Expression of the hypermethylated in cancer gene (HIC-1) is associated with good outcome in human breast cancer

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Summary A new cancer gene, HIC-1 (Hypermethylated in Cancer in Tumor) telomeric to p53 on chromosome 17p may be of clinical importance in sporadic breast cancer. Regional DNA hypermethylation of 17p13.3 resulting in suppression of gene expression has been shown to precede structural changes in human carcinogenesis. In addition, loss of heterozygosity studies have suggested clinically significant involvement of a gene on 17p13.3 associated with poor prognosis in breast cancer. Using RT-PCR analysis, we demonstrate that the MCF7 (wild type p53) cell line expressed HIC-1 transcripts but the MDAMB231 (mutant p53) cell line did not, suggesting loss of HIC-1 expression and p53 malfunction may be synergistic events in sporadic breast cancer. HIC-1 expression was examined using RT-PCR on RNA extracted from 50 primary untreated, human breast cancers and was detected in only 7/50 (14%) cancers. All seven patients with HIC-1 expression were alive without disease recurrence after 8 years follow-up and 5/7 had detectable p53 wild type mRNA expression. This suggests that retained HIC-1 expression may offer a survival advantage. However the seven cancers had 17p13.3 loss of heterozygosity (LOH; four patients), a feature previously associated with poor prognosis, or were homozygous (three patients) suggesting there may be two genes at 17p13.3 involved in breast carcinogenesis. Using a demethylating drug 5-aza-2′-deoxycytidine (DeoxyC), HIC-1 expression was restored in the MDAMB231 cells, also suggesting restoration of HIC-1 function by reversing hypermethylation may offer a therapeutic avenue in breast cancer. © 2001 Cancer Research Campaign

Keywords: HIC-1; hypermethylation; p53; prognosis; breast cancer

Abbreviations: HIC-1: hypermethylated in cancer gene 1; DNA: Deoxyribonucleic acid; mRNA: messenger ribonucleic acid; RT-PCR: reverse transcription-polymerase chain reaction; LOH: loss of heterozygosity; DeoxyC: 5-aza-2′-deoxycytidine.
also associated with an aggressive phenotype being significantly correlated with disease recurrence and death due to breast cancer (Thompson et al, 1998; Nagai et al, 1995).

Recently, a candidate tumour suppressor gene HIC-1 (hypermethylated in cancer) has been described on chromosome 17p13.3 (Makos-Wales et al, 1995) in the region with a high level of allelic loss in sporadic breast cancer (Phelan et al, 1998). HIC-1 was identified because of its association with a CpG-rich region or ‘CpG island’. These islands are short and dispersed regions of DNA with a high frequency of CpG dinucleotides relative to the genome generally. A minor fraction (about 2%) is associated with the 5′-ends and hence the promoters and transcription start sites of all housekeeping genes and of some tissue specific genes and have thus been postulated to be sites of interaction between transcription factors and promoters (Cross et al, 1994). The methylation state of the base cytosine modulates the overall genomic pattern of chromatin organization and gene expression and the CpG dinucleotide is the principal, but not exclusive, site of DNA methylation.

Methylation of DNA can lead to mutation through the conversion of methylated cytosine to thymidine by deamination. Hypermethylation of normally unmethylated CpG islands has been implicated as one alternative mechanism to chromosomal deletion for the functional inactivation of tumour suppressor genes. The HIC-1 gene, which is entirely encompassed within a CpG island, is aberrantly hypermethylated and transcriptionally inactivated in several types of human cancers including breast cancer (Fujii et al, 1998) and may play a critical role in mammalian development (Carter et al, 2000). Analysis of the putative primary protein sequence suggests the gene product has five Kruppel-type Cys2-His2 zinc-fingers and an N-terminal protein interaction domain characteristic of a subset of zinc-finger transcription factors (Deltour et al, 1998; Guerardel et al, 1999). Nucleotide sequence analysis has revealed a consensus p53 binding site in the 5′ flanking region 4 kb upstream from the transcription start site and HIC-1 is postulated to be a potential downstream target of p53. Functional studies have demonstrated activation of HIC-1 transcription by exogenous p53 in a colon cancer cell line and transfection of the HIC-1 gene into cultured colon cancer cells was found to suppress cell growth (Makos-Wales, 1995).

