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SHORT REPORTS

Fragile genes as biomarkers: epigenetic control of WWOX and FHIT in lung, breast and bladder cancer

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This study aimed to (a) determine if DNA methylation is a mechanism of WWOX (WW domain containing oxidoreductase) and FHIT (fragile histidine triad) inactivation in lung, breast and bladder cancers; (b) examine distinct methylation patterns in neoplastic and adjacent tissues and (c) seek correlation of methylation patterns with disease status. Protein expression was detected by immunohistochemistry, and methylation status by methylation-specific PCR (MSP) and sequencing, in lung squamous cell carcinomas and adjacent tissues, invasive breast carcinomas, adjacent tissues and normal mammary tissues and bladder transitional cell carcinomas. Wwox and Fhit expression was reduced in cancers in association with hypermethylation. Differential patterns of WWOX and FHIT methylation were observed in neoplastic vs adjacent non-neoplastic tissues, suggesting that targeted MSP amplification could be useful in following treatment or prevention protocols. WWOX promoter MSP differentiates DNA of lung cancer from DNA of adjacent lung tissue. WWOX and FHIT promoter methylation is detected in tissue adjacent to breast cancer and WWOX exon 1 MSP distinguishes breast cancer DNA from DNA of adjacent and normal tissue. Differential methylation in cancerous vs adjacent tissues suggests that WWOX and FHIT hypermethylation analyses could enrich a panel of DNA methylation markers.
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WWOX (WW domain containing oxidoreductase) is located at a common fragile region, *FRA16D*, on chromosome 16q23.3 (Bednarek *et al*., 2000) and exhibits genomic alterations in lung, breast, ovary and esophageal cancers (Paige *et al*., 2001; Driouch *et al*., 2002; Kuroki *et al*., 2002). Point mutations in the gene are infrequent but deletions occur frequently (Bednarek *et al*., 2000, 2001; Yendamuri *et al*., 2003). A recent study showed reduced Wwox expression in 63% of invasive breast tumors and 33% of adjacent normal tissues and Wwox and Fhit expression was strongly correlated, suggesting that genes at common fragile sites are likely to be coordinately inactivated in cancer (Ishii and Furukawa, 2004; Guler *et al*., 2004). The fragile histidine triad (*FHIT*) gene at chromosome 3p14.2 encodes a tumor suppressor and undergoes frequent hemi- and homozygous deletion in various types of cancer (Ohta *et al*., 1996; see for a review Huebner *et al*., 1998). Expression of Fhit protein is reduced or absent in the majority of human cancers (reviewed in Huebner and Croce, 2003). It has been shown that Fhit expression is lost in most lung SCCs, especially those from tobacco smokers (Sozzi *et al*., 1998), and is reduced in invasive breast carcinomas (Ingvarsson *et al*., 1999; Guler *et al*., 2004) and in transitional cell carcinomas of the bladder (Baffa *et al*., 2000; Skopelitou *et al*., 2001).

The absence of point mutations in *WWOX* and *FHIT* genes in cancers with highly reduced protein levels suggests that other mechanisms, in addition to deletions, might regulate their expression. Methylation of CpG islands, in 5′ regulatory regions of genes, has been associated with transcriptional inactivation of a number of suppressor genes in lung, breast and bladder cancers. Indeed, previous studies have shown that methylation is a mechanism of *FHIT* inactivation in breast (Gatalica *et al*., 2000), bladder (Maruyama *et al*., 2001) and non-small lung cancers (Zochbauer-Muller *et al*., 2001; Kim *et al*., 2004). A recent study showed that WWOX promoter hypermethylation was detected in pancreatic cancer cell lines and primary tumors and treatment with the demethylating agent 5-aza-2′-deoxycytidine caused an increase in Wwox expression (Kuroki *et al*., 2004).

We have been interested in mechanisms that control fragile gene expression and inactivation in human cancers and have assessed lung, breast and bladder cancers for control of expression of these genes by methylation of 5′ CpG islands. Frozen lung and breast tumors, adjacent non-neoplastic and normal tissue

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samples, and corresponding fixed archived tissues were used in this study. Specifically, 24 lung squamous cell carcinomas and corresponding noncancerous adjacent tissues, 28 invasive breast carcinomas, adjacent non-neoplastic mammary tissues and 26 normal mammary tissues were obtained from the Kimmel Cancer Center Pathology Core Facility at Thomas Jefferson University, after approval by the Institutional Review Board. DNAs and unstained paraffin sections from 27 transurethrally resected transitional carcinomas (TCCs) of the bladder and seven non-neoplastic urinary bladder tissues were obtained from the Department of Urology, Thomas Jefferson University. Peripheral blood from a healthy donor served as an internal control.

