HIN-1, a putative cytokine highly expressed in normal but not cancerous mammary epithelial cells

Ian E. Krop[a,b], Dennis Sgroi[b,c], Dale A. Porter[a,b], Kathryn L. Lunetta[d,e], Rebbecca LeVangie[c], Pankaj Seth[a,b], Carolyn M. Kaelin[b,f], Esther Rhei[b,f], Marcus Bosenberg[a,b,g], Stuart Schnitt[b,h], Jeffrey R. Marks[i], Zrinka Pagon[b,j], Drazen Belina[k], Jasminka Razumovic[l], and Kornelia Polyak[a,b,m]

Departments of [a]Adult Oncology and [d]Biostatistics, Dana–Farber Cancer Institute, Boston, MA 02115; Departments of [f]Surgery and [g]Pathology, Brigham and Women's Hospital, Boston, MA 02115; [h]Department of Pathology, Beth-Israel Deaconess Medical Center, Boston, MA 02115; [j]Department of Medicine, Children's Hospital, Boston, MA 02115; [c]Department of Pathology, Massachusetts General Hospital, Charlestown, MA 02129; [b]Department of Medicine, Harvard Medical School, Boston, MA 02115; [e]Harvard School of Public Health, Boston, MA 02115; [i]Department of Surgery, Duke University Medical Center, Durham, NC 27710; and Departments of [k]Cardiac Surgery and [l]Pathology, University Hospital Rebro and Zagreb Medical School, 10000 Zagreb, Croatia

Edited by Joan Massagué, Memorial Sloan-Kettering Cancer Center, New York, NY, and approved June 21, 2001 (received for review March 21, 2001)

To identify molecular alterations implicated in the initiating steps of breast tumorogenesis, we compared the gene expression profiles of normal and ductal carcinoma *in situ* (DCIS) mammary epithelial cells by using serial analysis of gene expression (SAGE). Through the pair-wise comparison of normal and DCIS SAGE libraries, we identified several differentially expressed genes. Here, we report the characterization of one of these genes, HIN-1 (high in normal-1). HIN-1 expression is significantly down regulated in 94% of human breast carcinomas and in 95% of preinvasive lesions, such as ductal and lobular carcinoma *in situ*. This decrease in HIN-1 expression is accompanied by hypermethylation of its promoter in the majority of breast cancer cell lines (>90%) and primary tumors (74%). HIN-1 is a putative cytokine with no significant homology to known proteins. Reintroduction of HIN-1 into breast cancer cells inhibits cell growth. These results indicate that HIN-1 is a candidate tumor suppressor gene that is inactivated at high frequency in the earliest stages of breast tumorogenesis.

The natural history of breast cancer involves a sequential progression through defined stages, starting with benign then atypical hyperproliferation, progressing to *in situ* then invasive carcinomas, and culminating in metastatic disease (1). Ductal carcinoma *in situ* (DCIS) is believed to be the true precursor of invasive ductal carcinoma, based on molecular and epidemiological studies as well as on studies in animal models of breast cancer (2). To gain a molecular understanding of the initiation of breast tumorogenesis, we determined the global gene expression profiles of normal and DCIS luminal mammary epithelial cells by using serial analysis of gene expression (SAGE; refs. 3 and 4). SAGE analyzes 14-bp tags derived from a defined position from the 3′-end of cDNAs (3). The tag numbers directly reflect the abundance of the transcript within the mRNA population studied. Because SAGE does not rely on preexisting databases of expressed genes, it provides a comprehensive and unbiased view of gene expression patterns. This feature is particularly important in the analysis of previously uncharacterized cell types, such as DCIS or normal luminal mammary epithelial cells, because transcripts expressed in these cells are unlikely to be represented in expressed sequence tag (EST) databases.

Using this approach, we isolated several differentially expressed genes. Here, we report the identification of a putative cytokine, HIN-1 (high in normal-1), which is highly expressed in normal luminal mammary epithelial cells and is hypermethylated and not expressed in the majority of breast cancers.

Materials and Methods

Generation and Analysis of SAGE Libraries. SAGE libraries were derived from two cases of normal and DCIS luminal mammary epithelium. Minced breast tissue was digested in DMEM/F12 medium (Life Technologies) supplemented with 1% FBS, 2 mg/ml collagenase I, and 2 mg/ml hyaluronidase at 37°C for 2 h. Cells were collected by centrifugation, trypsinized, and resuspended in PBS, 1% BSA, and 2 mM EDTA and purified by using Epithelial Enrich kit (Dynal, Great Neck, NY). SAGE libraries were generated after a modified microSAGE protocol, but including a 1% SDS washing/heating step after each enzymatic reaction to ensure complete inactivation of the enzymes (4).

Cell Lines, Tumor Specimens, RNA Preparation, and Northern Blot Analysis. Breast cancer cell lines were obtained from American Type Culture Collection or were generously provided by Steve Ethier (Univ. of Michigan), G. Tomlinson (Univ. of Texas), and Arthur Pardee (Dana–Farber Cancer Institute). Cells were grown in media recommended by the provider. To test the effect of methyl transferase inhibitors, cells were grown in the presence of 25 μM 5-aza-2′-deoxycytidine for 3–7 days, then harvested for RNA preparation. Primary tumors were obtained from the Brigham and Women's Hospital, Massachusetts General Hospital, and University Hospital Zagreb (Zagreb, Croatia), or were obtained from NDRI (National Disease Research Interchange) snap frozen on dry ice and stored at −80°C until use. All patient identifiers were removed before transport to the laboratory. Primary mammary epithelial cell cultures were initiated from reduction mammoplasty tissue, and cells were grown in MBEM medium (Clonetics, San Diego). Laser capture microdissection was performed as described (5), but mRNA was converted to double-stranded cDNA (dscDNA), quantitated with PicoGreen (Molecular Probes) by using a spectrofluorometer, and PCR was performed using 2.5 ng of dscDNA from each sample as template. The primer probe set used for real-time PCR was as follows: forward primer, 5′-GAGCATCTACACCTGAGGA-CAAGAC-3′; reverse primer, 5′-TTTTGCTCTTAAC-CACGTTTATTGA; Taqman probe, VIC-CAC CCG CGA GGG CTG AAA ACC-TAMRA. RNA isolation, reverse transcription (RT)-PCR, and Northern blot analyses were performed as described (6); human multiple tissue Northern blots were purchased from CLONTECH.