Thus, while mutation or LOH of tumour suppressor genes are common events in breast cancer, silenced gene transcription associated with hypermethylation is an alternate mechanism to chromosomal deletion for the inactivation of tumour suppressor genes. The aim of the present study was to determine the frequency of HIC-1 transcription in a cohort of 50 breast cancers and to compare this event with data on 17p allelic loss, p53 expression and clinical/pathological variables including disease outcome (Makos-Wales et al, 1995) in the region with a high level of allelic loss in sporadic breast cancer (Thompson et al, 1998; Nagai et al, 1995).

**Materials and Methods**

Samples of primary breast cancer tissue were collected from 50 female Caucasian patients with a histological diagnosis of invasive carcinoma of the breast. All 50 patients were clinically stage T1-T3, N0-N1 and M0, previously untreated and underwent mastectomy or wide local excision of the cancer. Histological type, tumour size and nodal involvement were recorded prospectively on the basis of pathology findings; tumour grading was not performed. Tumour samples were handled identically and snap frozen in liquid nitrogen prior to storage at −80°C until analysis. Following accrual of laboratory data, statistically significant associations were sought between HIC-1 expression and clinical/pathological variables using a two-tailed Fisher’s exact test.

**Extraction of RNA**

RNA was extracted as previously described (Thompson et al, 1990). Briefly, 500 mg frozen tissue was pulverized and disrupted in 3M lithium acetate/6M urea (2 ml/100 mg tissue) and incubated at 4°C overnight. DNA was sheared using a Soniprep 150 ultrasonic disintegrator (MSE Scientific Instruments, Crawley, UK) and the RNA recovered by centrifugation at 12000 rpm. The pellet was resuspended in 6 ml 10 mM Tris.HCl buffer pH7 containing 0.1% sodium dodecyl sulphate, 50 µg/ml of proteinase K (Boehringer, Mannheim, FRG) and incubated at 37°C for 20 min. Protein was extracted with phenol equilibrated with 0.1 M Tris.HCl pH7 followed by chloroform:isoamyl alcohol (24:1). Following ethanol precipitation at −20°C the RNA was recovered by centrifugation, dissolved in RNase-free distilled water and stored in aliquots at −70°C. The quantity and purity of the RNA was assessed by spectrophotometry.

In addition, RNA from the human breast cancer cell lines MCF7 and MDAMB231 was also examined in this study. The cells were grown at 37°C in a humidified atmosphere containing 5% CO2, in Dulbecco’s modified Eagle’s medium (Life Technologies, Paisley, UK) containing 2 mM glutamine, 0.1 mM non-essential amino acids, 10% fetal calf serum, 1000 U/ml penicillin and 100 µg/ml streptomycin. Confluent cultures were harvested by aspiration, the cells washed with phosphate buffered saline and RNA isolated with an RNA extraction kit (Qiagen, Crawley, UK) according to the manufacturer’s instructions.

**RT-PCR**

Aliquots of RNA were digested with 10 U/µg RNase-free DNase (Boehringer) for 1 h at 37°C to remove contaminating genomic DNA. An equal amount of each RNA sample was also incubated under identical conditions in the absence of the enzyme. Both enzyme-treated and untreated samples were purified with an RNA purification kit (Qiagen) according to the manufacturer’s instructions.

