Detection of promoter hypermethylation in serum samples of cancer patients by methylation-specific polymerase chain reaction for tumour suppressor genes including *RUNX3*

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Abstract. The purpose was to validate the use of *RUNX3* as a potential biomarker for detection of cancer in serum samples and to determine its sensitivity alone and in combination with *p16*, *RASSF1A* and *CDH1* using methylation-specific polymerase chain reaction (MSP). We examined the promoter methylation status of *RUNX3*, *p16*, *RASSF1A* and *CDH1* by MSP using the serum of 70 metastatic breast, non-small cell lung, gastric, pancreatic, colorectal or hepatocellular carcinomas. The DNA from 10 healthy serum controls was used to determine the specificity of methylation. According to our results, promoter hyper-methylation of *RUNX3* was detected in the serum of 44 patients comprising breast 9/19 (47%), non-small cell lung 11/20 (55%), gastric 4/4 (100%), pancreatic 2/2 (100%), colorectal 11/17 (65%) and liver 7/8 (88%) carcinomas. Comparative figures for the other genes were as follows: *p16* - 39/70 (7/19, 10/20, 2/4, 0/2, 12/17, 8/8); *RASSF1A* - 24/70 (8/19, 6/20, 1/4, 1/2, 4/17, 4/8); CDH1 - 10/70 (0/19, 4/20, 1/4, 1/2, 3/17, 1/8). Using a panel of four genes, hypermethylation of one or more genes was found in 62/70 samples (15/19, 19/20, 4/4, 2/2, 14/17, 8/8). A panel of three genes omitting *RUNX3* detected hyper-methylation in only 50/70 samples. No methylation was detected in the 10 healthy serum controls. Thus, *RUNX3* can be detected in the serum of a high proportion of advanced cancers. This suggests that serum hypermethylation of *RUNX3* is at least as, or possibly more sensitive a marker, than other tumor suppressor genes currently under investigation. Inclusion of *RUNX3* in gene panels can potentially increase the sensitivity of such panels for serum diagnosis of malignancies and warrants further study.

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Introduction

There has been considerable interest recently in the area of epigenetic changes contributing to the process of carcinogenesis. Although it has been evident for years that germ line or somatic mutations can enhance the function of oncogenes or down-regulate the activity of tumor suppressor genes leading to the development of cancer, investigations have shown the presence of epigenetic changes in driving the development of tumors from the earliest to the latest stages (1). Molecular evidence of aberrant methylation of cytosine in promoter CpG islands causing transcriptional silencing of vital tumor suppressor genes in cancer cells has come to be recognized as an important feature of human cancer (2,3). The methylation of tumor suppressor gene promoters contributes directly to the progression of some cancers (4). DNA methylation markers provide a unique combination of specificity, sensitivity, high information content and applicability to a wide variety of clinical specimens. Methylation markers are particularly suited for situations where sensitive detection is necessary, such as when tumor DNA is either scarce or diluted by excess normal DNA.

One of the most widely used methods for measuring methylation levels is methylation-specific polymerase chain reaction (MSP). MSP requires only a small quantity of DNA and is sensitive to 0.1% methylated alleles of a given CpG island locus (5). PCR-based methylation assays have been applied to the detection of tumor DNA in a variety of tissues and body fluids including serum, plasma, urine, sputum, stool, bile juice and lavage fluids (6). As current strategies for cancer detection are often costly, invasive and not well-defined, there remains a need to develop a reliable non-invasive test, preferably a simple venepuncture, for diagnostic purposes as well as for possible therapeutic monitoring. Hence using serum