Wwox and Fhit expression

We investigated Wwox and Fhit expression levels in the three types of cancer in a subset of our samples in order to verify accordance with previous results (Baffa *et al*., 2000; Yendamuri *et al*., 2003; Guler *et al*., 2004). Slides from archived tissues were available for a subset of the neoplastic and non-neoplastic tissues, including six lung tumors and adjacent tissues, five breast cancers and seven bladder TCCs. The lung tumors, three squamous and three adenosquamous (ASCCs) cell carcinomas, showed highly reduced Fhit expression (see, Figure 1a and b, for examples and Table 1 for summary); Wwox expression was moderately reduced in two and highly reduced in four cases (Figure 1c, for example). Wwox and Fhit exhibited similar patterns in adenosquamous tumors; in all three, the adenocarcinomatous differentiation regions showed stronger staining (Figure 1b). In adjacent normal lung tissue, bronchial epithelial cell layers and alveolar macrophages showed strong expression of both Wwox and Fhit (Figure 1d). Five invasive ductal carcinomas (IDCs) of the breast were evaluated for Fhit and Wwox expression. One tumor showed highly reduced Fhit expression (Figure 1e), two had moderately reduced (Figure 1f) and two cases showed strong expression of Fhit (summary in Table 1). Wwox was moderately reduced in four breast cancers (Figure 1g, for example) and strongly positive in one (Figure 1h). Among seven bladder TCCs, three showed highly reduced (Figure 1i), three moderately reduced (Figure 1j) and one case strong expression of Wwox (Figure 1k). There was adjacent normal transitional epithelium in the same sections in four cases; normal epithelium showed strong cytoplasmic Wwox expression in all four (Figure 1l, for example). Fhit was highly reduced in three bladder cancers, moderately reduced in two and highly expressed in two (reported in Skopelitou *et al*., 2001; summarized in Table 1).

FHIT and WWOX CpG islands

Epigenetic alterations of chromatin, such as DNA methylation and histone modification, are associated with transcriptional inactivation of many tumor suppressor genes (Tanaka *et al*., 1998; Esteller *et al*., 1999a, b; Yang *et al*., 2002). The methylation pattern of the FHIT 5′ CpG island was described by Tanaka *et al*. (1998) in a study showing that the region between nucleotides 195 and 283 (intron 1) is sensitive to methylation. Ensuing studies of FHIT methylation in non-small lung, breast and bladder cancers (Maruyama *et al*., 2001; Zochbauer-Muller *et al*., 2001) focused on assessment of methylation of intron 1 CpGs by MSP.

There have not been detailed studies of WWOX transcriptional regulation by epigenetic mechanisms, including CpG island methylation. Bednarek *et al*. (2000) suggested the presence of a CpG island in exon 1 of the *WWOX* gene. We used an interval including 1 kb upstream and downstream from the transcription initiation site for *in silico* CpG island identification (using the CpG plot program http://www.ebi.ac.uk/emboss/cpgplot) with the following parameters: 100 bp length, 50% C + G and 0.6 observed/expected ratio). A region 406 bp upstream of the *WWOX* transcription start site, exon 1 and a part of intron 1 (the first 125 bp) exhibited 66% C + G residues; specifically, the region from −183 to + 177 has the highest percentage (~70%) of CpG sites.

WWOX and FHIT methylation status

Genomic DNA was extracted from tissues and bisulfite modification of DNA was performed as reported

Figure 1 Immunohistochemical detection of Fhit and Wwox expression in lung, breast and bladder tissues. Immunohistochemical studies were performed as described using rabbit polyclonal anti-Fhit (Zymed, S. San Francisco, CA, USA) and anti-Wwox as primary sera (Guler *et al*., 2004). Detection was with strepavidin–biotin complex, using the LSAB2 system (DAKO) with diaminobenzidine as chromogen. In evaluation of Fhit and Wwox staining, intensity and extent of staining were scored. Intensity was graded as: 1, loss of staining, 2, moderate staining and 3, intense staining. Extent of staining was graded as: 1, <10%; 2, 11–25%; 3, 26–50%; 4, 51–75%; 5, 76–100%. Total staining scores were calculated by multiplying intensity and extent scores. Cases with staining scores between 12 and 15 were considered without loss, scores of 6–11, moderately reduced and 1–5 scores, and highly reduced expression. (**a**) Highly reduced Fhit staining in SCC of lung; (**b**) in an adenosquamous carcinoma, only tumor areas with adenoid differentiation showed strong cytoplasmic Fhit reaction, and the squamous portion exhibited highly reduced expression; (**c**) reduced expression of Wwox in lung SCC; (**d**) Wwox expression in normal lung tissue, and bronchial epithelial cells and alveolar macrophages showed strong cytoplasmic expression; (**e**) highly reduced Fhit staining in breast IDC, and residual normal ductal structures showed strong cytoplasmic reaction; (**f**) moderately reduced Fhit staining in an invasive breast tumor with both ductal and lobular features; (**g**) moderately reduced Wwox reaction in the same tumor, and both normal ductal structures showed strong cytoplasmic reaction in the luminal epithelial layer; (**h**) strong Wwox expression in an IDC, and adjacent normal breast lobules showed strong cytoplasmic expression; (**i**) highly reduced; (**j**) moderately reduced and (**k**) strong cytoplasmic Wwox expression in bladder TCC; (**l**) strong cytoplasmic expression of Wwox in adjacent normal epithelium of bladder

(Herman *et al*., 1996). Modified DNA was purified using Wizard DNA purification resin (Promega Corp., Madison, WI, USA). The MSP method was used to examine CpG islands in DNAs from the lung SCCs and adjacent nonmalignant lung tissues, breast IDCs, adjacent non-neoplastic mammary tissues and normal