1–5 µg of the RNA was reverse transcribed using oligo(dT)15 with superscript II reverse transcriptase (Life Technologies) according to the manufacturer’s instructions. HIC-1 transcripts were amplified in a Hybaid Touchdown thermal cycler from the cDNA in 50 µl AmpliTaq Gold buffer volumes containing 10% DMSO, 1.25U AmpliTaq Gold DNA polymerase (PE Biosystems), 200 µM of each dNTP, 2.5 mM MgCl2 and 1 µM of each primer. Primer sequences for HIC-1 (derived from Genbank Accession no. L41919) (Table 1) were designed to amplify a 242bp fragment from exon 2 of the HIC-1 gene. To activate the AmpliTaq Gold DNA polymerase the reaction mixture was incubated at 95°C for 12 min. Amplification conditions were 35 cycles of 95°C for 1 min (denaturation) and 72°C for 1 min (annealing and extension). PCR was also performed using primers specific for MDM2 and β-Actin as positive control reactions for successful cDNA synthesis (Table 1). MDM2 transcripts were amplified in a final volume of 50 µl AmpliTaq buffer containing 1.25U AmpliTaq (PE Biosystems), 200 µM of each dNTP, 1.5 mM MgCl2 and 1 µM of each primer. Amplification conditions were 95°C for 5 min (initial denaturation) followed by 35 cycles of 95°C for 1 min (denaturation), 65°C for
1 min (annealing) and 72°C for 1 min (extension). β-Actin transcripts were produced using RT-PCR control amplimers (Clontech) according to the manufacturer’s instructions. Following amplification, 5 μl of DNA loading buffer (bromophenol blue in 50% w/v glycerol) was added to each PCR reaction, PCR products were resolved on 1% agarose gels, containing 0.5 μg/ml ethidium bromide and visualized under UV illumination. A 1kb DNA ladder was used as size standards. PCR positive controls included the amplification of HIC-1 from genomic tonsil DNA, MDM2 from plasmid CMX-mdm2 containing full length human MDM2 cDNA (gifted from Christine Blattner) and human β actin control (Clontech). Negative controls consisted of all the components of the PCR excluding target sequences.

**p53 expression and 17p13.3 allele loss (LOH)**

From the same samples used for RT-PCR studies, total RNA for the 50 breast cancers was used for northern blots and p53 mRNA expression detected using the 2.1 kb cDNA clone php53Bam, the filters stripped then reprobed for alpha actin as internal control as previously described (Thompson et al, 1990). DNA was extracted from cancer tissue immediately adjacent to that used for the RNA studies and from the patient’s venous blood lymphocytes (Thompson et al, 1990). Lymphocytes were used in preference to apparently normal breast tissue given the adverse cosmetic impact exciting further breast tissue could have on breast conservation and the possibility of additional foci of cancer in mastectomy specimens. Allele loss (using a cut-off of 50% band strength) was detected using blood/tumour pairs for the 50 patients using Southern blots probed with YNZ22 which maps to 17p13.3 as previously described (Thompson et al, 1990).

**Reactivation of HIC-1 expression in MDAMB231**

MDAMB231 cells were seeded at 1 × 10^6 per 75-mm² flask in phenol red free Minimum Essential Medium (GibcoBRL) containing 2 mM glutamine, 10% fetal calf serum, 1000 U/ml penicillin and 100 μg/ml streptomycin. After 24 h the cells were incubated with freshly prepared medium containing 3 × 10⁻³ M 5-aza-2'-deoxycytidine (DeoxyC); (Sigma). DeoxyC-containing medium was replaced on days 5 and 8. The cells were harvested on day 10 for RNA extraction and RT-PCR as described above.

**RESULTS**

HIC-1 expression was detected in the MCF7 cell line and in 7/50 breast cancers (14%) by RT-PCR, but despite positive actin and MDM2 controls, no expression was detected in the MDAMB231 cell line nor in 43/50 (86%) of the cancers (Figure 1). The HIC-1 RT-PCR product was sequenced and the published HIC-1 sequence confirmed (data not shown). In the MDAMB231 cell line, constitutitional expression of beta actin and mdm2 genes was detected (Figure 2A) indicating the successful transcription of 1 min (annealing) and 72°C for 1 min (extension).

