H19 Overexpression in Breast Adenocarcinoma Stromal Cells Is Associated with Tumor Values and Steroid Receptor Status but Independent of p53 and Ki-67 Expression

Eric Adriaenssens,* Lionel Dumont,*
Séverine Lottin,* Domitille Bolle,* Alain Leprêtre,†
Alice Delobelle,‡ Fatima Bouali,§
Thierry Dugimont,*|| Jean Coll,¶ and
Jean-Jacques Cury*

From the Centre de Biologie Cellulaire,* Unité Dynamique des Cellules Embryonnaires et Cancéreuses, and Laboratoire d'Ecologie Numérique,† Université des Sciences et Technologies de Lille, Villeneuve d'Ascq, and the Laboratoire d'Anatomie et de Cytologie Pathologique,‡ Centre Oscar Lambret, and Régulation des Processus Invasifs, de l'Angiogenèse et de l'Apoptose§ and Immunopathologie Cellulaire des Maladies Infectieuses,¶ Institut de Biologie Moléculaire, Institut Pasteur, Lille, France; and Faculté des Sciences Jean Perrin,|| Université d'Antois, Lens, France

In a previous study we described the expression of the *H19* gene by *in situ* hybridization (ISH) in normal breast and in benign or malignant breast tumors (Dugimont T, Cury JJ, Wernert N, Delobelle A, Raes MB, Joubel A, Stéhelin D, Coll J: *Biol Cell* 1995, 85:117–124). In the present work, 1) we extend the previous one to a statistically useful number of adenocarcinomas, including 10 subclasses, 2) we provide information on the precise ISH localization of the *H19* RNA by using, on serial tissue sections, antibodies delineating specifically the stromal or the epithelial component of the breast, and 3) we consider relationships between the *H19* gene expression and various clinicopathological information as tumor values (T0 to T4), grades, steroid receptors, lymph node status, and molecular features as the p53 gene product and the Ki-67/MIB-1 protein, which is specific to proliferating cells. Data indicate that 1) in 72.5% of studied breast adenocarcinomas an overall *H19* gene expression is increased when compared with healthy tissues, 2) the *H19* gene is generally overexpressed in stromal cells (92.2%) and rarely in epithelial cells (2.9% only), 3) an up-regulation of the *H19* gene is significantly correlated with the tumor values and the presence of both estrogen and progesterone receptors, and 4) at the cellular level, the *H19* gene demonstrates an independent expression versus accumulation of both the p53 protein and the Ki-67/MIB-1 cell-cycle marker. (*Am J Pathol* 1998, 153:1597–1607)

H19 is a developmentally regulated gene. Thus, it is highly expressed in several fetal tissues, except in the nervous system and thymus,^{1–5} and repressed after birth in most of the organs. In adulthood, a basal *H19* gene expression has been detected only in mammary gland,^{4,6} cardiac and skeletal muscles,^{7,8} and to a lesser extent in kidney, adrenal gland, and lung.⁹

The *H19* gene codes for a capped, spliced, and polyadenylated RNA. It is highly conserved in vertebrates, as homologous sequences have been detected in rabbit,¹⁰ mouse,¹ chicken, monkey, and human.^{4,11} The protein-coding potential of *H19* RNA remains uncertain, and it has been proposed that this gene may act as an RNA.¹¹ However, introduction of deletions or point mutations into the 5'-untranslated region (5'UTR) of an ectopic *H19* gene, upstream of the largest open reading frame (ORF6), enabled the production of a 26-kd protein,¹² although this has not been detected in cells expressing an endogenous *H19* gene.

The *H19* gene is located at 11p15.5 and is imprinted with only the maternal allele being expressed.^{9,13} *H19* maps closely to another imprinted gene, *IGF-II*, but in the latter case it is the paternal allele that is transcribed. It has been reported that loss of heterozygosity (LOH) of a specific parental allele could be associated with the activation of a gene in cancers,¹⁴ and LOH of 11p¹⁵ was found in a wide variety of tumors, including some Wilms' tumor^{15–17} and lung,¹⁸ liver,¹⁹ ovarian, and breast cancers.^{20,21} Loss of imprinting of *IGF-II* has been described in a subset of Wilms' tumors. One hallmark of Wilms' tumors is the high levels of expression of the *IGF-II* gene, which has generated suggestions that an overdosage of the product of this gene contributes to Wilms' tumorigenesis.²² In some Wilms' tumors (approximately one-third)

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Address reprint requests to Dr. Jean-Jacques Cury, Centre de Biologie Cellulaire, DRED 1033, Université des Sciences et Technologies de Lille, Batiment SN3, Villeneuve d'Ascq Cedex 59655, France. E-mail cury@univ-lille1.fr.