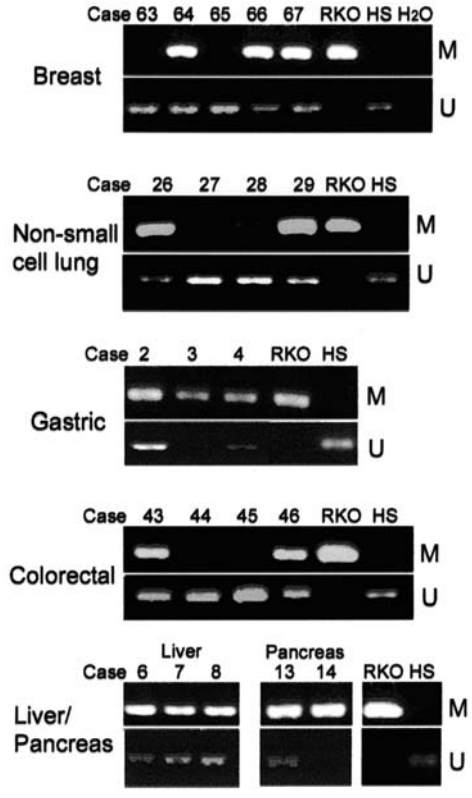

Figure 1. Representative gel electrophoresis pictures demonstrating aberrant methylation in breast, lung, gastric, liver, pancreas and colon serum samples. Lanes M and U; amplified product with primers recognizing methylated and unmethylated sequences respectively. DNA from RKO cell line and healthy serum (HS); positive control for methylated and unmethylated forms respectively.

for this purpose is ideal. Furthermore, the ability of serum from blood samples to be re-used after storage is advantageous.

The presence of tumor DNA in serum was reported more than two decades ago (7). Early studies have shown the presence of *K-ras* and *p53* mutations in the serum of patients with colorectal, pancreas and breast cancer (8-10). More recently, the presence of gene promoter hypermethylation in serum DNA has also been demonstrated in breast, gastric, lung and liver carcinomas (11-14). In recent years, a CpG island hypermethylation profile of human primary tumors has emerged showing differential methylation dependent on tumor type (15,16).

In this study, we sought to determine the feasibility and clinical correlations of detecting gene promoter hypermethylation utilizing the serum of patients with a variety of malignancies, namely breast, lung, gastric, pancreas, colorectal and liver, mainly focusing on the as yet unstudied tumor suppressor gene, *RUNX3*.

Inclusion of 3 other tumor suppressor genes which have been shown reliably in other studies to be down-regulated by methylation, namely *p16* (14,17,18), *RASSF1A* (19-21) and *CDH1* (22,23), was used to aid in confirming the presence and improving the sensitivity of tumor DNA detection.

Materials and methods

Sample collection. We examined 70 serum samples from patients who had histopathological evidence of cancer and radiological evidence of metastatic disease. Nineteen had breast, 20 had non-small cell lung, 4 had gastric, 2 had pancreatic, 17 had colorectal and 8 had confirmed hepatocellular carcinomas (HCCs). For the group of HCCs, they were diagnosed via histology or on a presumptive diagnosis based upon the following criteria (a) an α-fetoprotein (AFP) level >500 ng/dl (b) prior computed tomography or magnetic resonance imaging scans of the liver consistent with HCCs. Peripheral blood (5 ml) was obtained at the time of diagnosis before chemotherapy or any other form of treatment was instituted for >50% of the samples. For the remaining samples, their last cycle of chemotherapy was administered at least a month earlier, they were diagnosed radiologically to have progressed since then and were currently on conservative management. Serum samples from ten normal, healthy patients with no known cancer were used as controls. The study protocol was approved by the Institutional Review Board and all patients gave informed consent for the collection of blood samples. Serum was isolated by centrifugation at 3000 rpm and 4˚C for 10 min and stored at -80˚C until further processing.

DNA extraction. Genomic DNA from these samples was isolated using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) with certain modifications. 200 μl of sample was used together with 40 μl of Proteinase K, 200 μl of PBS and 1 μg of salmon sperm DNA. After incubating at 56˚C for 1 h, the genomic DNA was eluted using 100 μl of 0.5X AE buffer (Qiagen). To enhance efficacy, the DNA extraction was carried out 6 months or earlier after sample collection.