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: DCIS, ductal carcinoma *in situ*; SAGE, serial analysis of gene expression; RT, reverse transcription; HIN-1, high in normal-1; LCM, laser capture microdissection; LOH, loss of heterozygosity.

Data deposition: The sequence of the human HIN-1 cDNA reported in this paper has been deposited in the GenBank database (accession no. AY040564).

[m]To whom reprint requests should be addressed. E-mail: Kornelia_Polyak@dfci.harvard.edu.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Table 1. Most highly differentially expressed *HIN* and *HID* genes

SAGE tag	N1	N2	D1	D2	Unigene no.	Gene name
CTCCACCCGA	19	3	243	284	Hs.82961	Trefoil factor (intestinal)
AAGCTCGCCG	*69*	*19*	*0*	*0*	NA	*HIN-1*
GAGGGTTTAG	24	13	1	0	Hs.75498	Chemokine exodus-1
TTGAAGCTTT	67	106	1	1	Hs.75765	Chemokine GROβ
TTGAAACTTT	217	194	5	4	Hs.789	Chemokine GROα
TGGAAGCACT	203	151	4	13	Hs.624	IL-8 (CXCα chemokine)
GCCTTGGGTG	35	58	0	1	Hs.2250	Leukemia inhibitory factor
ACTCAGCCCG	36	14	0	1	Hs.101382	TNF-α-induced protein 2
GCTTGCAAAA	116	52	3	4	Hs.177781	Superoxide dismutase 2
CGAATGTCCT	29	21	0	0	Hs.335952	Keratin-6B

HIN-1 Methylation, Loss of Heterozygosity, and Mutation Analysis. To determine the location of methylated cytosines, genomic DNA was extracted from the cells, bisulfite treated, and purified as previously described (7). PCR amplification was performed by using primers designed to amplify the coding strand (nucleotides −340 to + 72) of bisulfite treated DNA, as follows: forward primer, 5′-GAGGGAAAGTTTTTTTTATTTGG-3′; and reverse primer, 5′-CAAAACTAACAAAACAAAACCA-3′. PCR reactions were performed as described (7). PCR products were subcloned into pZERO1.0 (Invitrogen), and four to six independent clones were sequenced for each PCR product. Based on sequence analysis, the following PCR primers were designed for the amplification of methylated or unmethylated DNA: methylated DNA forward primer (nucleotides −172 to −149), 5′-GGTACGGGTTTTTTACGGTTCGTC-3′; reverse primer R2 (nucleotides −37 to −58), 5′-AACTTCTTATACCCGATCCTCG-3′; unmethylated DNA forward primer (nucleotides −172 to −149), 5′-GGTATGGGTTTTTTATGGTTTGTT-3′; and reverse primer R2 (nucleotides −37 to −58), 5′-CAAAACTTCTTATACCCAATCCTCA-3′. PCR amplifications were performed as described (7). For loss of heterozygosity studies, forward and reverse primers were designed to amplify a polymorphic CA repeat present in the HIN-1 genomic clone. PCR amplifications were performed by using radioactive primers as described (8). Mutation screen was performed by using PCR-derived full-length cDNA or genomic fragments.

Generation of Recombinant HIN-1 Protein and Polyclonal Anti-HIN-1 Antibodies. The human HIN-1 cDNA was PCR amplified and subcloned into pQE-30 (Qiagen, Chatsworth, CA) in frame with an N-terminal hexahistidine tag and transformed into MJ15 [pREP4] bacteria. Recombinant HIN-1 protein was purified by using denaturing buffer and Ni-nitrilotriacetic acid (NTA) beads (Qiagen). Rabbit polyclonal antibody was generated by using full-length recombinant HIN-1 as immunogen (Custom antibody generation service, Zymed).

Generation of HIN-1 Mammalian Expression and Reporter Constructs and Recombinant Adenoviruses. For constitutive expression the HIN-1 cDNA was PCR amplified and subcloned into pCEP4 (Invitrogen). For the generation of a recombinant adenovirus, the HIN-1 cDNA was PCR amplified and subcloned into pAd-Track-CMV, followed by adenovirus generation using the Ad-Easy system (9). HIN-1 promoter luciferase reporter constructs were generated by subcloning a PCR-derived 2,800-bp fragment of the human HIN-1 promoter into pBR-pl-luc (6). Cells were transfected by using FuGene6 (Roche); luciferase and β-galactosidase activities were determined by using a luciferase assay system (Promega) and the Aurora GAL-XE reporter gene assay (ICN), respectively. Experiments were done in triplicate, and luciferase activity was normalized for transfection efficiency by using the ratio of luciferase to β-galactosidase activity.

Colony Assays and Western Blot Analysis. For colony assay experiments, cells were transfected with FuGene6 (Roche) followed by selection in hygromycin containing medium for 2 wk, after which colonies were visualized by crystal violet staining. For Western blot analysis, cells and media from 293 cells transfected with pCEP4 or pCEP4-His-HIN-1 constructs, and MCF10A or SUM159 cells infected with Ad-Track-GFP or Ad-Track-His-HIN-1, were lysed in denaturing buffer and purified as described above. Bound proteins were immunoblotted with rabbit anti-HIN-1 antibody.