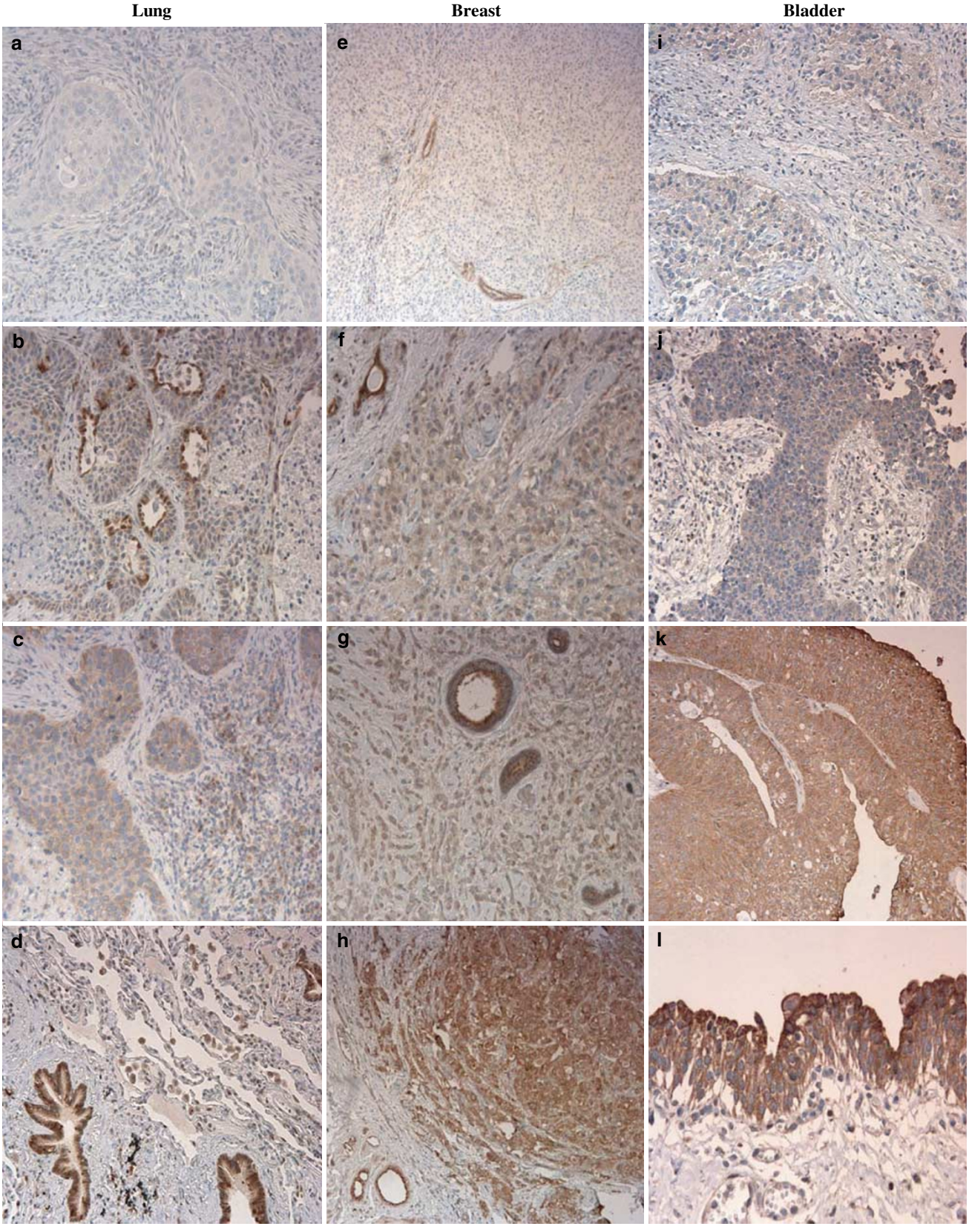


Table 1 Summary of WWOX and FHIT methylation status and expression

			WWOX		*FHIT*	
Tissue	*Samples*	*Diagnosis*	*Expression*[a]	*Methylation (%)*[b]	*Expression*[a]	*Methylation (%)*[b]
Lung	296T	SCC	—	24	—	24
	349T	ASCC	+	35	—	31
	370T	ASCC	—	24	—	21
	491T	ASCC	—	19	—	28
	523T	SCC	+	19	—	28
	564T	SCC	—	21	—	24
Breast	81T	IDC	+	27	—	100
	491T	IDC	+	36	+	24
	539T	IDC	+	13	+	32
	108T	IDC	+	20	+ +	7
	194T	IDC	+ +	6	+ +	3
Bladder	BLC22	TCC	+	24	—	100
	BLC25	TCC	—	24	—	21
	BLC4	TCC	—	56	—	21
	BLC21	TCC	—	18	+	4
	BLC26	TCC	+	11	+	10
	BLC20	TCC	+	11	+ +	0
	BLC15	TCC	+ +	0	+ +	0

[a]Protein expression assessed by immunohistochemistry: —, staining score of 1–5 represents highly reduced expression; + score of 6–11 represents reduced expression; + + score of 12–15 represents a normal level of expression. [b]% methylated CpG sites/ total CpG sites

mammary tissues, TCCs of the bladder and non-neoplastic urothelium tissues. Specific primers, for amplifying methylated or unmethylated products, were designed for the WWOX promoter and exon 1, and for FHIT intron 1 (Table 2). Representative results of MSP amplification of the WWOX promoter (347 bp), WWOX exon 1 (91 bp) and FHIT intron 1 (74 bp) are displayed in Figure 2a–c. The CpG islands in the WWOX promoter region were methylated in 62.5% of the lung SCCs, in WWOX exon 1 in 46% of the cases and *FHIT* intron 1 in 38% of the cases (Figure 2d). Only WWOX exon 1 was highly methylated in the adjacent non-neoplastic lung tissues. In breast IDCs, the *WWOX* promoter and exon 1 were methylated in 53 and 42% of cases, respectively, and *FHIT* intron 1 was methylated more frequently (57%). A large fraction of non-neoplastic mammary tissues adjacent to cancer (46%) showed WWOX promoter methylation. WWOX and FHIT DNA of normal mammary tissues were completely unmethylated in all cases. In TCCs of the bladder, the WWOX promoter and exon 1 were infrequently methylated (29 and 14% of cases, respectively), while FHIT intron 1 was methylated in 11% of cases. The non-neoplastic bladder tissue DNA was unmethylated for both genes (Figure 2d). In summary, WWOX and FHIT regulatory region CpGs are highly methylated in lung and breast cancer DNAs, less so in bladder cancers.