the transcriptionally silent maternal *IGF-II* allele is activated such that *IGF-II* expression occurs biallelically.^{23,24} There is evidence (enhancer deletion) that sequences flanking the *H19* gene in the mouse control the nearby *IGF-II* gene in *cis*.^{25,26} In the majority of Wilms' tumors the silencing of *H19* has been reported.²⁷⁻³¹ This transcriptional silencing was accompanied by DNA methylation of the maternal *H19* allele and activation of the maternal *IGF-II* allele.²⁷⁻²⁹ Loss of imprinting of *H19* and/or *IGF-II* has been described in various cancers, including lung carcinomas,³² rhabdomyosarcoma,³³⁻³⁵ hepatoblastoma,^{24,36} testicular germ cell tumors,³⁷ bladder carcinomas,³⁸ uterine cervix carcinomas,³⁹ and esophageal cancers.⁴⁰ On the contrary, in some tumors, maintenance of normal imprinting of the *H19* and/or *IGF-II* genes were observed (colorectal,⁴⁰ neuroblastoma,⁴¹ glioma,⁴² leiomyomata,⁴³ and breast⁴⁴).

H19 is overexpressed in a wide variety of cancers (breast,^{4,6} head and neck,^{4,39} papillary and follicular thyroid,⁴ uterine cervix,^{4,39} bladder,^{45,46} adrenal tumor,⁴⁷ trophoblast,⁴⁸ lung,^{4,32} and esophageal⁴⁰).

To date, the actual function of the *H19* gene in cancer is still a matter of debate. Hao et al⁴⁹ demonstrated that introduction of an *H19* cDNA construct into G401 cells or RD rhabdomyosarcoma cells (two embryonal tumor cell lines) caused morphological changes and growth retardation. These investigators also reported that one *H19*-transfected G401 clone no longer formed tumors when injected into nude mice and that many clones had reduced growth in soft agar. These results made the *H19* gene a good candidate to be a tumor suppressor gene. This function attributed to *H19* was supported by several well documented works demonstrating the silencing of the *H19* gene in several Wilms' tumors.^{27,28} However, Reid et al⁵⁰ reported that *H19* expression did not correlate with tumor suppression in their G401 cells (only two of the five nontumorigenic lines expressed *H19*). Otherwise, Cooper et al⁴⁶ demonstrated that *H19* is an onco-developmental marker during bladder tumor progression. Ariel et al⁵¹ examined the expression of *H19* in tumor arising from tissues that express this gene in fetal life, and Verkerk et al⁵² reported the expression pattern of *H19* in testicular germ cell tumors of adolescents and adults. These studies bring evidence that *H19* is not a tumor suppressor gene, and their authors proposed that its product is an oncofetal RNA. Recently, Lustig-Yariv et al⁵³ evaluated the level of *H19* expression in choriocarcinoma cell lines (JAr and JEG-3 cells) and in tumors formed by these cells after their injection into athymic nude mice; they concluded that their data assigned to the *H19* gene a role in contradiction with the tumor suppressor function proposed by others. Consequently, the role of *H19* is still enigmatic, and the question of the properties of the *H19* product, so far an RNA, remains open.

Other studies suggested that another locus on the short arm of chromosome 11 might be involved in tumor suppression, and the likely candidate is the cyclin-dependent kinase inhibitor, the *p57^{KIP2}* gene, in band 11p15.5, which causes G1 arrest⁵⁴⁻⁵⁸.

It has been frequently demonstrated that the *H19* gene is up-regulated *in vitro* in differentiating cells as well as

during growth arrest.^{1,7,59-61} A number of growth factors, such as insulin-like growth factor (IGF)-I and -II, epidermal growth factor (EGF), insulin, tumor necrosis factor (TNF)- α , interferon (IFN)- γ , and transforming growth factor (TGF)- β 1, and activators or inhibitors of protein kinase A and C modulated the *H19* gene expression level in different cell lines: vascular smooth muscle cells,¹⁰ fetal adrenal cells,⁶² and cultured adrenal cells.⁴⁷ Otherwise, Leibovitch et al⁶³ reported that the overexpression of c-mos protein in the muscle cell line C2C12 induces a concomitant increase of *H19* RNA expression, suggesting an interrelationship between these two gene products during muscle differentiation.

The mammary gland is a unique organ in that most of its growth, morphogenesis, and differentiation occur in the adult. During these periods, interactions between epithelial and mesenchymal cells and hormones and growth factors contribute to its development. Disorders of these interactions can result in a tumorigenic process.^{64,65}

Observations of the *H19* gene expression in normal breast and its overexpression in many tumors,^{4,6,44} despite the possible maintenance of genomic imprinting,⁴⁴ suggest that this gene is involved in both normal organogenesis and pathological events of the mammary gland.

