Overexpression of an ectopic H19 gene enhances the tumorigenic properties of breast cancer cells

Séverine Lottin1, Eric Adriaenssens1, Thierry Dupressoir2, Nathalie Berteaux1, Claire Montpellier1, Jean Coll1, Thierry Dugimon1 and Jean Jacques Curgy1,5

1Laboratoire de Biologie du Développement, UPRES-EA 1033, SN3, USTL, 59655 Villeneuve d’Ascq Cedex, 2Laboratoire de Pathologie Comparée, UMR5807, INRA/CNRS/UMII, cc101, 34095 Montpellier Cedex 5, 3Laboratoire Assemblage et Réplication du Virus de l’Hépatite C, FRE CNRS 2369, IBL, BP 447, 59021 Lille Cedex and 4Laboratoire d’Immunopathologie Cellulaire des Maladies Infectieuses, UMR 8527, IBL, BP 447, 59021 Lille Cedex, France

The maternally expressed H19 gene is transcribed as an untranslated RNA that serves as a riboregulator. We have previously reported that this transcript accumulates in epithelial cells in ~10% of breast cancers. To gain further insight on how the overexpression of the H19 gene affects the phenotype of human breast epithelial cells, we investigated the oncogenic potential of RNA that was abundantly expressed from MDA-MB-231 breast cancer cells stably transfected with the genomic sequence of the human H19 gene. The amount of H19 RNA did not affect cell proliferation capacity, timing of cell cycle phases or anchorage-dependent ability of H19-transfected clones in vitro. But in anchorage-independent growth assays the H19-recombined cells formed more and larger colonies in soft-agar versus control cells. To explore this phenotypic change, we analysed tumour development after subcutaneous injection of H19-recombined cells into scid mice. Results showed that H19 overexpression promotes tumour progression. These data support the hypothesis that an overload of H19 transcript is associated with cells exhibiting higher tumorigenic phenotypes and therefore we conclude that the H19 gene has oncogenic properties in breast epithelial cells.

Introduction

The mouse H19 gene was discovered in 1984 by virtue of its coordinate regulation with the alpha-fetoprotein gene under the control of the two raf and Rif loci (1). Its human counterpart is located at 11p15.5 within a cluster of imprinted genes, near the IGF2 gene (2). These two genes are oppositely imprinted, IGF2 being only paternally expressed (3), while H19 is exclusively transcribed from the maternal allele (4). H19 is highly expressed in the extraembryonic tissues (placenta), in the embryo proper and in most of fetal tissues but its expression is repressed or dramatically reduced after birth (5,6). Only few adult organs such as cardiac and skeletal muscles (1), lung (6), uterus (7,8) and mammary gland (9,10) retain a basal level of H19 expression.

The H19 gene is transcribed by the RNA polymerase II, producing a capped, spliced and polyadenylated RNA. This transcript, located in the cytoplasm and recently found to be associated with polyomes (11), is untranslated. Although a 26 kDa protein has been obtained after transient transfections of construct carrying deletions and/or point mutations, the 5' untranslated region prevents the H19 RNA from being translated in vivo (12). The comparison between mouse and human sequences did not show any conserved open reading frames (ORF) despite an overall 77% sequence homology (13). Theoretical translation of seven additional H19 mammal sequences confirmed the absence of conserved ORF of any size, but by aligning these sequences it was established that the secondary structure was conserved at the RNA level (14). It is now widely admitted that the H19 transcript belongs to the growing family of mRNA-like non-coding RNA (15).

As of today, the role of the H19 gene is still controversial. First, the H19 gene is reportedly associated with tumour suppression, as demonstrated by differential screening of cDNA of Syrian hamster embryo (SHE) cells that have either retained or lost the ability to suppress tumorigenicity of a highly malignant cell line in cell hybrid experiments (16). This putative tumour suppressor role was further sustained when Hao et al. (17) transfected the H19 gene in two embryonic tumour cell lines and showed that both cell lines demonstrated retarded growth. In one of these cell lines, they found that anchorage-independent growth and tumorigenicity were abrogated (17). Next, in SHE cells, the re-expression of H19 by stable transfection of the same construct as previously described had little effect on cellular growth in vitro, but did delay tumour growth in nude mice (18). Last, in non-experimental models, the inactivation of H19 in Wilms’ tumour concurs with a tumour suppressor role (19,20).

In contrast, many types of cancers such as breast (9,10,21), bladder (22–24), lung (9,25), oesophageal (26) and cervical carcinomas (27) exhibit an H19 overexpression. More importantly, in bladder carcinomas, H19 expression is statistically correlated with tumour grade and is a marker of early recurrence, suggesting that H19 may have oncogenic properties in this type of cancer (22,24). Likewise, the expression of the H19 gene was observed in tumours induced by bladder cancer cell lines with no detectable H19 mRNA before injection in nude mice (23). The same results were obtained with choriocarcinomas cell lines (JEG-3). But while cells isolated from the tumours produced by bladder cancer cells lost H19 gene expression after three passages of in vitro culturing, cells isolated from the JEG-3-derived tumours retained high H19 gene expression even after 15 passages. Not only did the high levels of H19 mRNA not alter the growth rate or the independent-anchorage growth of these cells, but also the latter were more tumorigenic than the original cells (28,29). In HeLa × normal human fibroblast hybrids, it has also been
shown that H19 was specifically expressed in the spontaneous tumorigenic segregants from non-tumorigenic hybrids. Nevertheless, stable transfections of H19 in the non-tumorigenic hybrids did not cause in vivo tumour growth in nude mice. This indicates that although H19 is necessary to the tumorigenic process, it does not induce it (30,31). Overall, in many models, data argue in favour of an implication of the H19 gene during tumorigenesis and contradict the tumour suppressor role of this gene.