Correlation of WWOX and FHIT methylation status and association with clinicopathological features

We sought correlation of the methylation status of the fragile genes with clinicopathological features (sex, age, clinical stage, lymph node metastasis and histology). Fisher's exact test was used to examine the association between WWOX and FHIT methylation and clinicopathological features. A two-sided *P*-value of less than 0.05 was considered statistically significant.

In lung SCCs, WWOX promoter and exon 1 methylation was observed very frequently in male patients (13/14) ($P=0.011$) and most of the cancers without methylation of WWOX promoter and/or exon 1 were from females (8/10). WWOX and FHIT methylation did not correlate significantly with known clinicopathological characteristics of breast cancers. On the other hand, 11 of 16 breast cancers methylated in WWOX exon 1 were also methylated in FHIT intron 1, showing a strong positive association between WWOX exon 1 and FHIT intron 1 ($P=0.002$) methylation, stronger than the association of WWOX promoter and FHIT intron 1 methylation ($P=0.02$).

Methylation patterns assessed by sequencing

To obtain detailed methylation profiles, we analysed 62 CpG sites spanning the WWOX promoter, exon 1 and a small region of intron 1, and 29 CpG sites in the FHIT promoter, exon 1 and intron 1, by sequencing amplified products of bisulfite-treated DNAs (representative results summarized in Figure 3). Bisulfite-modified genomic DNA (2 μl) was amplified for sequencing, using primers as indicated in Table 2 and products were analysed on 1% agarose gels. Sequencing of the amplified products was accomplished by BigDye terminator reaction chemistry for analysis on the ABI Prism 377 (Applied Biosystems, Foster City, CA, USA).

Table 2 *WWOX* and *FHIT* primers for MSP and genomic bisulfite sequencing

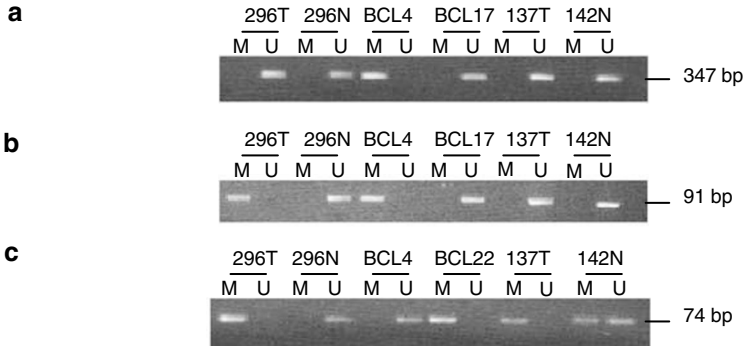
Primer	*Sequence*	*Annealing temperature (°C)*	*Product size (bp)*
WWOX MSP			
Promoter			
MFI	5′-TATGGGCGTCGTTTTTTTAGTT-3′	58	347
MR1	5′-CAATCTCCGCAATATCGCGACA-3′		
UF1	5′-TATGGGTGTTGTTTTTTTAGTT-3′	58	347
UR1	5′-CAATCTCCACAATATCACAACA-3′		
Exon 1			
MF2	5′-GCGAGTGGATTCGGTAGCGGGCGA-3′	62	91
MR2	5′-CCGTATCGTCCAACCCCGCGT-3′		
UF2	5′-GTGAGTGGATTTGGTAGTGGGTGA-3′	62	91
UR2	5′-CCATATCATCCAACCCCACAT-3′		
Genomic bisulfite sequencing			
First PCR			
Fl	5′-TAGAAGTTTAGGATAATAGTAT-3′	58	767
R1	5′-CTCCTTAACAATTACTTTCACT-3′		
Nested PCR			
FI	5′-TAGAAGTTTAGGATAATAGTAT-3′	58	690
R2	5′-TAAACTATACAAAATCCCAAAT-3′		
Sequencing			
S1	5′-TAGAAGTTTAGGATAATAGTAT-3′	55	
S2	5′-GTTTTTGTAGGATTGGTTAGAA-3′	55	
S3	5′-TAAACTATACAAAATCCCAAAT-3′	58	
S4	5′-CTACCATAACTAACTATAAA-3′	55	
FHIT MSP			
Methylated			
MF	5′-TTGGGGCGCGGGTTTGGGTTTTTACGC-3′	68	74
MR	5′-CGTAAACGACGCCGACCCCACTA-3′		
Unmethylated			
UF	5′-TTGGGGTGTGGGTTTGGGTTTTTATG-3′	68	74
UR	5′-CATAAACAACACCAACCCCACTA-3′		
Genomic bisulfite sequencing			
Region 1			
First PCR			
Fl	5′-GAAAAAGTTAAAGATTGTGCGA-3′	55	402
Rl	5′-AAACGACGCCGACCCCACTAAA-3′		
Nested PCR			
F2	5′-AGTTGTGTTTTGTGGTTAGTGTTTTT-3′	65	350
R2	5′-AAACTTACCTCCCCGCCCCTAC-3′		
Sequencing			
SI	5′-TAGGGTTATTGTTATTATTATGGT-3′	62	
Region 2			
First PCR			
F3	5′-GTTATTTAGTGGGTATATTTT-3′	52	398
R3	5′-CCCCAAAACCAAAAACTATA-3′		
Nested PCR			
F4	5′-GTTATTTAGTGGGTATATTTT-3′	54	347
R4	5′-TACCTCAATTTCCCCAATATA-3′		
Sequencing			
S1	5′-TAGGGTTATTGTTATTATTATGGT-3′	55	