Bisulfite treatment and MSP. The extracted DNA was treated with sodium bisulfite using the CpGenome DNA Modification kit (Chemicon, Temecula, CA), converting all unmethylated, but not methylated, cytosine to uracil. All bisulfite-modified DNA was resuspended in 20 μl of TE buffer [10 mmol/l Tris-0.1 mmol/l EDTA (pH 7.5)] and stored at -20˚C until subsequent MSP. PCR primers distinguishing between methylated and unmethylated DNA for *RUNX3* were used, the primer sequences being S (sense): 5'-GCGGTAAGATGGG CGAGAATA-3', AS (antisense): 5'-CACGAACTCGCCTA CGTAATC-3' and S: 5'- TGGTAAGATGGGTGAGAATA3', AS: 5'-CACAAACTCACCTACATAATCC-3' respectively. The primer sequences of *p16* (5), *RASSF1A* (24) and *CDH1* (25), annealing temperatures and the expected PCR product sizes had all been previously studied and can be found in the report referenced after each gene. For PCR amplification, 2.5 μl of bisulfite-modified DNA was added in a final volume of 25 μl of PCR mixture containing 10X PCR buffer, 10 mM each deoxynucleotide triphosphates, 10 μM each primers and 0.125 μl each of HotStarTaq (Qiagen). PCR amplification of template DNA was performed under the following conditions: 95˚C for 10 min; 55 cycles (50 cycles only for *RUNX3*) of 95˚C for 15 sec, the specific annealing temperature for each gene (60˚C/56˚C for methylated/unmethylated *RUNX3*) for 30 sec and 72˚C for 20 sec. PCR products (5 μl) were loaded onto a 2% non-denaturing agarose gel together with a 100-bp ladder, stained with ethidium bromide and visualized under UV illumination. The sample was scored as methylated when there was a visible band on the gel with methylated primers and

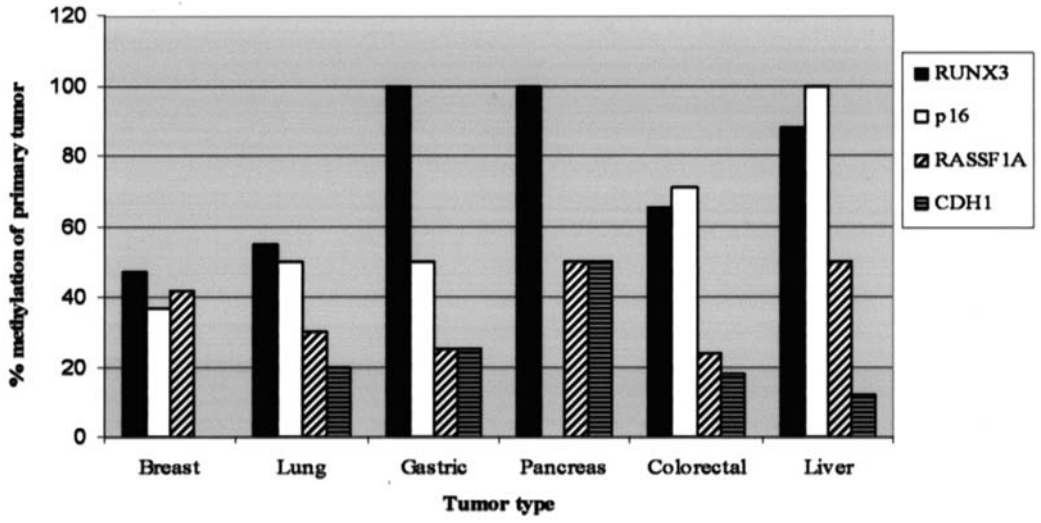

Figure 2. Methylation detection data of the various tumor types for *RUNX3*, *p16*, *RASSF1A* and *CDH1*.