In a previous study, we demonstrated that H19 was overexpressed in over 70% of breast adenocarcinomas. This overexpression was preferentially located in the stromal cells, though sometimes in epithelial cells. Although the small number of cases where H19 was activated in the sole epithelial cells could support no statistical correlation, we noticed that this pattern was associated with the absence of hormone receptors and the death of patients within 5 years after tumorectomy (21). The overexpression of the H19 gene in epithelial cells of ~10% of breast carcinomas and the controversial data about its oncogenic or anti-oncogenic role prompted us to evaluate the H19 gene status in breast cancer. To do so, we stably transfected MDA-MB-231 breast cancer cells with the genomic sequence of the human H19 gene and investigated the effects of H19 overexpression on growth rate, clonogenic ability and tumorogenicity of these cells.

Materials and methods

Cell culture

MDA-MB-231 breast cancer cells were originally obtained from the American Type Culture Collection (ATCC, Rockville, MD). Cells were maintained in minimal essential medium (MEM) supplemented with 20 mM HEPES, 2 g/l sodium bicarbonate, 2 mM L-glutamine, 100 U/ml of penicillin–streptomycin, 50 µg/ml gentamycin, 1% of non-essential amino acids (Biowhitaker) and 5% of fetal calf serum (FCS) in a humidified, 37°C, 5% CO2 incubator.

Stable transfection

MDA-MB-231 cells overexpressing H19 were obtained by stable transfection using the expression vector pRKCMV (Invitrogen) in which ligating a 6 kb genomic sequence containing the entire H19 gene was placed under the constitutive control of the Cyto/mediumator promoter after insertion in the HindIII. MDA-MB-231 cells were transfected using ExGen 500 (EuroMedex) according to the manufacturer’s instructions. Twenty-four hours before transfection, cells were plated into 100 mm dishes at a density of 1.5 x 105 cells per dish. Forty-eight hours after transfection, cells were diluted and divided into new culture dishes. Cells were selected after being grown 15 days in medium containing 1000 µg/ml of ganciclovir (GIBCO-BRL). Cell colonies were isolated by trypsinization onto small squares of sterile filter paper. All experiments were performed without G418 after culture for 1 week.

DNA isolation and PCR

Confluent monolayer cells were trypsinized, washed with PBS and lysed in a TNE solution (Tris-HCl 10 mM, NaCl 100 mM and EDTA 1 mM). Genomic DNA was extracted using proteinase K treatment (200 µg/ml, 30 min at 37°C) and phenol/chloroform extraction. For PCR, 50 ng of genomic DNA were used as template with 1 unit of AmpliTaq Gold (Applied Biosystems), 2.5 pmol of H19 primers and 1 pmol of TBP (TATA Binding Protein) primers. The H19 primers used were P2F, P3R previously described (32). The TBP primers were:

- gFL, 5'-AATGCCGTGCACTTCTTCTCC-3' and gBL, 5'-AGGAAACTTCCATCAGACG-3' (GIBCO-BRL).

DNA was first denatured for 7 min at 95°C, and then amplified by 40 cycles of 95°C for 1 min, 58°C for 1 min, 72°C for 1 min and finally extended for 7 min at 72°C. PCR products were electrophoresed through a 3% agarose gel and visualized with ethidium bromide under UV light.

Northern blot analysis

Total RNA was extracted using the guanidine isothiocyanate–CsCl gradient method (33). RNA (20 µg) was denatured, electrophoresed through a 1.2% agarose gel containing formaldehyde and transferred by capillarity onto a nitrocellulose membrane (Hybond-C-extra, Amersham). After being baked for 2 h at 80°C, the membrane was hybridized at 42°C with 32P-CTP-labeled random primed cDNA probes (Megaprime Labelling System, Amersham). The H19 cDNA probe was a 1.3 kb PstI digested fragment containing the end of the first exon and exons 2 to 5. A glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA probe consisting of a 1.2 kb PstI digested fragment was also used to normalize H19 signal.