FHIT MSP primers arc the same as those used in previous studies (23)

In all, 19–35% of the *WWOX* CpG sites in lung SCCs were methylated: two small groups of hypermethylated CpG sites in the promoter and one group in exon 1 were consistently methylated. In FHIT intron 1, two groups of CpGs were consistently methylated with 21–31% of CpGs methylated. The adjacent noncancerous lung tissue DNAs exhibited methylation in WWOX exon 1, but not FHIT methylation.

In breast carcinomas, 5–36% of the *WWOX* CpG sites were methylated, creating two CpG islands in the promoter and one in exon 1; the number of methylated *FHIT* CpG sites ranged from 3–100%, but intron 1 CpGs were always methylated. We also observed methylation in some CpG sites of WWOX promoter and FHIT intron 1 in adjacent non-neoplastic mammary tissues. WWOX and FHIT regulatory regions were unmethylated in normal mammary tissues.

In some TCCs, WWOX was methylated at 15–56% of CpG sites, mainly in the promoter and exon 1. We did



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Tissues	Description	WWOX promoter	WWOX exon1	FHIT intron 1
Lung	Squamous cell carcinomas	15/24 (62.5%)	13/24 (46%)	9/24 (38%)
	Non neoplastic adjacent tissues	1/24 (4%)	7/24 (29%)	2/24 (8.3%)
Breast	Invasive breast carcinomas	15/28 (53%)	12/28 (42%)	16/28 (57%)
	Non neoplastic adjacent tissues	13/28 (46%)	1/28 (3%)	4/28 (14%)
	Normal tissues	0/26 (0%)	0/26 (0%)	0/26 (0%)
Bladder	Transitional cell carcinomas	8/27 (29%)	4/27 (14%)	3/27 (11%)
	Non neoplastic adjacent tissues	0/7 (0%)	0/7 (0%)	0/7 (0%)

Figure 2 MSP amplification in WWOX and FHIT regulatory regions. Specific primers covering CpG sites were designed to distinguish between methylated and unmethylated sequences of bisulfite-modified DNA (Table 2). The genomic location of MF1 and UF1 primers is −387, MR1 and UR1 primers −41, MF2 and UF2 primers + 72 and MR2 and UR2 primers + 162, relative to the start site (+1) of exon 1 of the *WWOX* gene. For the *FHIT* gene, the genomic location of MF, UF and MR, UR primers is 39 and 112 relative to the start site of intron 1 of the *FHIT* gene. MSP analysis of 296T (lung SCC), 296N (normal lung), BLC4, BLC17, BLC22 (TCC), 137T (IDC) and 142N (non-neoplastic mammary tissue) in: (**a**) the promoter region of the *WWOX* gene (347 bp); (**b**) exon 1 of the *WWOX* gene (91 bp); and (**c**) intron 1 of the *FHIT* gene (74 bp). M and U lanes represent amplified products of primers recognizing methylated and unmethylated sequences, respectively. (**d**) High-frequency WWOX and FHIT methylation in lung and breast but lower frequency in bladder tissues. High frequency of methylation was detected in the WWOX promoter, exon 1 and FHIT intron 1 in SCCs, but the corresponding adjacent normal tissues showed a high frequency of methylation only in WWOX exon 1. The frequency of methylation for IDCs was similar to SCCs and interestingly the nonmalignant mammary tissues were methylated in the WWOX promoter. A very low frequency of methylation was observed in TCCs, and DNAs of non-neoplastic urothelia tissues were not methylated

not detect a specific methylation pattern for the *FHIT* gene in bladder cancer and WWOX and FHIT DNA methylation may be an infrequent mechanism of inactivation in bladder cancer.

WWOX and FHIT promoter methylation in cancers correlates with reduced protein expression

To examine the relationship between WWOX and FHIT methylation and protein expression, we assessed levels of expression of the proteins by immunohistochemistry in lung SCCs, breast IDCs and TCCs (Table 1). In lung SCCs, there was a correlation between methylation of WWOX and FHIT promoters and protein expression, confirming that promoter methylation is an important inactivation mechanism for Wwox and Fhit in lung cancer. WWOX and FHIT regulatory regions were methylated in all the lung tumors and protein expression was highly reduced. WWOX and FHIT DNA methylation was also associated with reduced protein expression in breast cancer, with most invasive breast carcinomas showing reduced expression of both. Apparently, methylation of 25–30% of WWOX and FHIT CpGs is sufficient for gene silencing in invasive breast cancer. WWOX and FHIT DNA methylation was also associated with reduced protein expression in bladder cancer, with most TCCs showing reduced expression for both (Table 1). WWOX and FHIT CpGs were unmethylated in normal tissues and both proteins were expressed.