Results

Generation and Analysis of SAGE Libraries. We generated SAGE libraries from two independent cases of immunomagnetic purified normal (N1 and N2) and DCIS (D1 and D2) luminal mammary epithelial cells by using a modified SAGE technique (3, 4). From the four SAGE libraries, 160,046 tags were obtained, enabling us to compare the expression levels of close to 30,000 unique transcripts. Pair-wise comparison of these SAGE libraries identified several differentially expressed tags. Ninety-seven tags were elevated at least 10-fold in one or the other DCIS library [HID genes (high in DCIS)], whereas 132 tags were at least 10-fold more abundant in the normal libraries [HIN genes (high in normal)]. There was only 1 tag that was highly elevated in both DCIS libraries, whereas there were 9 tags that were significantly (at least 10-fold) down-regulated in both DCIS libraries when compared with normal libraries (Table 1). Interestingly, the majority of these *HID* and *HIN* genes encode secreted proteins, including several chemokines (Table 1), suggesting the importance of extracellular factors in mammary tumorogenesis. One of the 9 HIN genes (HIN-1) appeared to be particularly interesting, because it was absent in 90 other SAGE libraries derived from a variety of normal and cancerous tissue types, including invasive and metastatic breast carcinomas (10, 11), suggesting normal luminal mammary epithelium-specific function. The full-length human HIN-1 cDNA is predicted to encode a small protein of 104 aa ($\approx$10 kDa) containing a putative signal peptide.

HIN-1 Expression in Normal Tissues and in Breast Carcinomas. Northern blot analysis was performed to evaluate HIN-1 expression levels in multiple independent normal breast organoids (uncultured breast ducts composed of luminal and myoepithelial cells), in mammary myoepithelial cell cultures, and in breast cancer cell lines (Fig. 1*A*). High levels of HIN-1 expression were detected in breast organoids, but not in myoepithelial cells or in breast cancer cell lines. Furthermore, we were unable to detect significant levels of HIN-1 cDNA by RT-PCR (30 cycles) in 96% (27/28) of breast cancer cell lines. The difference in HIN-1 mRNA levels in the three normal organoids may be due to differences in age, parity, or hormonal status of the three patients from whom the organoids were derived. HIN-1 expres-

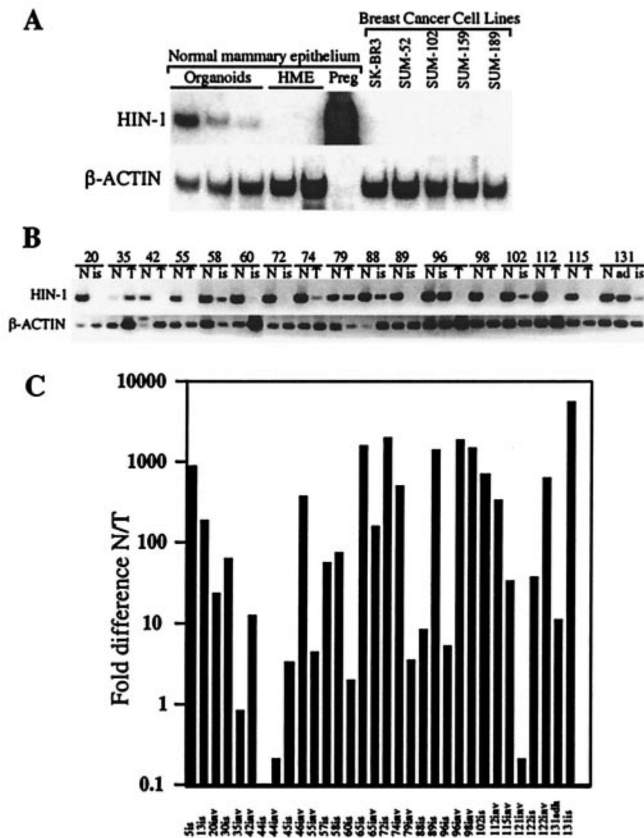

Fig. 1. HIN-1 expression in breast carcinomas and breast cancer cell lines. (*A*) Northern blot analysis of HIN-1 expression in normal human mammary organoids (freshly isolated breast ducts), myoepithelial cells (HME), mammary epithelium from a 25-wk-pregnant patient (Preg), and various breast cancer cell lines. The weak actin hybridization signal in the pregnant mammary tissue sample (Preg) is likely due to partial RNA degradation. (*B*) RT-PCR analysis of HIN-1 expression in LCM (laser capture microdissected) purified breast cancers and corresponding normal epithelium. Numbers indicate case numbers, whereas ''is'' and ''T'' denote *in situ* and invasive carcinomas, and ''ad'' atypical ductal hyperplasia. Amplification of the β-actin cDNA was used as control. (*C*) Real-time PCR analysis of HIN-1 expression in LCM-purified primary breast carcinomas and corresponding normal epithelium. Fold change indicates the ratio of HIN-1 mRNA levels in normal and cancerous epithelium. Numbers indicate case numbers, whereas ''is'' and ''inv'' denote *in situ* and invasive carcinomas, and ''adh'' atypical ductal hyperplasia.

sion was dramatically up-regulated in mammary epithelium derived from a 25-wk-pregnant patient (Fig. 1*A*, Preg).

To evaluate the expression of HIN-1 in primary breast carcinomas, we first performed RT-PCR analysis of 19 LCM (laser capture microdissection)-purified primary tumors and corresponding normal mammary epithelium (Fig. 1*B*; ref. 5). In most tumors (74%), we detected no or minimal HIN-1 cDNA even after 40 cycles of PCR, whereas some tumors had detectable, although decreased, HIN-1 expression (Fig. 1*B*). To determine the extent of decrease in HIN-1 mRNA levels quantitatively, we analyzed these and 10 additional cases (32 different normal/tumor pairs) of LCM-purified primary tumors and corresponding normal mammary epithelium by real-time PCR (Fig. 1*C*; ref. 5). Only four tumors were found to express HIN-1 mRNA at levels comparable to corresponding normal mammary epithelium. The majority of tumors (88%) had significantly decreased HIN-1 expression. These primary tumors included *in situ* and invasive ductal and lobular carcinomas, and one case of atypical ductal hyperplasia (ADH). HIN-1 expression was lost regardless of tumor stage and histological type, and, intriguingly, 18 of 19 (95%, including the 2 DCIS samples used for SAGE) preinvasive lesions had significantly decreased HIN-1 mRNA levels. Thus, down-regulation of HIN-1 expression is an early and frequent event in human breast carcinomas.