We indicated, in a preliminary study of the expression of *H19* gene by *in situ* hybridization (ISH) in 13 adenocarcinomas,⁶ that *H19* transcripts accumulate essentially within the stromal compartment of the mammary gland. The aim of the present work was 1) to extend the previous study on the expression of the *H19* gene to a statistically useful number of breast cancers (102), 2) to determine the level of *H19* gene expression in various subclasses of adenocarcinomas, including some which are rare, 3) to delineate the precise localization of the *H19* RNA, by using antibodies raised against specific stromal or epithelial components, 4) to establish the prognostic value of the *H19* RNA (localization and intensity of the *H19* signal were examined, and their relationships with histological grading system and various clinicopathological information were discussed), and 5) to correlate *H19* expression with molecular markers of growth activity of the tumor: steroid receptor content, Ki-67/MIB-1 antigen presence, and overexpression (abnormal) of *p53* gene product, which appears to be a common event in primary mammary carcinomas.

Materials and Methods

Biological Material and Clinicopathological Information

Breast carcinoma specimens were obtained from 102 patients (100 females and 2 males) treated by mastectomy at the Center Oscar Lambret (Lille, France) in 1990 and were selected on the basis of the first and unilateral cancer. In case of fatal issue, it was necessary to be sure that the cancer was only the more probable cause of death. For each tissue sample, the following clinicopathological information was obtained: histological subclassification (invasive ductal, invasive lobular, sarcomatoid,

epidermoid metaplastic, tubular, colloid mucillaneous, papillary, apocrine, intraductal, or lobular *in situ*), tumor (T) values (from the tumor/nodes/metastases (TNM) classification of the UICC, ranging from T0 to T4), histological grade according to Bloom and Richardson,^{66,67} the axillary lymph node status, and the hormone receptor status (estrogen receptor (ER) and progesterone receptor (PR)), determined in femtomoles of receptors per milligram of cytosolic proteins and considered positive above 15 fmol/mg. The presence of these receptors indicates the level of sensitivity of tumor cells to these hormones. Furthermore, the age and menopausal status of patients were known, and expectation of life was followed up until the end of 1995.

Fixation, Embedding, and Histological Staining

Immediately after resection, material was fixed with formalin (10%) for 24 hours and then dehydrated through increasing ethanol concentrations and embedded into Paraplast Plus. Five-micron sections were transferred to slides coated with 3-aminopropyl-triethoxysilane (TESPA, Aldrich) for immunohistochemical staining (IH) and to Esco Superfrost Plus (Polylabo) for *in situ* hybridization (ISH). For IH, tumor sections were fixed on slide by a glycerinated albumin (10%) solution.

Hemalun-phloxine-safran (HPS) staining was performed on one section of each tumor. This section indicated histological structures, and frequently this staining demonstrated heterogeneity of tumors. HPS allowed us to localize precisely the more interesting areas to be observed after various IH procedures or the ISH. As control, we analyzed normal healthy tissues from cosmetic surgery; resections originated from mature breasts of two premenopausal women.

Immunohistochemical Staining

Sections were treated with xylene to remove paraffin from tissues, which were then progressively rehydrated. Sections were preliminarily treated by a modified procedure of Balaton et al⁶⁸ to restore antigen specificity before immunostaining; slides were immersed for 7.5 minutes in citrate buffer (0.01 mol/L, pH 6) heated in a pressure-cooker, and the latter was then placed for 15 minutes under cold water.

To determine precisely which cells expressed the *H19* gene, four immunostaining reactions were performed in parallel: 1) monoclonal antibody named anti-KL1, anti-human cytokeratin specific for epithelial cells (1:200 dilution; Immunotech, Marseille, France), 2) monoclonal antibody anti-smooth-muscle- α -actin to define myoepithelial cells (1:2000 dilution; Sigma Chemical Co., St. Louis, MO), 3) anti-Ki-67/MIB-1 specific for a cell-cycle protein (prediluted; Immunotech), 4) anti-p53 protein raised against the amino-terminal amino acid sequence of both the wild-type and mutant versions of the protein (DO-1, prediluted; Immunotech). Immunoreactions were visualized with diaminobenzidine chromogen (Dako, Glostrup, Denmark), and sections were post-stained with hemalun.

In Situ Hybridization

Riboprobes

pSP65 plasmids were recombined with a 1.3-kb *Stul* fragment of *H19* cDNA at a *Sma*I site. cDNA fragments (5'→3' and 3'→5') were downstream of the SP6 promoter. Plasmids were linearized by *Hind*III digestion. Sense and antisense riboprobes were synthesized in the presence of [³⁵S]CTP and reduced to an average 150-bp length before use.