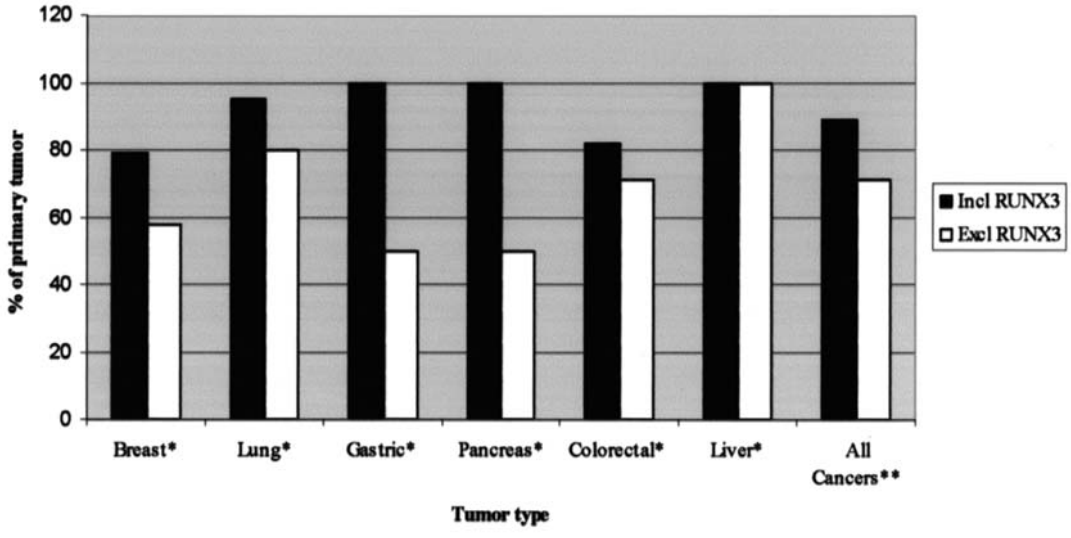

Figure 3. Percentage of 'each individual cancer' and 'all cancers' having at least one hypermethylated gene using *p16*, *RASSF1A* and *CDH1* including or excluding *RUNX3*. *p-value not significant for association of hypermethylation for *p16*, *RASSF1A* and *CDH1* with or without *RUNX3*, **p=0.01 for association of hypermethylation for *p16*, *RASSF1A* and *CDH1* with or without *RUNX3* considering all cancers collectively.

similarly for unmethylated primer sets. Representative results of methylation analysis by MSP for *RUNX3* are shown in Fig. 1.

Statistical analysis. The methylation status of the genes were tested for association with gender, smoking history and anatomical sites of metastases using the Chi-square test, while differences in sensitivity of cancer detection rate with and without *RUNX3* was analyzed by a 2-sample proportion test (SPSS 14.0, SPSS Inc.). The number of methylated genes were also assessed for correlation with age and survival duration of the patients using polyserial correlation coefficient (LISREL 8.72, Scientific Software International, Inc.). All tests were conducted at 5% significance level.

Results

Patient and tumor characteristics. A total of 70 peripheral blood samples were collected from patients with metastatic breast, non-small cell lung, gastric, pancreatic, colorectal and hepatocellular cancers from December 2004 to May 2006 under the Institutional Review Board guidelines. In total, we obtained 19 breast, 20 non-small cell lung, 4 gastric, 2 pancreatic, 17 colorectal, and 8 liver serum samples. Of the patients 38/70 (54%) were chemonaive at the point of blood sampling and 32/70 (46%) were free from chemotherapy for at least a month and had documented progressive disease since then.

Methylation status of four genes. The incidence of promoter hypermethylation of *RUNX3* was detected in the serum of 44/70 (62.9%) of patients; comparatively for *p16*, 39/70 (55.7%); *RASSF1A* 24/70 (34.3%) and *CDH1* 10/70 (14.3%). The frequency of hypermethylation of an individual gene in relation to individual cancer groups are summarized in Fig. 2 and Table I. Using a panel of four genes, aberrant methylation in one or more tumor-related genes was detected in the serum of 62/70 (88.6%) cancer patients. A panel of only three genes

Table I. Frequency of promoter hypermethylation of *RUNX3*, *p16*, *RASSF1A* and *CDH1* in sera (n=70).

Gene	Breast (n=19)	Non-small cell lung (n=20)	Gastric (n=4)	Pancreas (n=2)	Colorectal (n=17)	Liver (n=8)
RUNX3	9 (47%)	11 (55%)	4 (100%)	2 (100%)	11 (65%)	7 (88%)
p16	7 (37%)	10 (50%)	2 (50%)	0	12 (71%)	8 (100%)
RASSF1A	8 (42%)	6 (30%)	1 (25%)	1 (50%)	4 (24%)	4 (50%)
CDH1	0	4 (20%)	1 (25%)	1 (50%)	3 (18%)	1 (12%)

omitting *RUNX3* detected hypermethylation in only 50/70 (71.4%) of the samples. When comparing the sensitivity of the cancer detection rate with and without *RUNX3*, considering all the samples together regardless of cancer types, the percentage of patients having at least one hypermethylated gene using only *p16*, *RASSF1A* and *CDH1* was significantly different from that of including *RUNX3* to the panel of genes (p=0.01). This was not observed in the individual cancer types, which was probably due to the small sample size (Fig. 3). No methylated templates of the four genes were detected by MSP in all 10 healthy sera. There were 2 non-specific bands for healthy serum for *RASSF1A*.