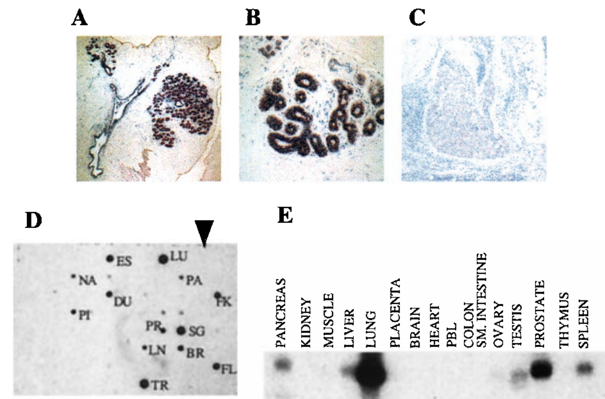

Fig. 2. HIN-1 expression in human tissues. (*A, B, C*) Representative mRNA *in situ* hybridization using digitonin-labeled human HIN-1 antisense ribo-probe on normal (*A*, ×20, *B*, ×200 magnification) and DCIS (C, ×200 magnification) mammary epithelium. Hybridization with the sense probe gave no signal (data not shown). (*D*) Evaluation of HIN-1 expression in 76 human tissues on a dot blot expression array. High level of expression was detected in breast (BR), lung (LU), esophagus (ES), duodenum (DU), trachea (TR), prostate (PR), salivary gland (SG), fetal lung (FL), and fetal kidney (FK), whereas lower level expression was seen in pancreas (PA), lymph nodes (LN), nucleus accumbens (NA), and pituitary gland (PI). Arrow indicates a column of cancer cell lines including leukemias, lymphomas, lung, colorectal, and cervical cancer cell lines. (*E*) Analysis of HIN-1 expression on multiple tissue Northern blots to confirm the size of the hybridizing RNA.

To confirm HIN-1 expression in the luminal mammary epithelial cells at the cellular level, we performed mRNA *in situ* hybridization (Fig. 2). HIN-1 is highly and specifically expressed in normal luminal epithelial cells of small (but not large) ducts and lobules irrespective of their proliferation and hormone receptor status (Fig. 2 *A* and *B*; and data not shown). In contrast, no hybridization signal was detected in DCIS (Fig. 2*C*). Analysis of HIN-1 mRNA levels in immunomagnetic-purified luminal and myoepithelial cells by RT-PCR also demonstrated that HIN-1 is expressed only in luminal epithelial cells (data not shown).

To further investigate HIN-1 expression, we hybridized the HIN-1 cDNA against a tissue expression array panel containing mRNA from 76 human adult and fetal tissue types (Fig. 2*D*). Besides mammary gland, HIN-1 is also highly expressed in other organs composed of branching ductal epithelia, raising the possibility that HIN-1 may be involved in regulating epithelial cell proliferation, differentiation, or morphogenesis (12, 13). We did not detect any HIN-1 expression in several cancer cell lines (Fig. 2*D*), nor did we detect significant expression in 39 of 40 primary lung cancers (data not shown). To verify the identity of the signal detected on the dot blots, we also hybridized multiple tissue Northern blots (Fig. 2*E*) and confirmed that the hybridizing band corresponds to a single HIN-1 mRNA.

Frequent HIN-1 Promoter Methylation in Cells with Decreased HIN-1 Levels. The loss of HIN-1 expression in the majority of breast cancers suggests a tumor suppressor role for HIN-1. To evaluate whether HIN-1 undergoes genetic alterations in breast cancers, we performed LOH (loss of heterozygosity) and mutational analyses of the HIN-1 gene. By fluorescence *in situ* hybridization (FISH) analysis, we localized HIN-1 to 5q35-tel, a region previously not implicated in breast cancer. However, LOH of 5q is frequent in lung, salivary, prostate, and pancreatic carcinomas (14–17). We identified a genomic clone that contains the entire

HIN-1 gene and an adjacent polymorphic CA repeat suitable for LOH analysis. Analysis of this CA repeat in 43 primary breast tumors showed LOH in 20% of the informative cases (5 of 25 cases), but sequence analysis of the remaining allele revealed no mutations. Similarly, PCR analysis of breast cancer cell lines detected no homozygous deletions, and sequence analysis of the HIN-1 coding region in six cell lines and in seven primary breast and four lung carcinomas unveiled no mutations. Therefore, the loss of HIN-1 expression in breast carcinomas is unlikely to be due to genetic events, and epigenetic mechanisms (such as methylation) might be responsible (18). This hypothesis was strengthened by the presence of an ≈1,500-bp CpG island containing 138 potential methylation sites in the promoter region, first exon, and first intron of the HIN-1 gene (Fig. 3*A*).

To investigate the potential role of hypermethylation in silencing HIN-1 expression, we analyzed the sequence of its promoter region after bisulfite treatment using genomic DNA isolated from normal mammary tissue and human breast cancer cell lines (19). We found that virtually all of the CpGs in the proximal promoter region (−304 to +31) were highly methylated in a breast cancer cell line with no HIN-1 expression (ZR-75-1), whereas no methylated CpGs were found in three independent cases of normal mammary epithelial cells (Fig. 3*B*).

To validate the consequence of promoter methylation on HIN-1 expression, we analyzed the effect of a DNA methyltransferase inhibitor (5-aza-2′-deoxycytidine═5azaC) on HIN-1 mRNA levels. 5azaC treatment of breast cancer cell lines led to marked expression of HIN-1 mRNA as determined by RT-PCR and Northern blot analysis, reaching levels found in normal mammary epithelial cells (Fig. 3*C*). This HIN-1 reexpression correlated with a decrease in the extent of promoter methylation (Fig. 3*B*). These data suggest that methylation is at least partially responsible for the loss of HIN-1 expression in breast cancer cell lines.

To determine whether the lack of HIN-1 mRNA and the methylation of the HIN-1 promoter were the consequence of deficiencies of certain transcription factors, we generated a HIN-1 promoter–luciferase reporter construct. Assaying luciferase activity of cells transiently transfected with this reporter construct revealed high levels of luciferase expression (Fig. 3*E*). We found no association between luciferase activity and endogenous HIN-1 promoter methylation status. These results, in combination with the 5azaC experiment, indicate that HIN-1-methylated breast cancer cells contain transcription factors required for HIN-1 expression.