ISH Protocol

Basic experiments were those previously described by Quéva et al.⁶⁹ After hybridization, slides were dipped in the NTB2 nuclear track emulsion (Kodak, Rochester, NY), heated at 45°C, and exposed for 3 weeks. Autoradiographic revelation (D19 revelator) and fixation (Unifix, Kodak) were performed at 12°C. A fluorescent post-staining of the nuclei was carried on (Hoechst 33258, Bisbenzimidine, Serva; λ = 340 nm). Coverslips were fixed by Dako-glycergel (Sebia). Observations were performed through an Olympus BH2 microscope.

Results

Patterns of the H19 Gene Expression

We previously reported that in normal breast resections signal for *H19* RNA was localized within both the epithelial and mesenchymal tissues.⁶ The mesenchymal compartment was rather focally labeled. Our observations of two other normal breast samples confirmed this initial report. However, it appears that the *H19* transcript abundance can vary from one sample to another and even in different areas of the same section (Figure 1A).

In 102 adenocarcinomas, we investigated the *H19* signal intensity and delineated regions where *H19* RNA was abundant. In 74/102 samples (72.5%), the *H19* gene was obviously more highly expressed than in normal breast. Figures 1 and 2 enabled us to compare the high difference of labeling with antisense *H19* riboprobe between normal (Figure 1A) and tumorigenic breast tissues (Figure 1, C and F; Figure 2, C and F). Based on this kind of representative observations, each tumor was classified as overexpressing the *H19* gene by a clear high-level labeling with the probe. Determinations were performed independently by two investigators (E. Adriaenssens and L. Dumont). If ambiguity of classification arose, the tumor was considered as exhibiting no more labeling than the control (normal breast). So, we admit that the pattern of overexpression, defined in this manner, in a given tumor, was objective. Frequently, in a given specimen we found a heterogeneous pattern of focal *H19* gene expression, which could be explained by histological diversity and/or differences in the grading of the carcinoma. Then, we focused our observations on the characteristic area, which was chosen for the clinical typing of the tumor. To delineate very precisely tumor compartments, we used two antibodies, anti-cytokeratin named anti-KL1 and anti-

Figure 1. Expression of the *H19* gene in normal breast and in adenocarcinomas. **A:** Normal breast; epithelial (arrows) and mesenchymal (arrowheads) cells are labeled by the riboprobe. **B and C:** Epidermoid metaplastic carcinoma; *H19* RNA was exclusively located in the epithelial cells. **D to F:** Invasive ductal carcinoma; *H19* RNAs were exclusively located in the stromal compartment. **B:** Anti-KL-1-immunostaining; **D:** HPS coloration; **E:** anti-smooth-muscle- α -actin immunostaining; **A, C, and F:** ISH. In these two tumors, *H19* transcripts accumulated at the epithelium/stroma boundaries, either in epithelial cells (**C**) or in stromal cells (**F**). Scale markers, 120 μ m (**A**) and 600 μ m (**B** to **F**).

smooth-muscle- α -actin raised against differentiation molecules specific for epithelial and myoepithelial cells, respectively. Only 3/102 tumors (2.9%) exhibited an exclusive but intense epithelial labeling (Figure 1, B and C), but in a large majority of tumors, 94/102 (92.2%), *H19* was expressed in the stromal component only (Figure 1, D to F and Figure 2, D to F). *H19* transcripts were present in both compartments in 5/102 specimens (4.9%); the epithelial signal was rather punctate (Figure 2C). Interestingly, an intense signal at epithelial-stromal boundaries was located quite frequently on epithelial or stromal cells (Figure 1, C and F, respectively). Whatever the

classification of a tumor, only a fraction of epithelial or stromal cells was stained. Table 1 indicates localization of *H19* RNA in ten histological subclasses of tumors.

Table 2 reports information concerning the three patterns of *H19* gene expression (stromal, epithelial, and both stromal and epithelial). Percentage of positivity, ie, *H19* RNA abundance, was indicated for the following clinicopathological factors or parameters: T values (T0 to T4), histological grade, estrogen and progesterone receptor status, age, menopausal and lymph node status, and cancer evolution for 5 years since tumorectomy. In the three specimens where *H19* RNA was exclusively