Semi-quantitative RT–PCR

RNA was isolated as mentioned above and treated for 1 h with an amplification grade Rnase free-Dnase I (GIBCO–BRL). cDNA was obtained by reverse transcription of total RNA (5 µg) using murine leukemia virus reverse transcriptase (Applied Biosystems). Aliquots of cDNA (1/20) were used for PCR amplification using AmpliTaq Gold (Applied Biosystems). For semi-quantitative analysis, β-Actin was co-amplified as an internal control. cDNA was first denatured for 7 min at 95°C, then amplified using 37 cycles of 30 s at 95°C, 30 s at 58°C and 30 s at 72°C, with a final extension of 7 min at 72°C. The forward and reverse primers (GIBCO-BRL) and expected size of the PCR products were: for IGF2, (25 pmol each): 5'-TCGATGCTGTTGTTTCTACCTT-3' and 5'-TGGACTGCTTCCAGGTGTCATA-3' 345 bp and for β-Actin (1.5 pmol each): 5'-CCAGGCAAAGGCAATCCTT-3' and 5'-GGTTGAAGGTTCCTAAACATGA-3' 219 bp. PCR products were electrophoresed through a 2% agarose gel and visualized with ethidium bromide under UV light.

Cell growth assay

To examine the growth rate on plastic surfaces, 35 mm diameter dishes were inoculated with 1×105 cells per dish in 2 ml of medium containing various percentages of FCS. The medium was changed every 48 h. Daily over 5 days, cells were trypsinized and counted using a hemocytometer.

Cell cycle analysis

Cells grown in chamber slides were fixed for 10 min in cold methanol at −20°C and rinsed with PBS. After hybridization in 5 N HCl for 1 h at room temperature, cell DNA was stained for 1 h with Schiff reagent. Image analysis of 300 randomly selected nuclei from each sample in duplicate was performed using a SAMBA 200 (Alcet-TITN-France). The proportion of cells in G0/G1, S, and G2/M phases was determined on computerized integrated optical density histograms (34).

Clonogenicity

Clonogenic ability on plastic surfaces was evaluated by plating 5×102 cells in 60 mm diameter dishes. The medium was changed every 48 h and after 1 week, cells were fixed with a 4% paraformaldehyde (PFA)phosphate buffer saline (PBS) solution, rinsed twice with PBS and finally stained with hematoxylin. Colonies consisting of >20 cells were counted using a light microscope.

Clonogenic ability in semi-solid medium was evaluated by seeding 5×104 well-separated cells in 0.37% bacto-agar/DMEM (Dulbecco modified MEM) containing 10% of FCS on a bottom layer consisting of the same medium containing 0.56% of bacto-agar (DIFCO). After 1 week, medium (DMEM-10% FCS) was added. Colonies of >20 cells were counted on the 15th day.

Adhesion assay

Culture dishes (35 mm diameter) were coated by incubation for 2 h at 37°C with 1 ml MEM containing 1 µg /cm² of rat tail type I collagen prepared according to Montesano et al. (35), mouse laminin (BD Biosciences) and human fibronectin (BD Biosciences). The wells were then incubated for 30 min at 37°C with MEM/5% of bovine serum albumin (BSA, Sigma) (v/v), rinsed once with MEM/0.03% BSA and immediately used. Cells suspended in MEM/0.03% BSA were transferred to the coated dishes (5×104 cells/dish). Cells were allowed to adhere for 10 min at 37°C and cell supernatant was collected. To remove non-adhered cells, dishes were rinsed twice with 1 ml of PBS that was pooled with the supernatant. Adherent cells were harvested using a 0.1% trypsin/EDTA solution. Both adherent and non-adherent cells were counted with a hemocytometer.

Tumorigenesis in nude mice

Exponentially growing cells were harvested by trypsinization, washed with serum- and antibiotic-free medium and resuspended in 1 ml MEM containing 1 µg /cm² of rat tail type I collagen prepared according to Montesano et al. (35), mouse laminin (BD Biosciences) and human fibronectin (BD Biosciences). The wells were then incubated for 30 min at 37°C with MEM/5% of bovine serum albumin (BSA, Sigma) (v/v), rinsed once with MEM/0.03% BSA and immediately used. Cells suspended in MEM/0.03% BSA were transferred to the coated dishes (5×104 cells/dish). Cells were allowed to adhere for 10 min at 37°C and cell supernatant was collected. To remove non-adhered cells, dishes were rinsed twice with 1 ml of PBS that was pooled with the supernatant. Adherent cells were harvested using a 0.1% trypsin/EDTA solution. Both adherent and non-adherent cells were counted with a hemocytometer.
were macroscopically and microscopically analysed for the presence of liver, gut or lung metastasis.

**In situ hybridization**

Soft-agar colonies and tumours were fixed in 4% PFA/PBS, washed with PBS, dehydrated by increasing ethanol concentrations, embedded in paraffin and serially cut. Seven μm sections were transferred onto SuperFrost coated slides (Polylabo), incubated for 1 week at 37°C and stored at 4°C until use. Sections were deparaffinized with toluene, and treated with proteinase K post-fixed with a 4% PFA/PBS solution. Slides were acetylated to reduce non-specific binding, dehydrated by increasing ethanol concentrations and hybridized with 35S-labeled riboprobes (36). To obtain the riboprobes, a pSP64 vector containing a sense or antisense Svu H19 cDNA fragment were linearized by Hind III, submitted to in vitro transcription in the presence of [35S]CTP and reduced to an average 150 bp length. After hybridization, slides were dipped in NTB2 nuclear track emulsion (Kodak) and exposed for 3 weeks at 4°C. Following development and staining with the intercalating agent Hoechst 33258, slides were analysed with epifluorescence for nuclei visualization and a dark-field condenser for silver grain detection using an Olympus BH2 photomicroscope.

