometrioid and mucinous	3	4	4	
Undifferentiated	2	0	0	
CPF 4 ($n = 46$)				
Histological grade				
I	5	5	6	$P = 0.575$
II	7	5	14	
III	2	0	2	
CPF 5 ($n = 48$)				
FIGO classification				
Ia	3	0	8	$P = 0.149$
Ib	8	9	15	
II	2	1	1	
IIIb	1	0	0	
CPF 6 ($n = 48$)				
Tumour invasion in genital organs				
Fallopian tubes not invaded	2	0	6	$P = 0.006$
One or two Fallopian tubes invaded	9	2	10	
Fallopian tubes and isthmus invaded	0	7	5	
Fallopian tubes, isthmus and/or cervix and ovaries invaded	3	1	3	
CPF 7 ($n = 47$)				
Myometrium invasion				
<50%	9	2	12	$P = 0.048$
⩾50%	5	8	11	

FIGO, International Federation of Gynaecology and Obstetrics.

The advent of immunohistochemical and molecular biological techniques have provided new tools that have the potential to help pathologists in the diagnosis of endometrial carcinoma. In our present study, we wanted to show the relevance of *H19* expression in endometrial tumours. We have also considered some hyperplastic proliferative diseases.

In a previous paper, we reported the localisation and level of *H19* gene expression in the mammary gland and uterus at various stages of normal development and

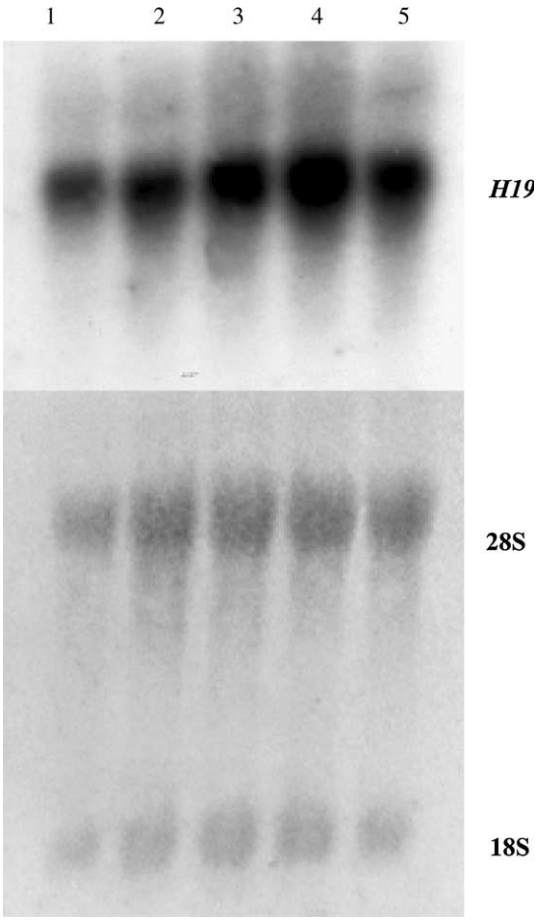

Fig. 4. Northern-blot analysis of *H19* gene expression in normal mammary fibroblast cells stimulated with medium conditioned by various epithelial cells. Lane 1: fibroblasts cultured in standard conditions. Lanes 2 to 5: fibroblasts cultured in medium conditioned by normal breast epithelial cells (NBEC), HBL-100, MDA-MB-231 and MCF-7, respectively.

demonstrated that the *H19* gene is controlled by steroid hormones and their specific receptors (upregulation by 17-β-oestradiol, downregulation by progesterone) [8]. Based on these findings, we investigated, by ISH, *H19* gene expression within endometrial adenocarcinomas and attempted to correlate *H19* gene activity in tumours with various clinicopathological factors usually used for the diagnosis and prognosis of neoplastic evolution in human endometrium. Weak and distinct differences in gene expression between pathological tissues *versus* normal ones were not detectable by ISH. Nevertheless, this semi-quantitative approach is the most appropriate method for the localisation of gene expression and determination of large differences in gene activity.