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**Table 1** Primer sets for HIC-1, MDM2 and β actin expression studies by RT-PCR

| Primer set | Sense primer | Antisense primer | Size bp |
|------------|--------------|-----------------|--------|
| HIC-1      | ATGGTGAGCCGCGGCCGTGTTCC | TAGCCGCCGCCGCCGCCGCCG | 242 |
| MDM2       | GGGAGATATGTTGTGAAA | AGATTCAACTTCAAATTC | 152 |
| β actin    | ATCTGGCACACACCTCTACA | CGTCTGATCCTGATGCTGATCC | 838 |
|            | ATGAGCTGCG       | ACATCTGC          |       |

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**Figure 1** HIC-1 expression in breast cancer cell lines MCF7, MDAMB231 and a human breast cancer. RT-PCR was used to detect HIC-1 mRNA expression (242 bp) and MDM2 expression (152 bp) in: (A) MCF7, (B) MDA-MB-231. (C) Breast cancer (patient number 38). +/− indicate whether reverse transcriptase (RT) was added to (+) or omitted from (−) the reaction. Lane 1–6: HIC-1 RT-PCR, Lane 7–12: MDM2 RT-PCR. Lane 1: DNase treated, Lane 2: DNase treated no RT, Lane 3: no DNase, Lane 4: no DNase, no RT, Lane 5: negative control for PCR reaction, Lane 6: positive control. Lane 7: DNase treated, Lane 8: DNase, no RT, Lane 9: no DNase. Lane 10: no DNase, no RT, Lane 11: negative control for PCR reaction, Lane 12: positive control.

**Figure 2** RT-PCR was used to detect actin mRNA (838 bp) expression in MDAMB231 (Figure 2A) and HIC-1 mRNA expression in DeoxyC treated MDAMB231 (Figure 2B). A Beta actin expression in MDAMB231. Lane 1: DNase treated, Lane 2: DNase treated no RT, Lane 3: negative control for PCR reaction. Lane 4: positive control, +/− indicate whether reverse transcriptase was added to (+) or omitted from (−) the reaction.

B Restoration of HIC-1 expression in MDAMB231. Lane 1: DNase treated, 5-aza-2’-deoxycytidine treated-MDAMB231: Lane 2: negative control for PCR reaction. Lane 3: positive control.
cDNA. Furthermore, HIC-1 expression was restored following treatment with the demethylating drug DeoxyC (Figure 2B). p53 mRNA expression was detected in 32/50 (64%) cancers, YNZ22 loss of heterozygosity (LOH) demonstrated in 21/50 (42%) with 16/50 (32%) patients constitutionally homozygous (Thompson et al, 1998).

Among the seven cancers where HIC-1 expression was detected using RT-PCR, 5/7 (71%) had detectable p53 expression which was wild type, (Thompson et al, 1992) (Table 2A) and 4/7 had YNZ22 LOH (3 were homozygous) (Table 2B). For the 43/50 cancers which tested negative for HIC-1 expression, 25/43 were positive for p53 expression and 17/43 (34%) had YNZ22 LOH (13 no loss, 13 homozygous).

All seven patients in whom cancer HIC-1 expression was detected were alive after 8 years follow-up (Table 3); 3/7 were oestrogen receptor (ER) positive and 1/7 was histologically node positive. For the remaining 43 patients in whom HIC-1 expression could not be detected, 28/43 (65%) were ER positive, 23/43 (54%) were node positive and 23/43 (54%) were alive without disease recurrence after 8 years follow-up (Table 3).

HIC-1 expression (detected in seven cancers) was associated with YNZ22 LOH ($P = 0.015$, two-tailed Fisher’s exact test) a result which concurs with that previously reported (Fujii et al, 1998). Given the link between YNZ22 LOH and poor prognosis in breast cancer (Thompson et al, 1998; Liscia et al, 1999) one might expect HIC-1 expression would correlate with poor prognosis. These differing associations suggest that HIC-1 may be distinct from a second gene involved in breast cancer marked by YNZ22. In addition HIC-1 may protect against the deleterious effects of YNZ22 LOH. Moreover, in our series, HIC-1 expression was not associated with p53 expression or with p53 mutation (Thompson et al, 1998) although activation of HIC-1 expression by p53 has been reported in colorectal cancer cell lines (Makos-Wales et al, 1995) and p53 mutation has been linked to HIC-1 methylation in four cancers (Fujii et al, 1998).