Figure 2. *H19* gene overexpression compared with accumulations of p53 protein and Ki-67/MIB-1, a protein specific to the cell cycle. **A to C:** Invasive ductal carcinoma; *H19* RNA was localized in epithelial and stromal cells. **D to F:** Invasive ductal carcinoma; *H19* RNA was exclusively localized in the stroma. Overlapping of the actin immunolabeling and the *H19* RNA patterns is seen. **A:** HPS coloration; **B:** anti-Ki-67/MIB-1 immunostaining; **D:** anti-smooth-muscle- α -actin immunostaining; **E:** anti-p53 immunostaining; **C and F:** ISH. **B and C:** No co-localization could be established between the overexpression of the *H19* gene and the presence of the Ki-67/MIB-1 cell cycle marker. **E and F:** The *H19* and p53 labelings do not overlap. Scale marker, 600 μ m.

observed in epithelial cells, one can notice that these tumors were of grade III, cells were devoid of hormone receptors, and patient death occurred within the 5 years after the surgery. This number of cases is too small to give a statistical meaning to these results, but it is intriguing that these 3 cases are a subset of the 21 deaths (21/102 cases) registered within this period. Indeed, we found only 10 tumors of grade III, showing no hormone receptors, which evolved fatally over the 5-year follow-up.

It can be noticed that when epithelial cells were capable of *H19* RNA synthesis, either in an epithelial pattern only (3 cases) or in an epithelial and stromal pattern (5

cases), then cells were completely devoid of hormone receptors.

H19 Gene Overexpression and Various Clinicopathological Factors

Table 2 shows the abundance of the *H19* RNA as a function of various clinicopathological indications. Proportions were compared by using a Fisher's exact probability test,⁷⁰ and the threshold *P* value of 0.05 was chosen. The *H19* overexpression, as defined above, exhibits a very high correlation with the T values (UICC classifi-

Table 1. *H 19* Transcript Localization in the Various Histological Subclasses of Tumors

Carcinomas	<i>n</i>	H19 transcript localization					
		Stromal		Epithelial		Stromal plus epithelial	
		<i>n</i>	%	<i>n</i>	%	<i>n</i>	%
Invasive ductal	55	51	92.73	1	1.82	3	5.45
Invasive lobular	15	15	100	0	0	0	0
Sarcomatoid	1	1	100	0	0	0	0
Epidermoid metaplastic	2	1	50	1	50	0	0
Tubular	2	2	100	0	0	0	0
Colloid mucillaneous	5	5	100	0	0	0	0
Papillary	1	0	0	1	100	0	0
Apocrine	12	11	91.67	0	0	1	8.33
Intracanalal	8	7	87.50	0	0	1	12.50
Lobular <i>in situ</i>	1	1	100	0	0	0	0
Total	102	94	92.16	3	2.94	5	4.90

cation; $P = 1.3 \times 10^{-5}$) but also with the presence of hormone receptors for estrogen and progesterone ($P = 0.0048$ and $P = 0.0159$, respectively). For other factors (age, menopausal and lymph node status, histological grade, and evolution at 5 years), P values were too high to be significant; as a consequence, the *H19* gene overexpression cannot be significantly correlated with these parameters.

H19 Gene Overexpression and Presence of a Cell Cycle Marker Protein, Ki-67/MIB-1

Proliferative-cell activity has been estimated by immuno-histochemical staining with Ki-67 antibody, and it has been shown that MIB-1, a monoclonal antibody, can react with an epitope of the Ki-67 protein in formalin-fixed, paraffin-embedded tissues processed by microwave

Table 2. *H19* RNA Localization and *H19* Gene Overexpression *versus* Various Clinicopathological Factors

