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Promoter hypermethylation in sentinel lymph nodes as a marker for breast cancer recurrence

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Abstract

Purpose—Promoter methylation of tumor suppressor genes in histologically negative sentinel lymph nodes (HNSN) of early stage breast cancer patients has not been extensively studied. This study evaluates the methylation frequency and pattern in HNSN to determine if detection of hypermethylation of one or more genes is associated with an increased recurrence risk in node negative breast cancer.

Experimental design—In 1998, a prospective study of patients with early stage breast cancer and HNSN was initiated in order to correlate sentinel node analysis with clinical outcome. Nodal tissue was selected from 120 HNSN patients for methylation analysis in at least one and up to six sentinel nodes using a panel of nine genes. Corresponding primary breast tumors from 79 patients were also evaluated for hypermethylation. Methylation analysis was performed using nested Methylation Sensitive PCR (n-MSP). Logistical regression was used to evaluate the relationship between clinical recurrence and methylation status.

Results—Over a median follow-up of 79 months, 13 of the 120 patients had clinical recurrence. Hypermethylation of genes was frequently observed in HNSN, but there was no correlation of methylation pattern and clinical recurrence. However, increased frequency of gene methylation of the primary tumor correlated with clinical recurrence.

Conclusions—Although hypermethylation of multiple genes occurs frequently in HNSN of breast cancer patients, it is not associated with breast cancer recurrence in the first 7 years of clinical follow-up.

Keywords

Bisulfite treatment; CpG Island; DNA methylation; Epigenetics; Methylation specific PCR; Nested MSP; Sentinel node

Introduction

Breast cancer is the most common malignancy among females in Western countries. In 2007, breast cancer will be diagnosed in over 200,000 women and result in death in over 40,000 [1]. Mammography and increased patient awareness of the need for breast cancer screening have resulted in a dramatic increase in the detection of early stage breast cancer [2, 3]. Of all the women who are initially diagnosed with breast cancer, approximately 62% have localized disease, where surgical resection is often curative. Regional lymph node status is the single most important prognostic factor, and accurate staging is essential for therapeutic decision making [4–6]. Sentinel lymph node (SLN) biopsy is a well accepted surgical procedure for the staging of early stage breast cancer patients.

While the majority of HNN patients are cured by local therapy alone, approximately 25% develop recurrent breast cancer and die of their disease [7, 8]. Traditional prognostic factors are not adequate to identify those patients at low risk of recurrence who can be spared systemic treatment with chemotherapy and its side effects, including the subsequent risk of secondary malignancies. Technologies such as DNA microarray analysis are at the forefront in attempting to identify those patients in the node-negative group who will have a poor outcome and thus, would benefit most from systemic adjuvant chemotherapy [9–11]. For example, *Oncotype DX* is a clinically validated, multi-gene assay that provides a quantitative assessment of the likelihood of distant breast cancer recurrence in women with estrogen receptor positive disease and it also assesses the potential benefit from chemotherapy [12]. The ability to accurately divide the group of HNN breast cancer patients into low risk and high risk groups could potentially benefit many patients.

Sentinel lymph node biopsy is a technique used to selectively identify and biopsy the first lymph node(s) draining a specified primary site. Histological status of the sentinel lymph node is predictive of the status of the regional nodal basin and is used to determine which patients can be spared an axillary dissection [13–15]. When multiple sections are cut through histologically negative lymph node(s), approximately 25% of patients otherwise deemed node negative have histologic evidence of micrometastasis [8, 16–18]. Advances in molecular techniques now make it possible to detect cancer cells at levels below the limit for routine histological examination. The significance of this microscopic disease in the sentinel lymph node is not well understood [19, 20]. As we improve our ability to identify early spread of cancer, it is likely that more patients may be considered for therapy. It may be equally important to use these markers to identify those patients who may be able to safely forego treatment.

Promoter hypermethylation of tumor suppressor genes represents a cancer specific alteration that might be used as a target for such molecular detection. These epigenetic alterations are among the most common molecular alterations in human neoplasia [21]. Epigenetic changes are heritable, yet not based on changes in the primary DNA sequence [22–24]. Methylation mediated silencing of gene expression involves the hypermethylation of CpG dinucleotides located within the promoter region of genes. Both DNA methylation and histone acetylation are major determinants of chromatin structure, which is a critical regulator of gene transcription [25].