HIN-1 Hypermethylation in Primary Tumors. To analyze HIN-1 methylation status in primary breast carcinomas, we developed a methylation-specific PCR assay (20). Using this approach, three independent normal breast tissues were found to be completely unmethylated, whereas the ZR75-1 cell line was completely methylated (Fig. 3*D*). Analysis of 28 breast cancer cell lines and 101 primary tumors determined that 89% of the breast cancer cell lines and 74% of primary breast tumors were completely or partially methylated (Table 2 and data not shown; representative examples Fig. 3*D*). This set of 101 tumors included 13 preinvasive lesions (DCIS). Of these, 8 of 13 were found to be methylated. We also analyzed 4 breast carcinomas used for SAGE (2 primary invasive and 2 lymph node metastasis), and found that all 4 were methylated. The analysis of 9 primary lung carcinomas revealed that 5 of 9 tumors were methylated (data not shown), indicating that methylation of HIN-1 occurs in other cancer types. Of the 101 breast tumors, we analyzed HIN-1 mRNA levels in 28 (20 methylated and 8 unmethylated) by RT-PCR and/or real-time PCR. All but one of the methylated tumors, and 6 of the unmethylated ones, lacked HIN-1 mRNA (Fig. 1 *B* and *C* and Table 2). These results suggest that HIN-1 hypermethylation and subsequent lack of

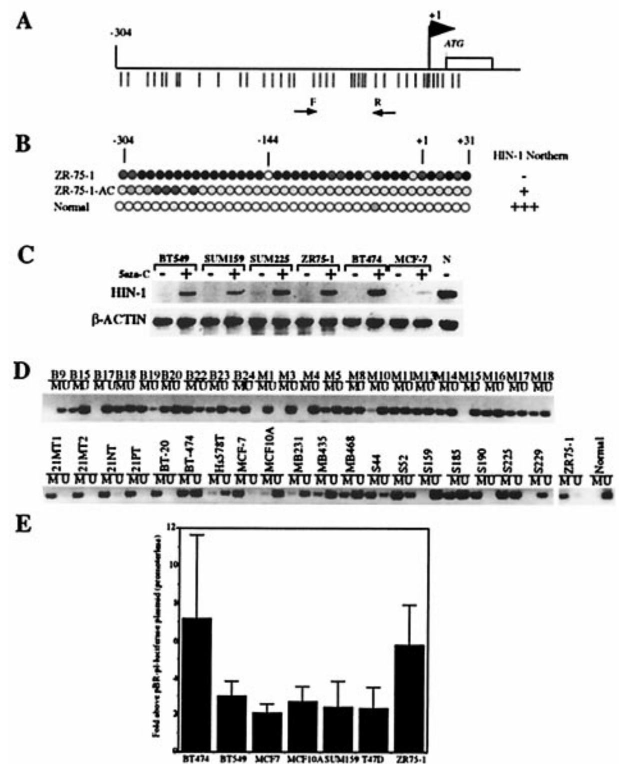

Fig. 3. Analysis of methylation patterns in the HIN-1 promoter region. (*A*) HIN-1 proximal promoter region and first exon. Transcription and translational initiation sites are indicated with arrow and ATG, respectively. Bars represent potential methylation sites (CpG). Arrows indicate the location of forward (F) and reverse (R) primers used in methylation-specific PCR reactions. (*B*) Results of sequence analysis of bisulfite-treated genomic DNA from the indicated cell types. ZR75-1-AC indicates 5azaC-treated cells. Circles represent potential methylation sites (CpG), whereas shading intensity indicates the frequency at which the site was found to be methylated in the clones analyzed (10–100%). HIN-1 mRNA levels are indicated by "+" and "−" signs; +++ denotes high level of expression detected only in normal luminal epithelial cells; + indicates mRNA levels detectable by Northern blot analysis of 5 μg of total RNA. (*C*) RT-PCR analysis of HIN-1 expression levels before and after 5-aza-deoxy-cytosine (5azaC) treatment in the indicated cell lines and in untreated/uncultured normal mammary epithelium (N). Amplification of the β-actin cDNA was used as control. (*D*) Methylation-specific PCR analysis of the HIN-1 promoter region in primary tumors (*Upper*) and breast cancer cell lines (*Lower*). M and U indicate amplification using methylated and unmethylated sequence-specific primers, respectively. DNA prepared from ZR-75-1 (ZR75-1) breast cancer cell line and normal (N) mammary tissue were used as controls. Most of these primary tumors were not microdissected; therefore amplification with the unmethylated primers could be due to contaminating normal tissue or could be due to tumor heterogeneity. (*E*) Luciferase activity in HIN-1-methylated and unmethylated cells after transient transfection with promoterless pBR-pl-luc or pBR-pl-HIN-1prom-luc plasmid.

expression are frequent and early events in breast carcinogenesis, but the possibility that other mechanisms or methylation of other sites not analyzed by this methylation-specific PCR are responsible for silencing HIN-1 cannot be excluded.

HIN-1 Is a Putative Growth Inhibitory Cytokine. The human and mouse HIN-1 cDNAs are predicted to encode a 104-aa protein containing a 20-aa signal peptide (Fig. 4*A*). We also identified a putative *Drosophila* HIN-1 homologue, and a related gene (HIN-1 related) from several different species (Fig. 4*B*). All of these homologues represent uncharacterized genes, suggesting that HIN-1 may be a member of a novel gene family. To confirm that HIN-1 is a secreted protein, we performed immunoblot

Table 2. HIN-1 expression and promoter methylation in breast carcinomas and cell lines

	HIN-1 methylation		
HIN-1 expression	Yes, no. (%)	No, no. (%)	Total
Primary tumors	23 (74)	8 (26)	31
Yes	1* (33)	2 (67)	3
No	22 (79)	6 (21)	28
Breast cancer cell lines	25 (89)	3 (11)	28
Yes	0 (0)	0 (0)	0
No	25 (89)	3 (11)	28

HIN-1 expression was evaluated by northern blot and RT-PCR analysis in the breast cancer cell lines and by RT-PCR and/or real-time PCR (or by SAGE) in primary tumors.