One section of each tumour sample was also coloured with hemalum–eosin.

**Ki-67 immunohistochemical staining**

Sections were deparaffinized with toluene and progressively rehydrated. Sections were preliminary treated by a modified procedure of Balaton et al. (37) to restore antigen specificity before immunostaining; slides were immersed for 5.5 min in citrate buffer (0.01 M, pH 6), heated in a pressure cooker, and the latter was then placed for 15 min under cold water. Immunostaining was performed with a monoclonal anti-Ki-67 antibody (clone MIB-1, 1:50 dilution, Immunotech). Immunoreaction was visualized with a diaminobenzidine chromogen (DAKO) and sections were post-stained with hemalum.

**Detection of apoptotic cells**

After deparaffining and rehydration, sections were treated with proteinase K (20 μg/ml in Tris/HCl pH 7.4) for 15 min at 37°C. Apoptotic cells were detected by the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) technique according to the manufacturer’s instructions (In Situ Cell Death Detection, POD kit, Roche). Immunoreaction was visualized with a diaminobenzidine chromogen (DAKO) and sections were post-stained with Gill’s hematoxylin.

**Results**

**Isolation and characterization of clones overexpressing the H19 gene**

To test the role of the H19 gene in breast cancer, the MDA-MB-231 cells were transfected with a vector containing the entire genomic H19 gene sequence placed under the control of the constitutive Cytomegalovirus promoter (pRC/CMV-H19 vector). G418-resistant clones were derived from either the control vector. The H19-transfected cells were isolated and expanded for further analysis. The presence of the transfected DNA in their genome was verified by PCR (Figure 1A), and the expression of the H19 transgene was examined by northern blot. Typical northern blot data are shown in Figure 1B. The H19-transfected clones (H19 S14 3 and H19 S14 4) exhibited a high level of the expected 2.3 kb transcript which corresponds to the correctly spliced and polyadenylated H19 mRNA. In contrast, no H19 signal was detected in parental (MDA) or control vector-transfected cells (Neo 2 and Neo 3), suggesting that H19 transcripts were poorly represented in these cells (Figure 1B). Nevertheless, H19 mRNA was not completely absent in these cells since H19 expression became detectable after the potent RT–PCR technique (data not shown). It has been demonstrated that H19 negatively regulates the transcription and the traducibility of the IGF2 gene (11,38). To validate this regulation in our model, we evaluated IGF2 mRNA levels in the transfectants. As expected, H19 overexpression in the H19-recombined cells decreased the IGF2 transcription levels in MDA-MB-231 cells (Figure 1C).

**Growth characteristics of H19-transfected MDA-MB-231 cells**

When MDA-MB-231 cells were transfected with the vector alone or the H19-vector, no morphological changes of the cells were observed. To evaluate the effect of an overexpression of the H19 gene on the growth of the MDA-MB-231 cell line, we examined the proliferative capacity of the various cell lines (parental, control vector-transfected and H19-transfected). Figure 2 shows that, in 5% FCS-containing medium, the H19-transfected cell lines grew at the same rate as both the parental and mock-transfected cell lines, doubling time being ~24 h for each cell line. Likewise, the growth of the H19-transfected and the control cell lines was also identical when cells are cultivated in medium containing either 10% or 0% of FCS (data not shown). These results were further confirmed by [3H]thymidine incorporation assays (data not shown). After stoichiometric Feulgen staining of DNA and measurement of the integrated optical density by image analysis, we evaluated the distribution of the cells in the G1, S and G2/M phases. Table I shows that regardless of the cell line, the percentage of cells in each cell cycle phase was unchanged.

Likewise, the response of MDA-MB-231 cells to a mitogenic growth factor such as nerve growth factor (39) and to a growth inhibitor such as sodium butyrate (40) remained unchanged when the cells overexpressed the H19 gene (data not shown). All these data suggest that an overexpressed H19 gene does not modify the growth characteristics of the MDA-MB-231 cell line in vitro.

**Clonogenic ability of H19-transfected cell lines**

One week after plating the cells at a very low density, we evaluated the percentage of colonies formed for each cell line. Figure 3A shows that both the vector alone and the H19-transfected cell lines exhibited the same clonogenic ability as the parental cell line. To thoroughly investigate the clonogenic capacity of the H19-transfected cell lines, we performed anchorage-independent growth assays. When the cells were grown in soft-agar, the H19-transfected cells showed a significant 3-fold increase in colony formation compared with the parental and the vector alone-transfected cell lines (Figure 3B). Figure 4A–F illustrates the increasingly rapid growth of the H19-transfected cells as the days progressed. On the 15th day, the colonies were counted: the H19-transfected cells exhibited significantly more (Figure 4G–H versus I) and larger colonies (Figure 4J–K versus L) overexpressing the H19 gene, as shown by in situ hybridization (Figure 4M–O). No obvious difference in the morphology of individual colonies was observed.