Correlations with clinical parameters. We correlated the methylation status of each of the genes with clinical characteristics from all the cancer patients. There was no significant association between the methylation status of each gene and clinical parameters including age, gender, distant metastases, smoking history and survival.

Discussion

The abnormal hypermethylation patterns of promoter site DNA causing gene silencing in cancer cells plays a pivotal role in tumorigenesis. The importance of promoter hypermethylation has been illuminated by the ability of demethylation drugs such as 5-azacytidine to reactivate the affected genes and restore production of the corresponding protein in cultured cancer cells (26).

One of the most widely used techniques for detecting and measuring methylation levels is MSP of which the level of sensitivity of conventional MSP can reach 1:1000 (5). Methylated serum DNA is a useful marker for cancer detection as serum is a readily accessible body fluid. DNA unlike mRNA is a stable molecule offering greater test stability and DNA methylation can be analyzed quantitatively. The mechanism leading to the presence of free tumor DNA in the serum remains unclear but may be related to cellular turnover, necrosis or apoptosis (27). On the other hand, serum tumor DNA may also originate from cells that have left the primary site and invaded the circulation, but are not yet capable of distant site metastases (28). However, aberrant methylation in the serum DNA has been reported to be detected more frequently in those who have developed distant metastases (28).

In recent years, the growing list of genes silenced by hypermethylation has given us ample opportunity to examine their role in various malignancies. In this present study, we determined the frequency of promoter hypermethylation, mainly focusing on *RUNX3,* as it had not been studied in serum yet. We included 3 other established genes; *p16*, *RASSF1A* and *CDH1* which have shown moderately high methylation frequencies in previous studies as detailed below. Epigenetic inactivation of these different genes may affect all of the molecular pathways involved in cell immortalization and transformation (15).

RUNX3 has a current and relevant role in carcinogenesis. Transforming growth factor-ß (TGF-ß) is a multifactorial growth factor that is crucial in many developmental and physiological processes. Polyomavirus enhancer binding protein/core binding factor (PEBP2/CBF) is a heterodimeric transcription factor composed of α and ß subunits and forms an important target for TGF-ß superfamily signaling essential for mammalian development (29). The α subunit contains a conserved region known as the *Runt* domain required for DNA binding and dimerization with the ß subunit. Three mammalian *runt*-related α subunit genes exist, namely *RUNX1/AML1*, *RUNX2/CBFA-1* and *RUNX3/PEBP2αC*, the former 2 genes being essential for granulocytic differentiation and osteogenesis respectively (29-31). Each *RUNX* forms complexes with Smad2 and Smad3 that transmit TGF-ß/activin signals (32) .

RUNX3 gene is located on human chromosome 1p36, where high frequency loss of heterozygosity (LOH) has been detected in various cancers. Thus *RUNX3* may be a tumor suppressor candidate in cancer development. This relationship between *RUNX3* and carcinogenesis has already been extensively studied in the context of gastric cancer where it was found that 45-60% of human gastric cancer cells do not express *RUNX3* due to hemizygous deletion and hypermethylation of the *RUNX3* exon 1 region (31). Tumorigenicity of human gastric cancer cell lines was found to be inversely related to the level of *RUNX3* expression, a mutation (R122C) within the Runt domain abolishing the tumor-suppressive effect of *RUNX3* (31). These results demonstrate that *RUNX3* plays a role as a tumor suppressor gene in gastric carcinogenesis. Furthermore, *RUNX3* expression has also been reported to be lost in the range of 19-50% of lung cancer cell lines and tissue samples (33-35), 70% of bile duct cancer cell lines, 75% of pancreatic cancer cell lines (36), 40-80% of liver cancer cell lines and tissue (37,38) and 50% of breast cancer tissue (39) due to hypermethylation of the CpG islands. Sporadic colon cancer associated with *RUNX3* methylation has also been reported in approximately 30-50% of tissue samples (40). The frequency of *RUNX3* in cancer serum has not been reported.