The renaissance of methylation studies has generated considerable interest for cancer researchers because: (1) methylation of CpG islands in gene regulatory regions, in combination with chromatin remodeling, is involved in downregulation of expression of genes, including tumor suppressor genes; (2) methylation of specific CpG islands is easily detected in tissues and body fluids of individuals with cancer (Esteller *et al*., 1999a, b; Sanchez-Cespedes *et al*., 2000; Palmisano *et al*., 2000; Soria *et al*., 2000; Muller *et al*., 2003), or at high risk for cancer development, so that specific gene methylation patterns can be useful in diagnosis or prognosis; and (3) epigenetic and chromatin remodeling marks can be reversed by specific agents or inhibitors, suggesting such inhibitors as therapeutic agents (McGregor *et al*., 2002; Hennessy *et al*., 2003; Takai *et al*., 2004). Methylation of regulatory regions of many genes has been reported in cancer cells, but which of these methylation marks will be most useful in diagnostic or prognostic clinical trials, or as surrogate markers in preclinical/clinical prevention and therapy trials of specific cancers, is only beginning to be defined.

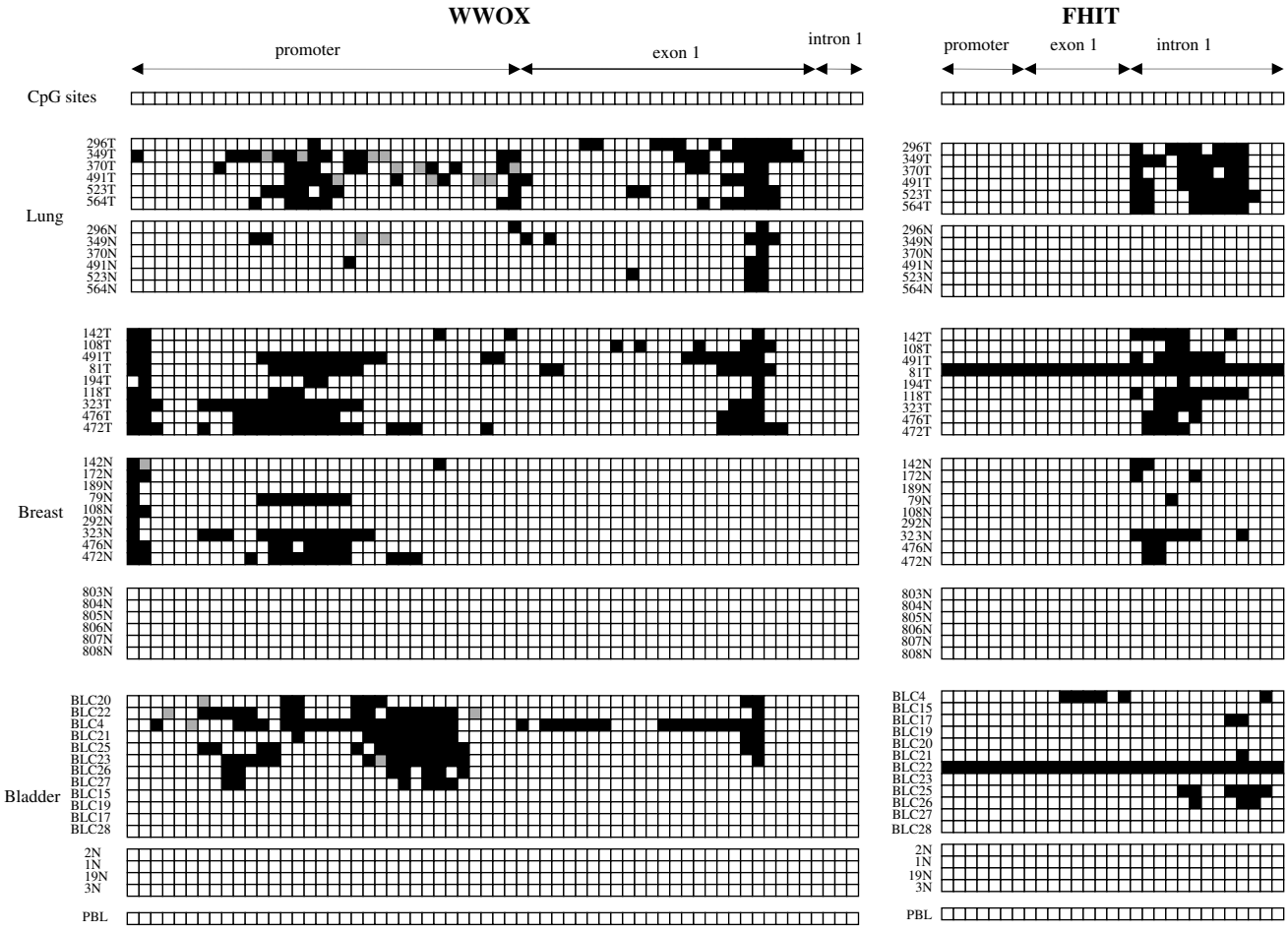

Figure 3 Methylation patterns in WWOX and FHIT 5′ regulatory regions. Sequencing of bisulfite-treated DNA revealed different methylation patterns for *WWOX* (**a**) and *FHIT* (**b**) genes. Black squares represent methylated CpG sites, white squares are unmethylated CpGs sites and gray squares are hemimethylated CpGs. The first *WWOX* CpG site is located −343 bp relative to the start of exon 1 and the first *FHIT* CpG site is located −94 bp relative to the start of exon 1

As genes at common fragile sites are likely to be inactivated by similar mechanisms (Ohta *et al*., 1996; Huebner *et al*., 1998), we have been interested in coordinate inactivation of fragile genes in cancer and have shown that loss or reduction of Fhit and Wwox expression is strongly associated in invasive breast cancers (Guler *et al*., 2004). Thus, we investigated the mechanism of inactivation of the two genes in three types of cancer: lung, breast and bladder. If the two genes should be coordinately inactivated in a large fraction of several types of cancers, they might serve as biomarkers for multiple cancer types, and eventually as surrogate markers for prevention and therapeutic trials.