**Adhesive properties of H19-transfected cells**

As the clonogenic ability of the H19-transfected cells depends on the anchorage, we have evaluated the adhesive behaviour of these cells on different substrates (Figure 5) i.e. plastic (culture treated dishes), type I collagen, laminin and fibronectin. On plastic, parental, control-transfected and H19-transfected cells adhere with the same efficiency (48% of adherent cells). On type I collagen, cells overexpressing H19 exhibited an increased adhesiveness (46% of adherent cells versus 27% for control cells). The same result was obtained on fibronectin but with a lower differential (43% of adherent cells versus 33% for control cells). MDA-MB-231 cells poorly attach on laminin-coated dishes (~0.5% of adherent cells) and this weak affinity for laminin was not increased by the overexpression of H19 in these cells. Finally, except for laminin substrate, the H19 overexpressing cells adhere with the same and better efficiency.
Fig. 1. Isolation and characterization of the H19-transfected cell lines. (A) PCR selection of H19-transfected cell lines harbouring H19 ectopic DNA. PCR experiments were performed using 50 ng of genomic DNA extracted from parental cell line (MDA) and from control-transfected (Neo 2, Neo 3) and H19-transfected (H19 S14 3, H19 S14 4) G418-resistant clones. TATA-binding protein (TBP) gene was co-amplified as an internal control. H2O lane corresponds to the same experiment performed without DNA; MW lane contains Puc19/MspI molecular weight marker. (B) Northern blot analysis of H19 gene expression. Membrane was hybridized with a H19 cDNA probe and a glyceraldehyde-3-phosphodehydrogenase (GAPDH) cDNA probe as an internal standard. (C) Expression of IGF2 gene by semi-quantitative RT-PCR. ß-actin cDNA was co-amplified as an internal control. For each sample, a RT–PCR experiment was performed without reverse transcription (–RT).

(∼45% of adhesion) whatever the substrate (plastic, collagen or fibronectin). On the contrary, the adhesion of control cells depends on the type of substrate.

Tumorigenicity analysis of the H19-transfected cells

The anchorage-independent growth results prompted us to further investigate the effects of a H19 gene expression change...
Oncogenic properties of the \textit{H19} gene

Fig. 2. Effects of \textit{H19} gene transfection on growth of the MDA-MB-231 cell line. Cells were plated at 1 \times 10^5 cells per dish in MEM supplemented with 5% of FCS. Cell numbers were counted daily. $\blacklozenge$, parental cell line; $\square$ and $\triangle$, control transfected cell lines; $\bigcirc$ and $\times$, \textit{H19}-transfected cell lines. Results represent the mean \pm standard deviation of three independent experiments.

Table I. Effects of \textit{H19} gene transfection on cell cycle phases of MDA-MB-231 cells

<table>
<thead>
<tr>
<th>Cell cycle phases</th>
<th>Controls</th>
<th>H19 S14 3</th>
<th>H19 S14 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>$G_1$</td>
<td>36.4 \pm 7.7</td>
<td>36.9 \pm 5.5</td>
<td>42.1 \pm 1.0</td>
</tr>
<tr>
<td>$S$</td>
<td>31.9 \pm 0.1</td>
<td>29.2 \pm 4.0</td>
<td>31.9 \pm 1.2</td>
</tr>
<tr>
<td>$G_2/M$</td>
<td>15.3 \pm 6.8</td>
<td>17.0 \pm 2.3</td>
<td>18.5 \pm 0.3</td>
</tr>
</tbody>
</table>

The percentage of cells in each cycle phase is evaluated after Feulgen staining of DNA and image analysis. Results represent the mean value \pm standard deviation of three independent experiments processed in duplicate.

Fig. 3. Effects of \textit{H19} transfection on anchorage-dependent and anchorage-independent clonogenic ability of MDA-MB-231 cells. (A) Cells were plated at low density (5 \times 10^2 cells/60 mm dish) in MEM supplemented with 5% of FCS, so that colonies appeared 1 week later. Colonies consisting of >20 cells were counted. (B) Cells (5 \times 10^4) were seeded in soft agar and colonies of >20 cells were counted 15 days later. MDA, parental cell line; Neo 2 and Neo 3, control vector-transfected cell lines; H19 S14 3 and H19 S14 4. \textit{H19}-recombined cell lines. Values are the mean of four determinations performed in triplicate; bars, standard deviation.