One of the most impressive features observed has been the continued absence of *H19* expression in neoplastic epithelial cells, as well as in healthy ones, within the adenocarcinoma cells of the 48 samples studied. This absence was also observed in hyperplastic epithelial cell tissues. Indeed, in carcinoma and hyperplastic cases, *H19* gene expression was limited to the stromal component,

particularly in cells in the vicinity of the pathological cells. Myometrial cells in the proximity of the tumours frequently possessed a high level of *H19* RNA. Elsewhere, we previously reported in breast adenocarcinomas that, 92% of cases analysed exhibited obviously high stromal labelling, and only 8% of adenocarcinomas showed *H19* overexpression in epithelial cells [7]. It is interesting to note that the neoplastic process in breast tissue was frequently accompanied by a loss of *H19* expression in cancer cells, whereas its expression is always observed in normal epithelial cells. This striking absence of *H19* gene expression suggests that the gene has a tumour suppressor function, as several authors have already proposed [26–28]. However, although the frequent absence of *H19* expression in breast cancer epithelial cells might suggest a tumour suppressor status for the gene, we have shown that a high level of *H19* RNA led to an oncogenic phenotype of breast cancer cells [20]. The latter phenomenon is in agreement with rare accumulations of *H19* transcripts in the epithelial cells of breast adenocarcinomas [5,7]. Taken together, our results suggest an imbalance in *H19* RNA synthesis plays a role during neoplastic processes. *H19* may have either a tumour-promoting activity or tumour suppressing activity, probably depending on the local environment.

As mentioned above, we show here that the *H19* gene in the endometrium was not expressed in normal epithelial cells, or in pathological cells. Observations in healthy epithelial cells are in agreement with the *H19* expression pattern previously noted in normal murine or human uterus [6,8]. Thus, an important finding of this study is that *H19* RNA is not conspicuously synthesised in epithelial cells, whatever the normal or pathological conditions. Consequently, it appears that the absence of *H19* expression cannot be overcome in human endometrial epithelial cells when endometrial proliferative events occur.

The present observations, in particular the continued striking absence of *H19* gene expression in neoplastic endometrial cells, are in apparent disagreement with the overexpression of the gene described in several other tumours under consideration (epithelial and stromal cells) [4,29,30]. In fact, there is no actual discrepancy between these accounts as the stromal part of these tumours contained most of the *H19* RNA. In addition, among the 34 specimens overexpressing *H19*, 28 were characterised by an increasing gradient of *H19* RNA accumulation from distal to proximal endometrial stroma. More accurately, we observed a *H19* RNA concentration preferentially localised at the epithelial/stromal boundary. This suggests that *H19* in uterine stromal cells can be targeted by one or several factor(s) released by the epithelium. We have previously described that in breast epithelial cells, *H19* gene expression could be stimulated by fibroblasts which release several growth factors, such as hepatocyte growth factor/scatters factor (HGF/SF) [25] into the culture medium. We show here that epithelial cells can exhibit the converse effect of increasing the *H19* expression level in stromal cells. Taken together, our results suggest crosstalking between the epithelium and mesenchyme. It has also been reported that abnormal fibroblast phenotypes observed *in situ* or in primary cultures may be explained as reversible physiological responses to abnormal signals transmitted by tumour epithelial cells. This is in agreement with the proposal that one tissue during tumour development could, by physical connection, cause the transformation of other tissues [31,32]. To investigate further the change in *H19* gene expression involved in the cell–cell communication that occurs during abnormal proliferation, we performed experiments involving culture medium conditioned by normal or tumoural breast epithelial cells. The comparatively higher abundance of *H19* transcripts in fibroblasts cultured in the presence of factors released (conditioned medium) by cancerous cells could account for the high labelling of the stroma when pathological proliferation of epithelial cells occurs. Singer and colleagues [33] described the overexpression of *IGF2* in stromal cells of breast cancers as the result of a paracrine stimulus released from epithelial cells, this stimulation playing a pivotal role in the maintenance of the tumours. Collectively, these data indicate that *H19* gene overexpression can be considered a stromal reaction marker. Moreover, Hashimoto and colleagues [34] reported a LOI and the upregulation of *H19* in malignant mixed Mullerian tumours of the uterus, which are thought to develop from the myometrium. This suggests that the widely varying levels of *H19* expression reflects the diverse functions of *H19* and probably depends, among other factors (developmental stage, local environment), on the tissue type examined.