Regional DNA hypermethylation at 17p13.3 has been shown to precede 17p structural changes and is thus an early event in the progression of renal tumours (Makos et al, 1993) but, conversely, is a late event in haemopoietic malignancies (Issa et al, 1997). In breast cancer the hypermethylation of HIC-1 has been demonstrated in cultured neoplastic cells (Makos-Wales et al, 1995) although other mechanisms of HIC-1 inactivation such as mutation may occur.

The failure to detect HIC-1 transcripts in MCF7 cells in an earlier study (Makos-Wales et al, 1995) may be attributable to their use of a less sensitive assay (RNase protection) or a consequence of phenotypic divergence of the cell lines maintained under separate culture conditions in different laboratories. It has been reported that one HIC-1 allele is methylated in normal breast epithelium and that such hemimethylation predisposes to increased risk of breast cancer through mutation of the other allele (Fujii et al, 1998). In contrast, methylation of the HIC-1 CpG

### Table 2 A HIC-1 expression, p53 expression and B YNZ 22 allele loss

| Table 2 | A HIC-1 expression, p53 expression and B YNZ 22 allele loss |
|---------|-------------------------------------------------------------|
| A       | p53mRNA expression | Yes | No |
| HIC-1 expression | yes | 5 | 2 |
| HIC-1 expression | no | 25 | 18 |
| B       | Allele | No allele loss | Homozygous |
| HIC-1 expression | yes | 4 | 0 | 3 |
| HIC-1 expression | no | 17 | 13 | 13 |

### Table 3 HIC-1 expression and clinical/pathological parameters

| Table 3 | HIC-1 expression and clinical/pathological parameters |
|---------|------------------------------------------------------|
|         | ER positive | ER negative | Node positive | Node negative | Disease recurrence |
| HIC-1 Expression | Yes ($n = 7$) | 3 | 4 | 1 | 6 | 0 |
|             | No ($n = 43$) | 28 | 15 | 23 | 20 | 20 |
island extends to a sequence between the intron 2 and exon 3 junction in normal haemopoietic cells but occurs beyond this boundary in newly diagnosed acute myeloid leukaemias (Melki et al., 1999). Thus the extent of methylation, as opposed to methylation per se may be a more reliable indicator of gene inactivation. In the present study we have investigated breast cancer RNA directly by RT-PCR for evidence of HIC1 transcription.

Promoter-associated CpG islands can be chemically modified by methylation of cytosine that prevents expression of the gene. This could be of particular relevance in breast cancer as oestrogen receptor genes may also become hypermethylated (Issa et al, 1997; Li et al., 1998), a potential mechanism for resistance of oestrogen receptor positive breast cancers to endocrine therapy, and gene methylation of the oestrogen receptor increases with age (Li et al., 1998), as does breast cancer. Thus, altering the methylation status of cancer cells through pharmacological inhibition of DNA methyltransferase with resultant DNA demethylation (Issa et al, 1997) offers a novel therapeutic approach to reactivating tumour suppressor function of genes such as HIC-1, as we have demonstrated here with MDA-MB231, but may also be relevant to reactivating the function of what are currently therapeutically useful genes such as oestrogen receptor. Indeed, the demethylation effects on gene expression (and hence putative reactivation) by the drug 5aza-2′-deoxycytidine are undergoing further study in our laboratories. Immunohistochemical analysis of 5aza-2′- deoxycytidine treated MDA-MB231 cells has demonstrated an increased expression of p53, the proto-oncogene erbB2, and the oestrogen responsive protein pS2 as well as re-expression of HIC-1 (data not shown).

Thus, HIC-1 is a prime candidate gene in the development of sporadic breast cancer since it is located in a chromosomal region of 17p distinct from p53 that frequently undergoes LOH. However, the relative contributions of mutation, deletion and methylation leading to HIC-1 inactivation in breast cancer remain unclear. In addition, the putative role for HIC-1 as a tumour suppressor gene, the relationship to p53 and the clinical importance of HIC-1 (with potential for therapeutic attack) in breast cancer require further elucidation. Our data suggest that events on chromosome 17p13 including HIC-1 and p53 may be of more interest than hitherto expected.

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