Clinicopathological factors	Localization patterns of <i>H19</i> RNA			<i>H19</i> gene overexpression	
	Stromal (<i>n</i> = 94, 92.2%)	Epithelial (<i>n</i> = 3, 2.9%)	Stromal and epithelial (<i>n</i> = 5, 4.9%)	Number of cases (%)	<i>P</i> values
Age					
<50 years	23 (24.5%)	1 (33.3%)	0 (0%)	16/24 (66.7)	0.4297
>50 years	71 (75.5%)	2 (66.7%)	5 (100%)	58/78 (74.4)	
Menopausal status					
Premenopausal	29 (32.2%)	1 (33.3%)	1 (20%)	21/31 (67)	0.4559
Postmenopausal	61 (67.8%)	2 (66.7%)	4 (80%)	50/67 (74.6)	
T values					
T0	7 (7.7%)	0 (0%)	2 (40%)	3/9 (33.3)	T1 to T3, 0.029 T0 to T3, 4.10^{-5} T0 to T4, $1.3.10^{-5}$
T1	13 (14.3%)	0 (0%)	1 (20%)	8/14 (57.1)	
T2	50 (54.9%)	1 (33.3%)	0 (0%)	43/51 (84.3)	
T3	15 (16.5%)	2 (66.7)	2 (40%)	12/19 (63.2)	
T4	6 (6.6)	0 (0%)	0 (0%)	5/6 (83)	
Lymph node status					
Negative	44 (50.6%)	1 (33.3%)	4 (100%)	40/49 (81.6)	0.2310
Positive	43 (49.4%)	2 (66.7%)	0 (0%)	32/45 (71.1)	
Estrogen receptor					
Negative	42 (46.7%)	3 (100%)	5 (100%)	30/50 (60.0)	0.0048
Positive	48 (53.3%)	0 (0%)	0 (0%)	41/48 (85.4)	
Progesterone receptor					
Negative	40 (44%)	3 (100%)	5 (100%)	29/48 (60.4)	0.0159
Positive	51 (56%)	0 (0%)	0 (0%)	42/51 (82.6)	
Histological grade					
Grade I	12 (14.1%)	0 (0%)	1 (20%)	9/13 (69.2)	I/II, 0.5426 I/III, 0.2498 II/III, 0.5059 I/II/III, 0.5635
Grade II	44 (51.8%)	0 (0%)	2 (40%)	35/46 (76.1)	
Grade III	29 (34.1%)	3 (100%)	2 (40%)	28/34 (82.4)	
Evolution over 5 years					
Alive	75 (80.6%)	0 (0%)	5 (100%)	57/80 (71.3)	0.4048
Dead	18 (19.4%)	3 (100%)	0 (0%)	17/21 (80.9)	
p53 immunoreactivity					
Negative	70 (79.5%)	2 (100%)	2 (40%)	54/74 (73)	0.4961
Positive	18 (20.5%)	0 (0%)	3 (60%)	17/21 (80.9)	

For a few patients, some clinicopathological evaluations failed; consequently, the total number of cases can differ from 102.

pretreatment.⁷¹ This protein is characterized by an accumulation at the transition between G2 and M phases, and its expression correlates with semiconservative DNA synthesis associated with the proliferating cell nuclear antigen (PCNA) expression but not with the DNA synthesis associated with DNA repair.⁷² Consequently, Ki-67 has a short half-life, and its concentration decreases rapidly after the mitotic phase; thus, it is considered as an accurate indicator of cell proliferation in histological material.⁷³ A majority of the studied tumors, 20/24 (83.3%), were mainly Ki-67/MIB-1 positive; but at the cellular level, no co-localization could be established between the overexpression of the *H19* gene and the presence of this marker (Figure 2, B and C).

H19 Gene Overexpression and p53 Protein Accumulation

Ninety-five carcinomas were examined for the presence of p53 protein by using an immunohistochemical method; twenty-one cases (23.3%) were positive. Accumulation of p53 protein was mainly inside nuclei (Figure 2E), although a weak cytoplasmic signal could not be excluded as described by Moll et al.⁷⁴ The *H19* and p53 labelings do not overlap (Figure 2, E and F). Anti-p53 immunoreaction was correlated with histological grade ($P = 0.0062$) but not with a high *H19* expression level ($P = 0.4442$) and clinical information, including T values, T0 to T4 ($P = 0.5732$), estrogen ($P = 0.3702$) and progesterone ($P = 0.2512$) receptors, age ($P = 0.4881$), and menopausal ($P = 0.8383$) and lymph node ($P = 0.7867$) status. Correlation of p53 index with the histological grade provided the following results: grades I/II, $P = 0.7571$; grades I/III, $P = 0.2001$; and grades II/III, $P = 0.0392$.

Discussion

The function of *H19* in mammary gland is of particular interest as it is one of the few tissues that continues to express detectable amounts of *H19* RNA in adulthood.^{1,6} Results reported in this work confirm that the *H19* gene is overexpressed in most cases of breast adenocarcinomas.^{4,6} The high level of expression of the *H19* gene observed in these cancers is probably not the consequence of the loss of imprinting, as Yballe et al⁴⁴ claimed that in their study *H19* was expressed monoallelically in all of the 18 informative breast tumors.

ISH detection of *H19* RNA, associated with immunohistochemical staining specific for epithelial or myoepithelial cells, allowed us to precisely detect the pattern of expression of this gene in tumors and, consequently, are complementary to previous results obtained by Northern blot.⁴ *H19* RNA was preferentially located in stromal cells only (92.2% of cases), whatever the considered histological subclasses of tumors (Table 1). Adipocytes were also highly labeled. Our data establish, too, that during breast tumorigenesis an overexpression of the *H19* gene within epithelial cells was rare: in 4.9% of specimens in both

epithelial and stromal cells and in 2.9% of them in epithelial cells only (Table 2).