*One case (T44) appeared to be heterogeneous with respect to HIN-1 expression because some areas expressed HIN-1 (Fig. 1*C*), whereas other areas of the same block had no detectable HIN-1 mRNA (data not shown).

analysis of cell extracts and media of cells transiently transfected with a mammalian expression construct or infected with a recombinant adenovirus expressing a hexahistidine-tagged human HIN-1 protein (Fig. 5*A*). Using a rabbit polyclonal anti-human HIN-1 antibody, we detected an ≈8-kDa protein both intra- and extracellularly in HIN-1 expressing, but not in, control cells.

HIN-1 hypermethylation and its lack of expression in a large fraction of breast carcinomas suggest a tumor suppressor role for HIN-1. To test this hypothesis, we transfected a mammalian expression construct with no insert (pCEP4) or expressing the HIN-1 or p53 cDNA (pCEP4-HIN-1 and pCEP4-p53, respectively) into various breast cancer cell lines and assessed colony growth after two weeks of selection. HIN-1 expression led to a significant decrease in colony numbers in BT549 cells compared with control pCEP4-transfected cells. HIN-1 expression had a lesser effect on colony numbers in MDA-MB-435 cells. In contrast, p53 effectively inhibited the growth of both cell lines (Fig. 5*B*). HIN-1 expression had no effect in Chinese hamster ovary (CHO), COS, and 293 cells (data not shown). These results indicate that HIN-1 is a putative growth inhibitory cytokine that may act in a concentration-dependent autocrine manner.

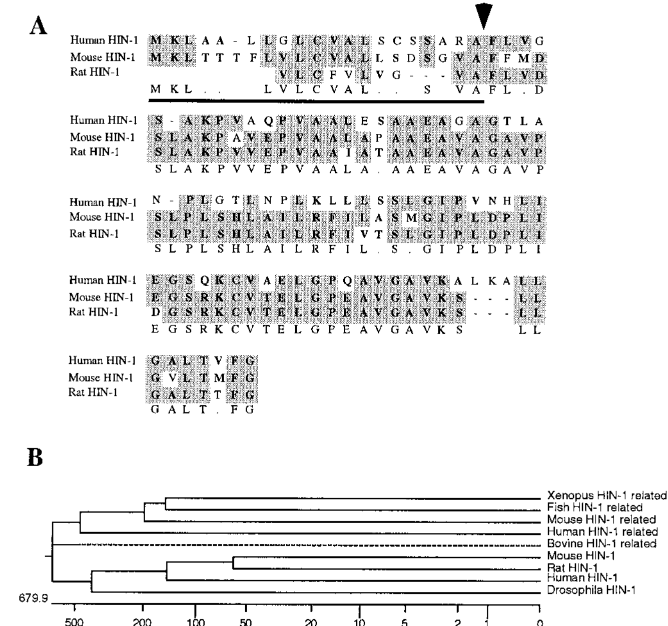

Fig. 4. Putative HIN-1 homologues. (*A*) Amino acid alignment of human, mouse, and rat HIN-1 proteins. Identical and conserved amino acids are highlighted; shading intensity correlates with homology. N-terminal signal peptide and predicted signal peptidase cleavage site are indicated by underlining and arrow, respectively. (*B*) Phylogenetic comparison of HIN-1 homologues. Comparisons were made by using DNASTAR and the Jotun Hein algorithm. Most of the HIN-1-related proteins do not have full-length sequence; therefore the degree of similarity may be imprecise.

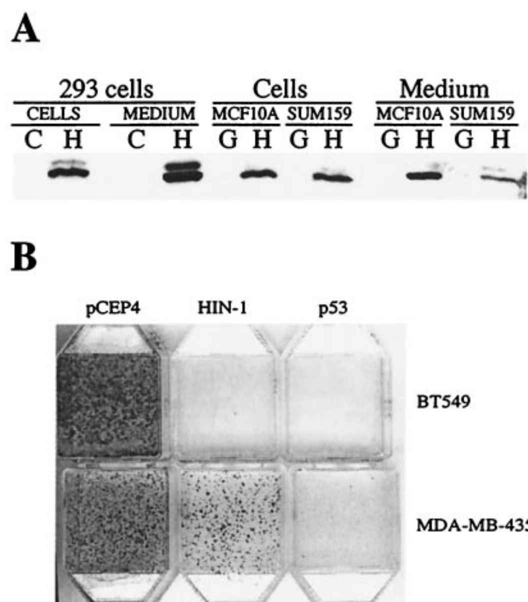

Fig. 5. HIN-1 is a secreted protein that negatively regulates cell growth. (*A*) Western blot analysis of HIN-1 protein expression. Cells and media from 293 cells (first four lanes) transfected with pCEP4 (C), pCEP4-His-HIN-1 (H) constructs, and MCF10A or SUM159 cells infected with Ad-Track-GFP (G) or Ad-Track-His-HIN-1 (H) were lysed in denaturing urea buffer and incubated with Ni-nitrilotriacetic acid (NTA) beads. Bound proteins were resolved by PAGE and immunoblotted with polyclonal rabbit anti-human-HIN-1 antibody. (*B*) Results of a representative colony growth assay experiment in BT549 and MDA-MB435 cells. Cells transfected with empty vector (pCEP4), HIN1, or p53 expression constructs were selected for 2 weeks, followed by crystal violet staining. Hundreds of colonies were observed in control (pCEP4) flasks in both cell lines; no colonies were detected in p53 transfectants. HIN-1 expression significantly suppressed colony growth in BT549 cells and to a lesser degree in MBA-MD435 cells.