in the neoplastic process. To address this issue, we inoculated exponentially growing parental, control, or \textit{H19}-transfected cells in \textit{scid} mice and followed the progression of tumour genesis. Regardless of the cell line injected, almost all the mice developed tumours (Table II). Mice injected with the parental and control-transfected cell lines showed a palpable tumour at a median time of 52 and 48 days after inoculation respectively, whereas only 27 days were necessary to make the same observations in the mice injected with the \textit{H19}-transfected cells. Sixty days after injection, mice were killed and the average diameter of \textit{H19}-transfected cell-derived tumours was twice that of the control cell line-derived tumours (Table II and Figure 6A and B). Regardless of the cell line injected, no metastasis was observed in lung and liver. According to the histological study of the various cell-derived tumours, these were undifferentiated carcinoma of high grade with atypical cells having very chromatic nucleus, regardless of the cell lines inoculated (Figure 6C and D). Finally, we assessed \textit{H19} gene expression in the tumours derived from the various cell lines using \textit{in situ} hybridization. In the parental and mock-transfected cell derived tumours, \textit{H19} gene expression was lacking, in concordance with the difficulty in detecting \textit{H19} gene expression in the cells before injection (Figure 6E). \textit{H19}-transfected cell-derived tumours exhibited a high \textit{H19} gene expression level. In these tumours, \textit{H19} expression was not uniform, being in some cases very localized to certain parts of the tumour (Figure 6F) and more dispersed in others (Figure 6G). However, in both cases, the \textit{H19} signal appeared in clusters, indicating that only certain cells overexpressed the \textit{H19} gene, while many others had lost this capability. As enhanced tumour growth may be the result of increasing proliferation or decreasing apoptosis, we have studied the contribution of these two processes in the highest tumorigenicity of \textit{H19}-transfected cells. The proliferation rate was evaluated by immunohistochemistry with a monoclonal anti-Ki-67...
Fig. 4. *H19*-transfected cells rapidly formed numerous and larger colonies in soft agar. Light microscopy photographs, on the 5th, 7th and 12th day, of colonies in soft agar of control-vector transfected cells (A, B, C respectively) and *H19*-transfected cells (D, E, F respectively). G, H and I, light microscopy photographs on day 15 of parental, control vector-transfected and *H19*-transfected cell colonies, respectively. J, K and L, light microscopy photographs on day 15 of parental, control vector-transfected and *H19*-transfected individual colony, respectively. M, N and O, in situ hybridization with an antisense *H19* riboprobe on parental, control vector-transfected and *H19*-transfected colony, respectively. A to F and J to L, magnification ×100; G to I, magnification ×40; M to O, magnification ×200.

antibody (MIB-1) while apoptotic cells were detected using the TUNEL technique. Regardless of the cell line injected, no difference was observed both in the Ki-67/MIB-1 immunostaining (Figure 7A versus B) and in the number of apoptotic cells (Figure 7C versus D).

**Discussion**

The *H19* gene is highly expressed during embryogenesis and in subsequent fetal development, but repressed postnatally in most organs. While *H19* is only marginally transcribed in adult tissues, it is re-expressed in a wide range of tumours derived from tissues that formerly expressed it during fetal life. This pattern of expression, typical of oncofetal RNA (41), suggests that the gene may be implied in both normal development and tumorigenesis. Despite many years of research, the function of *H19* has not yet been clearly established. Since Hao et al. (17) demonstrated that the transfection of *H19* in G401 cells...
Our results show that when H19-transfected cells were inoculated to scid mice, the time elapsed before the appearance of tumours was shortened and the tumours grew twice as large. MIB-1 immunostaining and TUNEL reaction on the induced tumours indicate that the increased tumorigenicity of MDA-H19 cells was not due to an imbalance of proliferation/apoptosis ratio, which is in agreement with the absence of modification of the growth characteristics of MDA-H19 cells cultured as monolayer. However, as the overexpression of the H19 gene in MDA-MB-231 cells result in a significant increase of anchorage-independent growth and tumorigenicity in vivo, but have no effect on the anchorage-dependent growth, we have studied the adhesive properties of these cells on different substrates. When cultured on fibronectin or collagen, H19 overexpressing cells adhere more efficiently than control cells. Such a difference of adhesion capacities could explain the enhanced in vivo tumorigenicity in regulating the ability of a small number of cells to initiate tumour formation. Indeed, it has been reported that increased adhesive properties are associated with an increase of tumorigenesis of many tumour cells (42–44), probably in allowing a better survival and a better colonization by tumour cells of their immediate environment. In a recent study, we have demonstrated that the H19 gene expression is regulated by anchorage and cell shape, being upregulated when cells were cultured between two collagen layers (45). Likewise, Plisov et al. have shown that H19 and adhesion molecule genes are among the earliest genes activated at the time of the conversion of metanephric mesenchyme to the epithelium of the nephron (46). Taken together these data support the idea that the H19 gene behaves as an oncogene in MDA-MB-231 cells not in increasing the growth capacities of these cells but rather in promoting optimal adhesive properties.

Fig. 5. Adhesiveness of MDA-MB-231 cells overexpressing H19. Adhesion assays were performed with parental, control-transfected and H19-transfected cells seeded onto non-coated culture-treated dishes (plastic) or culture-treated dishes coated with 1 μg/cm² of type I collagen, laminin or fibronectin. Ten minutes after seeding the number of adherent and non-adherent cells was evaluated. The percentage of adhesion represents the number of adherent cells divided by the total number of cells (adherent and non-adherent). The parental and control-transfected cell lines give the same results, only the control-transfected cells data are indicated on the graphic.