Spanakis and Brouty-Boyé⁷⁵ tested the hypothesis that predicts that the stroma also progresses along with the epithelium in a breast tumor. They asked what characteristics were likely to change in a permanent manner during tumor development, and they screened a large number of transcripts. They concluded that stromal cells from normal and pathological breast tissues present multiple irreversible differences in gene expression. During the desmoplastic reaction, cells constructing the stroma originated mainly in fibroblasts and smooth muscle cells, the so-called myofibroblasts, which correspond to a significant percentage of cells present in breast tumors, as high as 45%.^{76,77} Thus, when tumorigenesis occurs, quantitative (cellular proliferation) and qualitative (disappearance of myoepithelial cells) modifications arise, and the stroma is transformed in a fibrous tissue.⁷⁸ Several genes are expressed specifically in the stromal part of a breast cancer, ie, hepatocyte growth factor,⁷⁹ urokinase plasminogen activator,^{80,81} thrombospondin-1,⁸² tissue factor,⁸³ aromatase,⁸⁴ and *c-ets-1* transcription factor.⁸⁰ Through reciprocal exchanges between epithelial and stromal cellular types, products of the latter genes interfere in the tumor growth when proliferation, angiogenesis, and invasion occur. The study of Singer et al⁸⁵ is worth mentioning in this context; indeed, these authors reported a paracrine influence, mediated by soluble factors released by epithelial cells, which are able to increase expression of the *IGF-II* gene in stromal cells; *IGF-II*-expressing fibroblasts are selected specifically in the stroma of breast cancers by the malignant cells. As *IGF-II* and *H19* genes are co-regulated by the same set of enhancers, although they are oppositely imprinted,^{9,13} we propose that the stromal *H19* up-regulation described in the present work could be induced by paracrine factors involved in the activation of the *IGF-II* transcription within the same mesenchymal cells. Furthermore, the epithelium-mesenchyme interactions also play a key role in proliferation and differentiation mechanisms during normal breast development.^{80,86-89}

Otherwise, the *H19* RNA concentration observed at the epithelium/mesenchyme boundary can reflect one issue of the interactions or the dialogue between cancerous and stromal cells. The conversion of fibroblast to myofibroblast is the consequence of epithelial stimuli.⁹⁰ The closer the cells are to the tumor epithelial cells, the more they are stimulated. In another respect, kinetics of *H19* RNA paralleled the accumulation of muscle-specific markers (α -actin, MLC1/MLC3),⁶³ and the expression of the rabbit *H19* homologue was found in nonproliferative actin-positive cells.⁹¹ This could explain the overexpression of the *H19* gene at the epithelium/stroma boundary in benign and malignant tumors (see also Ref. 6). This pattern of expression can be put in parallel with that one observed for *c-ets-1*, *uPA*, and *collagenase* expression detected in mesenchyme cells facing invasive epithelial cells; these data suggest that epithelial cells send signals to mesenchymal cells, which react by expressing these genes.^{92,93}

Owing the few number of cases (2.9%) where *H19* transcripts were localized exclusively in epithelial cells, the question of the importance of this observation is posed. We can notice that this rare *H19* expression pattern matches with the absence of hormone receptors and the death of patients within the 5 years after tumorectomy. These rare situations could be explained by the general deregulation of genes, which can be encountered in the advanced tumor phase of cells.

In the healthy breast, epithelial cells synthesize a basal level of *H19* transcripts depending on the specimen and even the area within a given section (this study and Ref. 6); this indicates that in the majority of carcinomas (~92%), the tumor development is accompanied by the complete loss of *H19* gene expression in cancer cells. This striking silencing of the *H19* gene does not establish the final evidence of the tumor suppressor function of the gene, but our statement on *H19* expression patterns fit well with this role proposed by several authors.^{17,27,28,30,49,94} The *H19* product (a RNA) could be implicated in some differentiation (or proliferation arrest) mechanisms. Moreover, Leibovitch et al⁶³ demonstrated that both the *H19* gene and *c-mos* oncogene are involved in myogenic differentiation and even are necessary in the maintenance of this status. The fact that the *H19* gene is expressed during embryogenesis and then turned off in almost all adult tissues, except in breast, heart, and skeletal muscles, could suggest a dual function: one in proliferative events and the other one in differentiation.