Discussion

Breast cancer is a heterogeneous group of tumors with no unifying molecular alteration yet identified. The BRCA1 and -2 genes involved in hereditary breast cancers do not appear to play a role in sporadic cases, whereas amplification or overexpression of oncogenes [c-myc, erbB2, cyclin D1, and epidermal growth factor receptor (EGF-R)] and loss of tumor suppressor genes [p53, PTEN (phosphatase and tensin homolog deleted on chromosome 10), PTCH (patched), MKK4 (MAP kinase kinase 4)] occur in only a fraction of cases (2). Here, we describe the identification of HIN-1, a candidate breast tumor suppressor gene that is not expressed and is hypermethylated in the majority of breast carcinomas. HIN-1 was identified as one of the most abundant transcripts in normal luminal mammary epithelial cells that is absent in DCIS, based on comprehensive gene expression profiling using SAGE. Subsequently, we detected a significant decrease of HIN-1 mRNA levels in 94% (including 2 DCIS, 2 pairs of invasive ductal carcinoma, and lymph node metastasis used for SAGE) of primary breast carcinomas and in 95% of preinvasive lesions (16 DCIS, including 2 DCIS lesions used for

SAGE, 2 lobular carcinoma *in situ* (LCIS), and 1 atypical ductal hyperplasia).

In the mammary gland, HIN-1 is highly expressed in luminal epithelial cells of small ducts and lobules irrespective of their proliferation and hormonal status, whereas large ducts were mostly HIN-1 negative. Because mammary carcinomas are thought to arise from the terminal duct-lobular units (21, 22), where HIN-1 expression is the highest, it is unlikely that breast cancers lack HIN-1 expression because of different cell type of origin. However, the possibility that HIN-1 is not expressed and is methylated in mammary epithelial stem cells, from which tumors may arise, cannot be excluded. The dramatic induction of HIN-1 during pregnancy (Fig. 1*A*) suggests a possible role for *HIN-1* in lobulogenesis and/or terminal differentiation, which occur only during pregnancy, and may also indicate a link between hormonal factors and HIN-1 signaling (23, 24). The high expression of HIN-1 in organs that are composed of branching ductal epithelia (breast, lung, prostate, and salivary gland) raises the possibility that HIN-1 may be involved in regulating epithelial cell proliferation, differentiation, or morphogenesis (12, 13). Loss of HIN-1 expression in 39 of 40 primary lung carcinomas, accompanied with promoter methylation in 5 of 9 lung tumors, suggests that the elimination of the HIN-1 signaling pathway may play a role in multiple cancer types (unpublished data).

HIN-1 is a putative cytokine with no significant homology to known proteins (Figs. 4 and 5). Preliminary *in vitro* and *in vivo* evidence indicates that mammary epithelial cells express a high affinity HIN-1 binding protein (unpublished data). Moreover, reintroduction of HIN-1 into breast cancer cells inhibits their growth. These data suggest that HIN-1 may act in an autocrine manner. Therefore, it is reasonable to hypothesize that loss of an autocrine growth inhibitory pathway would be advantageous for tumorogenesis.

Despite the putative tumor suppressor function of HIN-1, we were unable to identify somatic genetic changes in the HIN-1 gene in breast cancers. However, we found that the HIN-1 promoter is hypermethylated in the majority (>70%) of breast carcinomas, including preinvasive lesions. Because the PCR-based methylation assay we used is inherently restricted to the detection of methylation within the short region recognized by the primers, this number may be underestimated. Although the dramatic reexpression of HIN-1 after 5azaC treatment and the high correlation between lack of HIN-1 expression with promoter methylation strongly suggest that HIN-1 expression is silenced because of methylation in the majority of breast carcinomas, other mechanisms cannot be excluded. Several other genes have been demonstrated to be hypermethylated in breast carcinomas, including p16, E-cadherin, BRCA1 (breast cancer 1), estrogen receptor, GSTP1 (glutathione *S*-transferase P1), MDGI (mammary-derived growth inhibitor), HoxA5, and 14-3-3σ (25–34). However, among these, only 14-3-3σ is methylated in more than 50% of primary invasive breast carcinomas, and none of these genes have been implicated in preinvasive lesions.

In summary, we identified a candidate tumor suppressor gene, HIN-1, that is not expressed and is hypermethylated in a majority of breast carcinomas and may be in other cancer types as well. Because HIN-1 is inactivated in preinvasive tumors, such as DCIS and lobular carcinoma *in situ* (LCIS), the elimination of the HIN-1 signaling pathway may be a pivotal step in the initiation of breast tumorogenesis. In addition, the methylation of HIN-1 in a high fraction of early-stage tumors makes it an excellent molecular marker for early detection. Moreover, because HIN-1 is a putative cytokine and breast carcinomas appear to express a putative HIN-1 receptor (unpublished data), the HIN-1 signaling pathway may provide a new target for cancer prevention and treatment.

We thank Susan Lester, Carmen Tam, Massimo Loda, Matthew Meyerson, and Arindum Bhattachajee for technical assistance and specimen acquisition, Matthew Meyerson, Jeffrey Sklar and Mark Ewen for critical review of the manuscript, and Bert Vogelstein for his continuous support. This work was supported by grants from the National Cancer Institute-Cancer Genome Anatomy Project (NCI-CGAP) (contract S98-146A), National Cancer Institute-Specialized Programs of Research Excellence (NCI SPORE) in Breast Cancer, the American Society of Clinical Oncology, the Sydney Kimmel Foundation (to K.P.), and by the Dana–Farber Cancer Institute (to K.P. and I.E.K.).