Table II. Effects of H19 gene transfection on the tumorigenicity of MDA-MB-231 cells

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Tumour incidence (no. tumour/no. mice)</th>
<th>Median latency (Days)</th>
<th>Tumour diameter (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parental</td>
<td>5/6</td>
<td>52</td>
<td>6.0 ± 0.5</td>
</tr>
<tr>
<td>Control-transfected</td>
<td>5/6</td>
<td>48</td>
<td>6.2 ± 0.6</td>
</tr>
<tr>
<td>H19-transfected</td>
<td>6/6</td>
<td>27</td>
<td>13.4 ± 0.9</td>
</tr>
</tbody>
</table>

Exponentially growing cells (2 × 10⁶) were injected subcutaneously in the flank of six scid mice for each cell line. Tumour incidence represents the number of tumours formed per number of injected mice. Median latency indicates the median day of apparition of a manually palpable tumour. Tumour diameter represents the mean value ± standard deviation of two diameter measurements of each tumor in each of the six mice.

causd growth retardation and suppressed clonogenicity in soft agar and tumorigenicity in nude mice (17). H19 has been considered as a tumour suppressor gene. In contrast, in other models such as bladder carcinomas (22,24) and choriocarcinoma cells (28,29), H19 expression was associated with the tumorigenesis process.

In breast cancers, we have previously demonstrated that H19 is overexpressed in ~72% of breast adenocarcinomas. While overexpression is preferentially located in the stromal cells, in some cases H19 is overexpressed in both the epithelial and the stromal cells, and in others it is limited to the sole epithelial cells (21). Breast is of particular interest since it belongs to the few organs in which H19 is not completely repressed after birth. In order to study the role of H19 in breast cancer, we stably transfected human cancerous mammary epithelial cells with the genomic DNA of the entire H19 gene. The MDA-MB-231 cell line was chosen because it exhibits a very weak H19 gene expression, so that its transcript is hardly detectable in this cell line. Our results show that when transfected with H19, MDA-MB-231 cells did not exhibit any difference in growth pattern on plastic surfaces regardless of

the percentage of fetal calf serum or of the presence of mitogenic growth factor or growth inhibitor, nor was their distribution over the various phases of cell cycle modified. These results are concordant with our observations in breast adenocarcinoma, in which H19 overexpression was not correlated with the presence of Ki-67, a marker that is specific to cycling cells (21). They suggest that the H19 gene does not play a major role in cell cycle regulation.

The H19-transfected cells demonstrated unchanged anchorage-dependent clonogenic ability, as measured by colony formation on plastic surface, but exhibited significantly increased anchorage-independent growth, as measured by colony formation in soft agar. Since anchorage-independent growth in semi-solid media is a characteristic of cancerous cells, we assessed the effects of H19 overexpression on the tumorigenicity of MDA-MB-231 cells in immune-deficient animals. Although the incidence of tumours was identical when the H19-transfected cells were inoculated to scid mice, the time elapsed before the appearance of tumours was shortened and the tumours grew twice as large. MIB-1 immunostaining and TUNEL reaction on the induced tumours indicate that the increased tumorigenicity of MDA-H19 cells was not due to an imbalance of proliferation/apoptosis ratio, which is in agreement with the absence of modification of the growth characteristics of MDA-H19 cells cultured as monolayer. However, as the overexpression of the H19 gene in MDA-MB-231 cells result in a significant increase of anchorage-independent growth and tumorigenicity in vivo, but have no effect on the anchorage-dependent growth, we have studied the adhesive properties of these cells on different substrates. When cultured on fibronectin or collagen, H19 overexpressing cells adhere more efficiently than control cells. Such a difference of adhesion capacities could explain the enhanced in vivo tumorigenicity in regulating the ability of a small number of cells to initiate tumour formation. Indeed, it has been reported that increased adhesive properties are associated with an increase of tumorigenesis of many tumour cells (42–44), probably in allowing a better survival and a better colonization by tumour cells of their immediate environment. In a recent study, we have demonstrated that the H19 gene expression is regulated by anchorage and cell shape, being upregulated when cells were cultured between two collagen layers (45). Likewise, Plisov et al. have shown that H19 and adhesion molecule genes are among the earliest genes activated at the time of the conversion of metanephric mesenchyme to the epithelium of the nephron (46). Taken together these data support the idea that the H19 gene behaves as an oncogene in MDA-MB-231 cells not in increasing the growth capacities of these cells but rather in promoting optimal adhesive properties.

It has been reported that tumours derived from H19-transfected Syrian hamster embryo (SHE) cells lacked H19 expression even though they retained the exogenous gene (18). Consequently, we analysed H19 expression in our MDA-transfected induced tumours using in situ hybridization. We also observed that although some cells had retained high H19 gene overexpression, others had lost it. By comparing in situ hybridization with histological colorations, we established that necrosis cannot explain the absence of H19 in certain cells. In transfection experiments, it is not rare that the expression of the transgene declines with time, and this extinction is not always solely the result of a lost transcription. For it has been recently demonstrated that while the human genome contains stable integration sites responsible for long-term expression of
the transgene, it also contains some unstable integration sites that eliminate the transgene from the host genome (47). Also, the $H19$ gene is submitted to genomic imprinting, and one of the epigenetic features consistently implied in this allele-specific expression is DNA methylation of CpG dinucleotides. Both the upstream sequence of $H19$ and the structural gene itself are differentially methylated, being hypermethylated on the silent paternal chromosome (48). Thus, $H19$ may be unexpressed in the $H19$-transfected derived tumours because of the methylation of the ectopic sequence, as it was shown in $H19$-transfected SHE cells (18). Recently, Milligan et al. (49) have demonstrated that expression modulations were not only regulated transcriptionally, but also post-transcriptionally. Indeed, during muscle cell differentiation, the $H19$ gene
Oncogenic properties of the H19 gene