Breast cancer is considered to result in part from the accumulation of multiple genetic alterations leading to oncogene over-expression or tumor suppressor loss. Promoter methylation is a common mechanism for silencing tumor suppressor genes and occurs frequently in breast cancer [26, 27]. Genes reported to be hypermethylated in breast cancer include those involved in cell cycle repair (p16, cyclin D), DNA repair (GSTP1, BRCA-1), cell adhesion (CDH1, AP2-alpha), cytokine signaling (SOCS1), and other functions (HIN-1, RASSF1, FHIT). Thus, aberrant hypermethylation appears to significantly contribute to the malignant phenotype of breast cancer. Hypermethylation of primary breast tumor genes is associated with pathologic prognostic factors and may have utility in assessing patient prognosis and predicting early regional metastasis [2]. Methylation specific PCR (MSP) allows for sensitive detection of altered patterns of methylation and may identify regional lymph nodes metastasis that is not detected histologically [28]. If this molecular tool can reliably identify breast cancer patients at higher risk of recurrence, despite negative histologic evaluation of the sentinel lymph node, it may be useful in guiding breast cancer therapy. In this study histologically negative sentinel nodes in patients with early breast cancer are evaluated to determine if methylation frequency or patterns are associated with clinical risk of recurrence.

Patients and methods

Study population

From February 1998 to September 2006, 450 patients with T1 or T2 breast cancer without clinical evidence of nodal metastasis were enrolled in a prospective study at Johns Hopkins Hospital done in order to correlate findings in the sentinel nodes with clinical outcomes. This population of early stage breast cancer patients was chosen for study because the patients were likely to have HNN disease. This would offer an opportunity to study the utility of molecular markers in histologically negative sentinel nodes as a means of identifying patients at increased risk of recurrence.

Patients underwent lumpectomy or mastectomy as clinically indicated, as well as sentinel node biopsy. Post-operatively, patients received standard radiation, hormonal therapy and

chemotherapy as clinically indicated. Patient characteristics and clinical data were collected, including age, sex, race, smoking history, tumor size, number of sentinel nodes resected, therapy (i.e., hormonal, radiation, chemotherapy) and recurrence. Information was collected through routine follow-up care at Johns Hopkins Hospital and via annual phone calls to participants through February 2007. This study was approved by the Johns Hopkins Medicine Institutional Review Board with all patients providing written informed consent.

Collection and processing of samples

Sentinel nodes were processed for clinical care by routine hematoxylin and eosin staining in the Department of Pathology. Of 381 patients with minimum of 2 year follow-up, 121 were histologically node positive and 260 patients were HNN. From this group of 260 node negative patients, specimens from 120 patients were evaluated for methylation analysis. This group of 120 patients was chosen based upon specimen availability, a minimum known clinical follow-up time of 2 years, and also intentional inclusion of patients with known recurrences due to small number of clinical relapses. Methylation analysis was performed on 79 available primary tumors and 227 sentinel nodes from 120 patients. In cases where multiple sentinel nodes were harvested, every node was evaluated. Additionally, 32 histologically positive sentinel nodes (HPSN) were also chosen for an initial feasibility study. Samples were processed in a blinded fashion such that laboratory and clinical data were deliberately separated.

Tissue samples and DNA isolation

Formalin-fixed, paraffin embedded tissue samples were obtained ($N=306$). For each primary tumor ($N=79$), the paraffin block was sectioned for adequate sampling. Sentinel nodes ($N=227$) were processed, in entirety, for DNA extraction. All tissue sections were deparaffinized and then extracted in 50 μ l PK buffer (Invitrogen) and 1.2 $\times$ PCR buffer (19.5 mM ammonium sulfate, 78.8 mM Tris, pH 8.8/7.9 mM MgCl₂/11.7 nM 2-mercaptoethanol) at 60°C. The tissue extract was heat inactivated at 100°C for 10 min and DNA was purified by phenol–chloroform extraction. The quality and quantity of DNA was determined by A_{260}/A_{280} ratio.

Breast and axillary lymph node samples were acquired postmortem from cancer-free subjects for use as normal controls for evaluation of methylation in axillary lymph nodes. In vitro methylated DNA (IVD) was obtained from treatment of leukocyte DNA with *SSSI* methyltransferase as a methylated control for PCR. Peripheral blood leukocytes (NL) were isolated from normal volunteers as an unmethylated control. IRB approval was obtained for all samples.