MEDICAL SCIENCES

1. Beckmann, M. W., Niederacher, D., Schnurch, H. G., Gusterson, B. A. & Bender, H. G. (1997) *J. Mol. Med.* **75,** 429–439.
2. Silverstein, M. J. (1997) *Ductal Carcinoma In Situ of the Breast* (Williams & Wilkins, Baltimore).
3. Velculescu, V. E., Zhang, L., Vogelstein, B. & Kinzler, K. W. (1995) *Science* **270,** 484–487.
4. St Croix, B., Rago, C., Velculescu, V., Traverso, G., Romans, K. E., Montgomery, E., Lal, A., Riggins, G. J., Lengauer, C., Vogelstein, B. & Kinzler, K. W. (2000) *Science* **289,** 1197–1202.
5. Sgroi, D. C., Teng, S., Robinson, G., LeVangie, R., Hudson, J. R. & Elkahloun, A. G. (1999) *Cancer Res.* **59,** 5656–5661.
6. Polyak, K., Xia, Y., Zweier, J. L., Kinzler, K. W. & Vogelstein, B. (1997) *Nature (London)* **389,** 300–305.
7. Herman, J. G., Umar, A., Polyak, K., Graff, J. R., Ahuja, N., Issa, J. P., Markowitz, S., Willson, J. K., Hamilton, S. R., Kinzler, K. W., *et al*. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 6870–6875.
8. Thiagalingam, S., Lengauer, C., Leach, F. S., Schutte, M., Hahn, S. A., Overhauser, J., Willson, J. K., Markowitz, S., Hamilton, S. R., Kern, S. E., *et al*. (1996) *Nat. Genet.* **13,** 343–346.
9. He, T. C., Zhou, S., da Costa, L. T., Yu, J., Kinzler, K. W. & Vogelstein, B. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 2509–2514.
10. Velculescu, V. E., Madden, S. L., Zhang, L., Lash, A. E., Yu, J., Rago, C., Lal, A., Wang, C. J., Beaudry, G. A., Ciriello, K. M., *et al*. (1999) *Nat. Genet.* **23,** 387–388.
11. Lal, A., Lash, A. E., Altschul, S. F., Velculescu, V., Zhang, L., McLendon, R. E., Marra, M. A., Prange, C., Morin, P. J., Polyak, K., *et al*. (1999) *Cancer Res.* **59,** 5403–5407.
12. Pohl, M., Stuart, R. O., Sakurai, H. & Nigam, S. K. (2000) *Annu. Rev. Physiol.* **62,** 595–620.
13. Warburton, D., Schwarz, M., Tefft, D., Flores-Delgado, G., Anderson, K. D. & Cardoso, W. V. (2000) *Mech. Dev.* **92,** 55–81.
14. Fong, K. M., Zimmerman, P. V. & Smith, P. J. (1995) *Cancer Res.* **55,** 220–223.
15. Saric, T., Brkanac, Z., Troyer, D. A., Padalecki, S. S., Sarosdy, M., Williams, K., Abadesco, L., Leach, R. J. & O'Connell, P. (1999) *Int. J. Cancer* **81,** 219–224.
16. Yamano, M., Fujii, H., Takagaki, T., Kadowaki, N., Watanabe, H. & Shirai, T. (2000) *Am. J. Pathol.* **156,** 2123–2133.
17. Yamamoto, Y., Kishimoto, Y., Virmani, A. K., Smith, A., Vuitch, F., Albores-Saavedra, J. & Gazdar, A. F. (1996) *Hum. Pathol.* **27,** 782–786.
18. Baylin, S. B. & Herman, J. G. (2000) *Trends Genet.* **16,** 168–174.
19. Frommer, M., McDonald, L. E., Millar, D. S., Collis, C. M., Watt, F., Grigg, G. W., Molloy, P. L. & Paul, C. L. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 1827–1831.
20. Herman, J. G., Graff, J. R., Myohanen, S., Nelkin, B. D. & Baylin, S. B. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 9821–9826.
21. Russo, J. & Russo, I. H. (1999) *Oncol. Res.* **11,** 169–178.
22. Goldstein, N. S., Vicini, F. A., Kestin, L. L. & Thomas, M. (2000) *Cancer* **88,** 2553–2560.
23. Rudland, P. S., Fernig, D. G., Leinster, S. & Lunt, G. G., (1998) *Mammary Development and Cancer* (Portland Press, London).
24. Howard, B. A. & Gusterson, B. A. (2000) *J. Mammary Gland Biol. Neoplasia* **5,** 119–137.
25. Graff, J. R., Herman, J. G., Lapidus, R. G., Chopra, H., Xu, R., Jarrard, D. F., Isaacs, W. B., Pitha, P. M., Davidson, N. E. & Baylin, S. B. (1995) *Cancer Res.* **55,** 5195–5199.
26. Herman, J. G., Merlo, A., Mao, L., Lapidus, R. G., Issa, J. P., Davidson, N. E., Sidransky, D. & Baylin, S. B. (1995) *Cancer Res.* **55,** 4525–4530.
27. Esteller, M., Silva, J. M., Dominguez, G., Bonilla, F., Matias-Guiu, X., Lerma, E., Bussaglia, E., Prat, J., Harkes, I. C., Repasky, E. A., *et al*. (2000) *J. Natl. Cancer Inst.* **92,** 564–569.
28. Esteller, M., Corn, P. G., Urena, J. M., Gabrielson, E., Baylin, S. B. & Herman, J. G. (1998) *Cancer Res.* **58,** 4515–4518.
29. Ottaviano, Y. L., Issa, J. P., Parl, F. F., Smith, H. S., Baylin, S. B. & Davidson, N. E. (1994) *Cancer Res.* **54,** 2552–2555.
30. Foster, S. A., Wong, D. J., Barrett, M. T. & Galloway, D. A. (1998) *Mol. Cell. Biol.* **18,** 1793–1801.
31. Huschtscha, L. I., Noble, J. R., Neumann, A. A., Moy, E. L., Barry, P., Melki, J. R., Clark, S. J. & Reddel, R. R. (1998) *Cancer Res.* **58,** 3508–3512.
32. Huynh, H., Alpert, L. & Pollak, M. (1996) *Cancer Res.* **56,** 4865–4870.
33. Raman, V., Martensen, S. A., Reisman, D., Evron, E., Odenwald, W. F., Jaffee, E., Marks, J. & Sukumar, S. (2000) *Nature (London)* **405,** 974–978.
34. Ferguson, A. T., Evron, E., Umbricht, C. B., Pandita, T. K., Chan, T. A., Hermeking, H., Marks, J. R., Lambers, A. R., Futreal, P. A., Stampfer, M. R. & Sukumar, S. (2000) *Proc. Natl. Acad. Sci. USA* **97,** 6049–6054. (First Published May 16, 2000; 10.1073/pnas.100566997)