Fig. 7. The enhanced tumour growth is due neither to an increase of proliferation nor to a decrease of apoptosis. (A and B) Ki-67 immunostaining of tumours induced by control and H19-transfected cells, respectively. (C and D) Apoptotic cell detection by the TUNEL technique in tumours induced by control and H19-transfected cells, respectively.

only accumulates because its transcript is stabilized (49). Consequently, the extinction of the H19 signal in the H19-transfected derived tumour may be due to destabilized H19 mRNAs subsequent to the proliferation process that goes with tumour development.

When choriocarcinoma-derived cell lines (JEG-3) were injected in nude mice, the resulting tumours exhibited an increased H19 expression compared with the cells before injection (28,29). These results suggest that H19 expression may promote tumorigenesis, or that H19 expression is a result of the carcinogenic process. In HeLa × normal human fibroblast hybrid cells, it has recently been demonstrated by differential display screening that H19 was specifically expressed in tumorigenic hybrids. But, the re-expression of H19 in these hybrids was not sufficient to restore a tumorigenic phenotype (30,31). In our experiments, while H19-transfected cells enhanced the tumorigenic capacity of MDA-MB-231 cells, tumour formation did not induce H19 gene expression in the parental cells. Therefore we can conclude that in breast cancer cells, H19 expression is not necessarily concomitant with the tumorigenic process, but is responsible for an increased tumorigenesis of these cells.

Others have found that H19 and IGF2 exhibit coordinate reciprocal regulation in many situations. The explanation of this is found in the H19 flanking sequences, which are implied in the regulation of IGF2 imprinting in cis (50). Knockout experiments have shown that mice with a targeted deletion of the H19 normally active maternal allele have only displayed an overgrowth phenotype, typical of an IGF2 overproduction (51,52). A trans-function of H19 in IGF2 regulation has also been suggested, since IGF2 mRNA level and translatability are correlated with the H19 mRNA level in a Wilms’ tumour (11). This trans-function was further confirmed in hepatocellular carcinoma cells, where H19 sense and antisense transgenes modulate IGF2 mRNA levels (38). Based on these data, it has been anticipated that the role of H19 in tumorigenesis could be directly linked with its effect on IGF2 expression. In our study, we showed that H19 overexpression in MDA-MB-231 cells decreased the IGF2 mRNA level, thereby confirming the negative trans-riboregulator function of H19 on IGF2.
expression in breast cancer cells. Moreover, the down-regulation of IGF2 expression associated with the increased tumorigenicity of H19-transfected MDA-MB-231 cells in scid mice argued against an oncogenic activity of H19 in breast cancer cells via an upregulation of IGF2.

Since the H19 transcript is an mRNA-like non-coding RNA acting as a riboregulator, we studied the proteome to investigate protein pattern changes in H19-overexpressing cells. After two-dimensional electrophoresis and mass spectrometry analysis, we identified thioredoxin as being positively regulated and showed that this regulation occurred at the post-transcriptional level (53). Thioredoxin is a key protein of the reduction–oxidation metabolism (54). It has been demonstrated that thioredoxin-transfected breast cancer cells exhibited unaltered growth on plastic surfaces, but several-fold increased colony formation in soft agar. When the same cells were transfected with a redox-inactive mutant of thioredoxin, they almost completely inhibited tumour formation when they were inoculated to immune-deficient mice (55). Based on all these findings, we propose that the increased level of thioredoxin in H19-transfected MDA-MB-231 cells may be involved in the neoplastic phenotypes exhibited by our H19-recombined clones.

In conclusion, although the H19 gene is a tumour suppressor according to some models, our study establishes that H19 may be involved in the neoplastic phenotypes exhibited by our H19-recombined clones.

Acknowledgements

We thank L.Brunet and G.Courtrand for their help in manuscript illustrations. We also thank I.Pollet and S.Ruault for technical assistance. This work was supported by grants from the Association pour la Recherche sur le Cancer (ARC), the Groupement des Entreprises Francaises dans la lutte contre le Cancer (GEFLUC) and the Ligue Nationale Contre le Cancer (Comité du Nord). S.Lottin was financially supported by the ARC and the Fondation pour la Recherche Médicale (FRM). E.Adriaenssens was recipient of an ARC fellowship.

References

36. Que, C., Leprince, D., Sëhelín, D. and Vandenbunder, B. (1993) p54c-ets-1 and p68c-ets-1 the two transcription factors encoded by the c-ets-1 locus are differentially expressed during the development of the chick embryo. Oncogene, 8, 2511–2520.

Received December 27, 2001; revised July 18, 2002; accepted July 24, 2002