# Methylation of the Homeobox Gene, *HOPX*, Is Frequently Detected in Poorly Differentiated Colorectal Cancer

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**Abstract.** *Background: Homeodomein only protein x (HOPX) gene methylation has frequently been detected in cancer tissues. The methylation status of the HOPX gene in colorectal cancer was examined and compared to the clinocopathological findings. Materials and Methods: Eighty-nine tumor samples and corresponding normal tissues were obtained from colorectal cancer patients who underwent surgery at our hospital. The methylation status of the HOPX gene in these samples was examined by quantitative methylation-specific PCR (qMSP). Subsequently, the clinicopathological findings were correlated with the methylation status of the HOPX gene. Results: HOPX gene methylation was found in 46 (52%) out of the 89 colorectal carcinomas, suggesting that it was frequently observed in colorectal cancer. A significant increase of methylation was observed in the poorly differentiated carcinomas (p=0.0049). Conclusion: HOPX gene methylation could play an important role for the development of colorectal cancer and is closely related to the histological type.*

There is now solid evidence that a series of genetic alterations in both dominant oncogenes and tumor suppressor genes are involved in the pathogenesis of human colorectal cancer. The activation of oncogenes such as the *RAS* gene, and the inactivation of tumor suppressor genes such as adenomatous polyposis coli (*APC*) and *p53*, have been identified in colorectal cancer (1-3). In addition, we have also found several other genes to be related to the pathogenesis of colorectal cancer (4-8). It is also known that gene promoter hypermethylation is involved in the development and progression of cancer (9). An investigation of genetic changes is important in order to clarify the tumorigenic pathway of colorectal cancer (10).

The homeodomain only protein x (*HOPX*) gene, also known as *NECC1* (not expressed in choriocarcinoma clone 1), *LAGY* (lung cancer-associated gene Y), and OB1 (odd homeobox 1 protein), was initially identified as an essential gene for the modulation of cardiac growth and development (11). Three spliced transcript variants, *HOPX-α, HOPX-β* and *HOPX-γ*, encode the same protein, which contains a putative homeodomain motif that acts as an adapter protein to mediate transcriptional repression (12). Only the *HOPX-β* promoter harbors CpG islands, which the frequency of the CG sequence is higher than other regions and 'p' simply indicates 'C' and 'G' are connected by a phpsphodiester bond, encompassing the first exon and intron (13). *HOPX* gene expression is ubiquitous in diverse types of normal tissue (14), but the molecular mechanisms responsible for the loss of *HOPX* gene expression are not very well known. The *HOPX* gene also acts as a tumor suppressor gene (15-16), however, the correlation between the *HOPX* gene and colorectal cancer remains unknown. Recently, it has been shown that *HOPX* gene expression is epigenetically silenced in gastric cancer (13).

In the present study, the methylation status of the *HOPX* gene was examined in primary tumors derived from 89 patients with colorectal cancer and the correlation between the *HOPX* gene methylation and the clinicopathological findings was evaluated.

## Materials and Methods

*Sample collection and DNA preparation.* Eighty-nine tumor samples were obtained at the time of surgical resection from patients with primary colorectal cancer at Showa University Fujigaoka Hospital from 2007 to 2008. All the tissue specimens were confirmed

*Abbreviations:* qMSP: quantitative methylation-specific PCR.

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*Key Words: HOPX*, colorectal cancer, gene methylation.

Table I. *Clinicopathological findings and methylation of HOPX gene in colorectal tumors.*

| Clinicopathological findings | Variable | No. of cases | *HOPX* gene methylation | | *p*-Value |
|---|---|---|---|---|---|
| | | | + | – | |
| Gender | Male | 49 | 24 | 25 | 0.5718[a] |
| | Female | 40 | 22 | 18 | |
| Age (years, mean±SD) | | 89 | 65.7±1.70 | 67.8±1.76 | 0.3799[b] |
| Maximal tumor size (mm, mean±SD) | | 89 | 51.0±4.25 | 46.4±4.39 | 0.4599[b] |
| CEA (ng/ml) | <5 | 53 | 26 | 27 | 0.5471[a] |
| | ≥5 | 36 | 20 | 16 | |
| CA19-9 (U/ml) | <37 | 71 | 35 | 36 | 0.3703[a] |
| | ≥37 | 18 | 11 | 7 | |
| Extent of tumor | <mt | 25 | 15 | 10 | 0.3266[a] |
| | mt ≤ | 64 | 31 | 33 | |
| Pathological type | Well | 72 | 32 | 40 | 0.0049[a] |
| | Poorly | 17 | 14 | 3 | |
| Tumor site | C, A, T | 38 | 20 | 18 | 0.8775[a] |
| | D, S, R | 51 | 26 | 25 | |
| Lymph node metastasis | + | 38 | 20 | 18 | 0.1497[a] |
| | – | 51 | 26 | 25 | |
| Liver metastasis | + | 11 | 6 | 5 | 0.8393[a] |
| | – | 78 | 40 | 38 | |
| Peritoneal dissemination | + | 7 | 3 | 4 | 0.6263[a] |
| | – | 82 | 44 | 39 | |
| Distant metastasis | + | 6 | 2 | 4 | 0.3516[a] |
| | – | 83 | 44 | 39 | |
| Dukes stage | A,B | 44 | 19 | 25 | 0.1124[a] |
| | C,D | 45 | 27 | 18 | |
| Total | | 89 | 46 | 43 | |

[a]Chi-square test; [b]Student's *t*-test; mt: muscular tunic; CEA: carcinoembryonic antigen; CA19-9: carbohydrate antigen 19-9; Well: well- or moderately differentiated adenocarcinoma; Poorly: poorly differentiated adenocarcinoma or mucinous carcinoma; C: cecum; A: ascending colon; T: transverse colon; D: descending colon; S: sigmoid colon; R: rectum.

histologically. Written informed consent, as required by the Institutional Review Board, was obtained from all the patients. All the tumors and corresponding normal tissue samples were immediately frozen and stored at –80˚C until analysis. The clinicopathological findings of the patients enrolled in the study are shown in Table I.