We confirmed that WWOX and FHIT are frequently inactivated in lung, breast and bladder cancer, and that expression is reduced or lost coordinately with promoter region methylation. Wwox expression was highly reduced in lung cancer and reduced in breast and bladder cancers. Yendamuri *et al*. (2003) showed that WWOX alterations occur in a significant fraction of lung cancers; in addition, in a previous study of 94 invasive breast cancers, we observed reduced Wwox and Fhit expression in 63 and 55% of the cases (Guler *et al*., 2004). Thus, *WWOX* and *FHIT*, two genes that are located in fragile sites in different chromosomes, are coordinately inactivated in lung and breast cancers. An interesting aspect of these results is that WWOX and FHIT methylation patterns for the three types of cancers were relatively consistent for a given type of cancer, but differed among cancers from different organs. The pattern that was shared by tumors with hypermethylation was consistent methylation of two regions in the promoter and one region in exon 1 of WWOX and a region in FHIT intron 1, in accord with previous methylation studies for the *FHIT* gene (Esteller *et al*., 1999a, b; Yang *et al*., 2002; McGregor *et al*., 2002).

Sparse methylation in WWOX exon 1 in non-neoplastic lung tissue adjacent to cancer may be an early marker of carcinogen exposure; perhaps, when the extent of methylation reaches a critical level (~20%), or when an additional epigenetic alteration, such as Fhit inactivation occurs, preneoplastic changes appear.

Interestingly, WWOX promoter and FHIT intron 1 were methylated in DNA from non-neoplastic

mammary tissues adjacent to cancer, but not in mammary tissues from noncancer patients. In this study, we showed that FHIT regulatory region CpGs outside intron 1 could also be methylated, as shown for the promoter and exon 1 in some breast cancers. WWOX exon 1 methylation differentiates breast cancer from non-neoplastic tissue adjacent to cancer and normal mammary tissue.

FHIT and WWOX methylation was less frequent in bladder cancer, and when it occurred, the number of methylated FHIT CpGs was high. According to WWOX methylation status, it seemed that there are two different bladder tumor groups. In one group, WWOX promoter region is heavily methylated and in the other WWOX is not methylated. The findings in breast and bladder cancer showed that comprehensive examination of entire 5′ regions of a gene may be useful in identifying regulatory regions correlating with epigenetically mediated loss of gene function, and in molecular marker studies, to distinguish neoplastic from normal cells. A total of 29 methylated CpG sites, as observed in FHIT for several breast and bladder cancers, is among the highest number of methylated CpGs reported for a regulatory region of a tumor suppressor gene. As we found that methylation in FHIT intron 1 was sufficient for total loss of Fhit expression in lung and some bladder tumors, it would be interesting to investigate whether heavy methylation of the *FHIT* gene is related to morphological features, environmental exposure and clinical outcome, or could affect response to methylation inhibitors.

The application of molecular markers specific for lung, breast or bladder cancer offers possibilities for early detection (Palmisano *et al*., 2000; Soria *et al*., 2000; Tang *et al*., 2000; Muller *et al*., 2003). WWOX can be included as a biomarker for early detection of lung cancer, as well as for monitoring the response to chemopreventive agents. WWOX exon 1 MSP may be used as a marker for early lung carcinogenesis, while WWOX promoter MSP differentiates lung cancer from adjacent non-neoplastic lung tissue. The high frequency of WWOX and FHIT methylation in association with loss of expression suggests that these fragile genes could enrich a hypermethylation panel for lung, breast, bladder and probably other cancers.

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References

Baffa R, Gomella LG, Vecchione A, Bassi P, Mimori K, Sedor J, Calviello CM, Gardiman M, Minimo C, Strup SE, McCue PA, Kovatich AJ, Pagano F, Huebner K and Croce CM. (2000). *Am. J. Pathol.*, **156,** 419–424.

Bednarek AK, Keck-Waggoner CL, Daniel RL, Laflin KJ, Bergsagel PL, Kiguchi K, Brenner AJ and Aldaz CM. (2001). *Cancer Res.*, **61,** 8068–8073.

Bednarek AK, Laflin KJ, Daniel RL, Liao Q, Hawkins KA and Aldaz CM. (2000). *Cancer Res.*, **60,** 2140–2145.

Driouch K, Prydz H, Monese R, Johansen H, Lidereau R and Frengen E. (2002). *Oncogene*, **21,** 1832–1840.

Esteller M, Hamilton SR, Burger PC, Baylin SB and Herman JG. (1999a). *Cancer Res.*, **59,** 793–797.

Esteller M, Sanchez-Cespedes M, Rosell R, Sidransky D, Baylin SB and Herman JG. (1999b). *Cancer Res.*, **59,** 67–70.