These data suggest that *RUNX3* plays an important part in various types of cancers. Based on these promising results, we thought that methodogically it would be an ideal approach to analyze *RUNX3* with blood samples of patients with cancer. With regards to technological issues such as sample preparation, assay design and primer selection, we have attempted to improve and optimize the conditions in order to obtain the best possible experimental conditions. Our results were comparable with methylation frequencies obtained from cell lines and tissue samples in the other studies as previously mentioned. The higher than expected percentages for gastric and pancreatic patients were possibly due to small sample sizes.

Since its discovery as a cyclin-dependent kinase inhibitor (CDKI) in 1993, the tumor suppressor *p16* located on chromosome 9p21 has gained widespread importance in cancer. Loss of function of *p16* occurs in at least three ways: homozygous deletion, methylation of the promoter and point mutation (the first two mechanisms comprising the majority of inactivation events in most primary tumors). *p16* is a major target in carcinogenesis, rivaled in frequency only to that of the *p53* tumor suppressor gene. Its mechanism of action as a CDKI has been elucidated and involves binding to and inactivating the cyclin-dependent kinase 4 (or 6) complex and thus rendering the retinoblastoma protein inactive. This effect blocks the transcription of important cell-cycle regulatory proteins and results in cell-cycle arrest (41). Our results for lung, gastric, colorectal and liver were comparable to, or even better in the case of breast, to previous quoted percentages (13,14,42-44). The small sample number for pancreatic cancer specimens possibly limited our ability to adequately detect methylation.

A new 3p21.3 tumor suppressor gene, the *Ras* association domain family 1A gene (*RASSF1A*) from a minimal 120 kb region defined by overlapping homozygous deletions in lung and breast cancers has also been recently identified (19,45,46). *RASSF1A* may participate in the DNA damage response or in DNA damage-induced regulation of other cell signaling events (45,47). In our study, we observed a methylation rate of ~20-50% in all our cancer groups.

CDH1 (*E-cadherin*) is localized on chromosome 16q22 and down-regulation via methylation of the promoter region is observed in a wide variety of tumors from epithelial cells, hence its role in promoting cellular carcinogenesis. *E-cadherin* mediates cell-cell adhesion by association with intracellular molecules of α, ß, and γ-catenins (48). Reduction of *E-cadherin* induces cell mobility and promotes tumor cell invasion (49). In this study, we generally did not find a high proportion of methylation among our specimens. Although we did not detect any *CDH1* methylation for the breast samples, 15/19 (79%) had methylation of at least one of the three remaining genes.

It is important to note that certain tumor suppressor genes taken alone such as *RUNX3* or *p16* in hepatocellular carcinoma appear to give good detection frequencies in our study. Although this is suggestive of a possible histopathological correlation, further studies which include a panel of methylated markers are needed in order to increase the specificity.

Using *RUNX3* as a candidate gene in our panel increased the methylation detection frequency by 17%. Although further evaluation is essential in a larger study, the results of this study indicate the substantial usefulness of *RUNX3* in combination with other tumor suppressor genes for the detection of various malignancies as it appears to increase the sensitivity over and above other more well-established tumor suppressor genes. It has been shown that the loss of expression of *RUNX3* increases from the early stages of gastric carcinomas from 40% to almost 90% as cancer stage progresses (31). Thus MSP for *RUNX3* may also prove to be a powerful diagnostic and prognostic tool for early detection of neoplastic and pre-neoplastic states for example intestinal metaplasia and liver cirrhosis in the future. Potential therapeutic applications are also possible, such as reactivation of *RUNX3* tumor suppressor genes in established tumors. Therefore, there is scope for further studies into the utility of *RUNX3* as a promising diagnostic and prognostic marker as well as its potential clinical applications in the future.

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