Several studies have shown that Ki-67 gives a rapid and reliable estimate of the neoplastic cell growth fraction in various tumours, i.e., lymphoma, breast cancer and colorectal cancer. In this study, we found a positive Ki-67/Mib-1 reaction (20 of the 48 cases investigated) in only some epithelial cells (less than 50% of cells), and never in stromal cells. The statistical test established no correlation between the level of stromal *H19* expression and the Ki-67/Mib-1 status of the epithelial cells. Similarly, in normal systems, we previously reported that the *H19* gene expression was independent of Ki-67 labelling in the uterus of steroid-stimulated mice [8].

The tumour suppressor protein p53 acts as a molecular switch that activates a checkpoint in the G1 phase of the cell-cycle. Point mutations of the *p53* gene, leading to the overexpression of a mutant p53 protein, are the most common molecular alterations described in human cancers to date. It has been shown that normal cells have low levels of p53 protein because it is rapidly degraded, but mutant p53 proteins are resistant to degradation

and hence accumulate in the nucleus. Consequently, mutated p53 protein can be reliably detected by immunohistochemical techniques. In HeLa cells, we have previously demonstrated down- and up-regulation of the *H19* gene by the wild-type and mutated p53 protein, respectively [35]. In the present study, p53-positive areas were found only in epithelial cells in 25 of 48 endometrial adenocarcinomas. As *H19* gene transcripts were absent from the tumour cells, we conclude that whatever the p53 status (wild-type or mutated) of the epithelial cells, *H19* gene is not regulated by p53.

Furthermore, various diagnostic and/or prognostic factors were considered in our study. No significant correlations were observed between the age and menopausal status of the patients, the histological tumour type, the histological grade and the FIGO classification. By contrast, the level of *H19* expression was significantly correlated with tumour invasion and myometrium invasion of female genital organs. Further, a high *H19* labelling seems to be significantly correlated with one subclass of adenocarcinomas (adenocarcinomas with an epidermoid component, according to a S-Plus 4 probability test). Collectively, these considerations indicate that the overexpression of *H19* (34 out of 48 carcinomas) is a reflection of tumour extension.

In conclusion, *H19* RNA accumulation in stromal cells and the absence of *H19* gene expression in epithelial cells was observed, even though the gene was frequently overexpressed (approximately 70% of endometrial adenocarcinomas). This duality forms part of the general stromal reaction arising during uterine tumour genesis. Interestingly, numerous genes are differentially expressed in stromal cells under the influence of cancerous epithelial cells. For example, Wang and Tetu [36] showed that stromelysin-3 (ST3) expression by stromal cells was cancer-specific. Indeed, ST3 expression by stromal cells was induced by three malignant breast cancer cell lines, whereas no ST3 was expressed under normal mammary epithelial cell stimulation. In a more comprehensive study, differential modulation of stromal characteristics induced by epithelial cells has been reviewed, as well as the tumour stroma influencing reciprocally the differentiation status of tumour cells [37]. *H19* gene expression could be regulated by this crosstalk between the epithelium and stroma. Thus, it seems important to consider mesenchymal cells, in addition to cancer cells, and our results argue in favour of considering *H19* gene overexpression as a stromal reaction during abnormal endometrial cell proliferation.

Conflict of interest statement

None declared.

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