*Sodium bisulfite modification*. One microgram of the genomic DNA extracted from each tumor and corresponding normal colorectal tissue specimen was subjected to bisulfite treatment using an Epitect Bisulfite Kit (Qiagen, Hilden, Germany). Briefly, the DNA was denatured by NaOH and modified by sodium bisulfate. The DNA samples were then purified using the kit-attached column, again treated with NaOH, precipitated with ethanol and resuspended in water.

*Quantitative methylation-specific polymerase chain reaction (qMSP)*. The bisulfite-treated DNA was amplified with qMSP, using a Thermal Cycler Dice® Real-Time System TP800 (Takara Bio Inc., Otsu, Japan). Thermocycling was performed in a final volume of 25 μl containing 1.0 μl of the DNA sample, 100 nM each of the *HOPX* gene or β-actin primers (forward and reverse) and 12.5 μl of SYBR Premix Ex Taq II (Takara Bio Inc.), which consists of Taq DNA polymerase, reaction buffer and a deoxynucleotide triphosphate mixture. The *HOPX* gene primers for qMSP have been described elsewhere (13) and were: *HOPX* MS (sense), 5'-TTTGGAG AGGGTTTTAAAGCG-3', and *HOPX* MAS (antisense), 5'-AAC AAACTTAACAAATCGCGAA-3'. The PCR amplification consisted of 40 cycles (95˚C for 5 s and 55˚C for 30 s) after an initial denaturation step (95˚C for 10 s). The bisulfite-treated DNA obtained from L132 cells that were fully methylated by *Sss*I methylase was used as a positive control. To correct for differences in both quality and quantity between samples, β-actin was used as an internal control. The targets were obtained from the same bisulfite-treated DNA.

*HOPX methylation score*. The relative levels of *HOPX* gene-methylated DNA in the colorectal tumors and the corresponding normal tissues that were normalized to the internal control β-actin were calculated. The *HOPX* gene methylation score in each tissue was defined as follows: relative level of methylated *HOPX* gene in tumor/relative level of methylated *HOPX* gene in all the corresponding normal tissues. *HOPX* gene methylation was defined as being positive when the methylation score was more than 3.0.

*Statistical analysis*. The associations between *HOPX* gene methylation and clinicopathological findings were analyzed using Chi-square tests or Student's *t*-test. A $p$-value<0.05 indicated statistical significance.

## Results

Methylation was found in 46 (52%) of the colorectal carcinomas.

Correlation of the clinicopathological findings with the methylation status of the *HOPX* gene are shown in Table I. No significant correlations were observed between the presentation of methylation and patients' gender, age, maximal tumor size, tumor markers, tumor extent, tumor site, lymph node metastasis, liver metastasis, peritoneal dissemination or distant metastasis. A significant increase of methylation was observed in the poorly differentiated compared to the well-differentiated carcinomas ($p$=0.0049).

## Discussion

Colorectal cancer, one of the most aggressive carcinomas, occurs with a high incidence in most countries (17) and is treated by resection and then with chemotherapy and radiotherapy. The determination of genetic alterations as a new parameter for assessment might help in the treatment of such tumors. Colorectal carcinomas are classified histopathologically as either differentiated carcinomas forming tubular or papillary structures, or poorly differentiated carcinomas, including mucinous adenocarcinoma, in which such structures are inconspicuous.

Poorly differentiated colorectal carcinomas are quite rare, comprising only 3-5% of all colorectal carcinomas. It is well known that mucinous carcinoma is frequently observed in colorectal cancer with genetic instability, but the difference in genetic pathways between these histological types is mostly unknown because of the very small number of cases (18). In this study, *HOPX* methylation was frequently observed in colorectal cancer and was closely related to the histological type. Most (82%) of the poorly differentiated colorectal carcinomas presented *HOPX* gene methylation, while only 44% of the well-differentiated tumors did so. We previously found a significant difference ($p$=0.0053) when the *CDH13* (H-cadherin) methylation of poorly-differentiated colorectal carcinomas was compared to that of better differentiated tumors (7). These results suggested that inactivation of the *HOPX* gene, as well as the *CDH13* gene, could play an important role in directing colorectal tumors towards poor differentiation.

Recent studies have shown that it is possible to reverse epigenetic changes and restore gene function to a cell. Treatment with DNA methylation inhibitors might restore the activities of the *HOPX* gene and reduce the growth rate of cancer cells. The administration of drugs such as cytosine analogs might soon enable the functional restoration of these tumor suppressor genes and slow the rate of colorectal cancer progression.

## Acknowledgements

We would like to thank M. Ogata for her technical assistance.

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*Received April 20, 2011*
*Revised June 22, 2011*
*Accepted June 23, 2011*