Gatalica Z, Lele SM, Rampy BA and Norris BA. (2000). *Cancer*, **88,** 1378–1383.

Guler G, Uner A, Guler N, Han SY, Iliopoulos D, Hauck WW, McCue P and Huebner K. (2004). *Cancer*, **100,** 1605–1614.

Hennessy BT, Garcia-Manero G, Kantarjian HM and Giles FJ. (2003). *Expert Opin. Invest. Drugs*, **12,** 1985–1993.

Herman JG, Graff JR, Myohanen S, Nelkin BD and Baylin SB. (1996). *Proc. Natl. Acad. Sci. USA*, **93,** 9821–9826.

Huebner K and Croce CM. (2003). *Br. J. Cancer*, **88,** 1501–1506.

Huebner K, Garrison PN, Barnes LD and Croce CM. (1998). *Ann. Rev. Genet.*, **32,** 7–31.

Ingvarsson S, Agnarsson BA, Sigbjornsdottir BI, Kononen J, Kallioniemi OP, Barkardottir RB, Kovatich AJ, Schwarting R, Hauck WW, Huebner K and McCue PA. (1999). *Cancer Res.*, **59,** 2682–2689.

Ishii H and Furukawa Y. (2004). *Int. J. Hematol.*, **79,** 238–242.

Kim H, Kwon YM, Kim JS, Lee H, Park JH, Shim YM, Han J, Park J and Kim DH. (2004). *J. Clin. Oncol.*, **22,** 2363–2370.

Kuroki T, Trapasso F, Shiraishi T, Alder H, Mimori K, Mori M and Croce CM. (2002). *Cancer Res.*, **62,** 2258–2260.

Kuroki T, Yendamuri S, Trapasso F, Matsuyama A, Aqeilan RI, Alder H, Rattan S, Cesari R, Nolli ML, Williams NN, Mori M, Kanematsu T and Croce CM. (2004). *Clin. Cancer Res.*, **10,** 2459–2465.

Maruyama R, Toyooka S, Toyooka KO, Harada K, Virmani AK, Zochbauer-Muller S, Farinas AJ, Vakar-Lopez F, Minna JD, Sagalowsky A, Czerniak B and Gazdar AF. (2001). *Cancer Res.*, **61,** 8659–8663.

McGregor F, Muntoni A, Fleming J, Brown J, Felix DH, MacDonald DG, Parkinson EK and Harrison PR. (2002). *Cancer Res.*, **16,** 4757–4766.

Muller HM, Widschwendter A, Fiegl H, Ivarsson L, Goebel G, Perkmann E, Marth C and Widschwendter M. (2003). *Cancer Res.*, **63,** 7641–7645.

Ohta M, Inoue H, Cotticelli MG, Kastury K, Baffa R, Palazzo J, Siprashvili Z, Mori M, MuCue P, Druck T, Croce CM and Huebner K. (1996). *Cell*, **84,** 587–597.

Paige AJ, Taylor KJ, Taylor C, Hillier SG, Farrington S, Scott D, Porteous DJ, Smyth JF, Gabra H and Watson JE. (2001). *Proc. Natl. Acad. Sci. USA*, **98,** 11417–11422.

Palmisano WA, Divine KK, Saccomanno G, Gilliland FD, Baylin SB, Herman JG and Belinsky SA. (2000). *Cancer Res.*, **60,** 5954.

Sanchez-Cespedes M, Esteller M, Wu L, Nawroz-Danish H, Yoo GH, Koch WM, Jen J, Herman JG and Sidransky D. (2000). *Cancer Res.*, **60,** 892–895.

Skopelitou AS, Gloustianou G, Bai M and Huebner K. (2001). *In vivo*, **15,** 169–173.

Soria JC, Rodriguez M, Liu DD, Lee JJ, Hong WK and Mao L. (2000). *Cancer Res.*, **62,** 351–355.

Sozzi G, Pastorino U, Moiraghi L, Tagliabue E, Pezzella F, Ghirelli C, Tornielli S, Sard L, Huebner K, Pierotti MA, Croce CM and Pilotti S. (1998). *Cancer Res.*, **58,** 5032–5037.

Takai N, Desmond JC, Kumagai T, Gui D, Said JW, Whittaker S, Miyakawa I and Koeffler HP. (2004). *Clin. Cancer Res.*, **10,** 1141–1149.

Tanaka H, Shimada Y, Harada H, Shinoda M, Hatooka S, Imamura M and Ishizaki K. (1998). *Cancer Res.*, **58,** 3429–3434.

Tang X, Khuri FR, Lee JJ, Kemp BL, Liu D, Hong WK and Mao L. (2000). *J. Natl. Cancer Inst.*, **92,** 1511–1516.

Yang Q, Nakamura M, Nakamura Y, Yoshimura G, Suzuma T, Umemura T, Shimizu Y, Mori I, Sakurai T and Kakudo K. (2002). *Clin. Cancer Res.*, **8,** 2890–2893.

Yendamuri S, Kuroki T, Trapasso F, Henry AC, Dumon KR, Huebner K, Williams NN, Kaiser LR and Croce CM. (2003). *Cancer Res.*, **63,** 878–881.

Zochbauer-Muller S, Fong KM, Maitra A, Lam S, Geradts J, Ashfaq R, Virmani AK, Milchgrub S, Gazdar AF and Minna JD. (2001). *Cancer Res.*, **61,** 3581–3585.