Table 2 shows that among several clinicopathological factors considered in this study, only the T values classification (UICC) and the presence of hormone receptors (ER and PR) gave a positive significant correlation with *H19* gene overexpression. Interestingly, the T value is one of the three elements of the TNM classification usually used to determine evolution and prognosis of the tumors. *P* values indicate that this important clinicopathological factor is highly correlated with *H19* gene overexpression. No less interesting is the positive significant correlation between *H19* overexpression and the presence of hormone receptors, which can be put together with the established feature that the estimate (in femtomoles) of these receptors indicates the level of sensitivity of tumor cells to these hormones.⁹⁵ Otherwise, it is known that aromatase is involved in estradiol synthesis, and the expression of the aromatase gene increases in fat tissue adjacent to the tumor.⁹⁶⁻⁹⁸ Interestingly, *H19* transcripts were abundant in adipocytes, mostly in those located near the tumor. The latter cells synthesize estrogens, particularly those located in this area,⁹⁹ and these hormones could account for *H19* overexpression, as it has been proposed that estrogen could play a role in modulations of *H19* expression.¹⁰⁰ Consequently, variations of the estrogen levels during the menstrual cycle could account for the observed differences in *H19* RNA abundance detected in various healthy breast resections and eventually also in pathological tissues.

Now we have to discuss ISH data in parallel with information on two physiological properties of the cells overexpressing the *H19* gene. Do these cells accumulate the p53 protein and are they in cycle? Relationships

between p53 accumulation and pathological factors, such as the histological type and grade and the status of the ER and PR is still in dispute.¹⁰¹⁻¹⁰⁶ The prognostic and predictive value of p53 overexpression in breast carcinomas appears weaker than hoped.¹⁰⁷ Nevertheless, accumulation of p53 is usually associated with tumor grades and negative ER status.¹⁰⁷ In our series of breast tumor resections, no positive correlation was provided by the comparison between p53 protein accumulation and *H19* gene overexpression. Nevertheless, in another study we have demonstrated a down-regulation of the *H19* promoter by the wild-type p53 protein, but not by one p53 mutant (the 143 Ala mutant).¹⁰⁸ Discrepancies between our previous data and those reported in the present work can be explained by several outlines, not mutually exclusive. Our previous study¹⁰⁸ was concerned with a cell line (HeLa cells) transiently or stably transfected with a p53 recombinant vector, and it was focused on relationships between p53 protein and the *H19* promoter and displayed the effect of an accurate p53 mutation, exhibiting a thermosensitive phenotype. On the contrary, in the present study we considered *H19* gene expression in tissues originated from primary breast cancers, of which the causes are necessarily multifactorial. Furthermore, one must keep in mind that although any accumulation of p53 protein can be generally the consequence of a genetic or an epigenetic outcome, we have no indications that all of the p53 mutations induce necessarily an overexpression of the *H19* gene. Otherwise, p53 protein was located exclusively in epithelial cells, and positive correlation between p53 accumulation and a mutation of the p53 gene in breast cancers varied from 62% to 92%.¹⁰⁹ Finally, one must remember that *H19* gene overexpression was anyway rare in epithelial cells.

We consider now ISH data and a feature specific of cells in cycle. A monoclonal antibody, Ki-67, has been used to demonstrate that cells are in cycle. Ki-67 identifies a nuclear nonhistone protein of 395 and 345 kd present in the nucleoli of proliferative interphase cells as well as the condensed chromatin in mitotic cells. On the contrary, cells in quiescent phase G0 lack this antigen.^{110,111} In this study, we used MIB-1, which is a monoclonal antibody raised against a recombinant part of the Ki-67 antigen.⁷¹ As for p53 protein detection, the MIB-1 immunostaining labeled frequently tumor cells, independently of any ISH signal specific of the *H19* RNA equipment. Once more, we must remember that epithelial cells express rarely the *H19* gene. Consequently, the *H19* RNA seems to be not crucial in the maintenance of cells in cycle.

In conclusion, 1) *H19* gene overexpression is significantly correlated to the T values (TNM classification) and the presence of hormone receptors, but with neither the p53 tumor suppressor gene product nor with a protein indicating that cells are in cycle, 2) the frequent (92.2% of adenocarcinomas) overexpression of the *H19* gene in stroma could be one of the responses of mesenchymal cells to paracrine factors released by tumor epithelium (this is stressed by abundance of *H19* transcripts in mesenchymal cells adjacent to epithelial tissue), 3) *H19*

RNA accumulates rarely in epithelial cells (7.8% of cases, but in 2.9% in malignant cells only); the general silencing of *H19* in invasive cells is in agreement with considerations on which this gene has been proposed as a tumor suppressor candidate, and 4) the fold increase of a basal level of *H19* gene expression in the normal breast during adulthood, as the loss of regulation inducing a frequent but complex overexpression pattern of this gene in carcinomas, seems a result of puzzling processes, reflecting the fundamental relationships between cells with different phenotype. It is unlikely that any simple mechanism will explain all of the changes of the *H19* expression level that occur as the mammary gland differentiates, ages, or undergoes a neoplastic development.

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