Bisulfite modification

Bisulfite modification was performed as previously described [29]. Tissue, control and cell line DNA (1 μ g) in a volume of 50 μ l was denatured by adding 5.5 μ l of NaOH (2 M) for 10 min at 37°C. Subsequently, 30 μ l of 10 mM hydroquinone (Sigma-Aldrich) and 520 μ l of 3 M sodium bisulfite at pH 5 were added and mixed, and samples were incubated for 16 h at 55°C. Bisulfite-modified DNA was purified using the Wizard DNA CleanUp System (Promega) according to the manufacturer's instructions. Bisulfite modification was completed by incubation with 5.5 μ l of NaOH (3 M) for 5 min at 20°C, followed by ethanol precipitation. DNA was resuspended in 20 μ l of Tris EDTA buffer (Low TE, pH 8.5) and stored at –20°C.

Nested methylation-specific polymerase chain reaction (n-MSP)

Primer pairs described in Tables 1, 2 were purchased from Integrated DNA Technologies. The n-MSP procedure required two PCR reactions and was performed as previously described [30, 31]. In the first PCR reaction (the multiplex step), 4 μ l of bisulfite-treated DNA was added to 21 μ l of reaction buffer containing 1 $\times$ PCR buffer (16.6 mM ammonium sulfate/67 mM Tris, pH 8.8/6.7 mM MgCl₂/10 nM 2-mercaptoethanol), dNTPs (Continental Lab Products, each at 1.25 mM), 2–4 sets of primers (50 ng each per reaction), and 1 μ l of Red-Taq polymerase (Sigma). Conditions were 95°C for 5 min, followed by 35 cycles of 30 s at 95°C, 30 s at 56°C, and 30 s at 72°C, with a final extension cycle of 72°C for 5 min. Two μ l of the PCR products were then diluted to 500 μ l of water to use in the second PCR reaction. For the second PCR reaction, 2 μ l of the diluted PCR product was added to 23 μ l of reaction buffer containing 1 $\times$ PCR buffer, dNTPs, MSP primers from the gene of interest (300 ng each per reaction), and 1 μ l of Red Taq polymerase. Conditions were 95°C for 5 min, followed by 35 cycles of 30 s at 95°C, 30 s at the annealing temperature listed in Table 2, and 30 s at 72°C, with a final extension cycle of 72°C for 5 min. Optimization of annealing temperatures was performed using the normal node samples from postmortem samples obtained from cancer-free patients. Known unmethylated controls (normal lymphocytes), known methylated controls (IVD), and water controls were performed. Multiplex PCR products (7.5 μ l) were loaded onto a non-denaturing 6% polyacrylamide gel, stained with ethidium bromide, and directly visualized under UV illumination. MSP products (7.5 μ l) were loaded onto 2% agarose gels containing GelStar nucleic acid stain solution (Cambrex), and directly visualized under UV illumination. The presence of visible amplification in the methylation reaction was scored as positive, while amplification in the unmethylated reaction without visible amplification in the methylated reaction was scored as negative. Failure to amplify with either PCR reaction signified inadequate DNA and was scored as non-informative.

Statistical analysis

Baseline characteristics of patients with and without recurrence were examined using *t* tests and Fisher's exact test. For patients with multiple sentinel node samples evaluated, two summary methylation values were calculated using the percentage of nodes methylated per gene. If at least one node (>0%) was methylated for a specific gene, that gene was called methylated at a 0% cutoff. The second summary value was calculated similarly using a 50% cutoff. For example, a patient with 2/3 (67%) nodes for one gene would have that gene called methylated, but if 1/3 (33%) nodes were methylated for another gene, that gene would be considered unmethylated. These summary values were used to describe the frequency distributions of methylation by genes and across genes in the nodes. To examine the variability of methylation in the nodes within patients, the distributions of mean methylation values per gene were plotted Supplemental data, Fig. 1. This was performed to ensure that collapsing across nodes within a patient was justifiable; that is, if there was substantial variability within the nodes, these data could not be collapsed with confidence. Ultimately, the 50% cutoff was used as the standard for presenting methylation of node data.

Among patients with both a tumor and node sample, the percent agreement in methylation assessment between tumor and node(s) was calculated for each gene and cutoff. The total number of genes methylated for each patient separately in the tumor and node was calculated and compared by recurrence status and cutoff using nonparametric Wilcoxon rank sum tests. Logistic regression was used to describe the relationship between recurrence and methylation in each gene within tumor and nodes separately. In this analysis, the non-summarized, complete set of nodes and generalized estimating equations were used in estimation. REMARK criteria are used in the presentation of data [32].

Results

Study population

From February 1998 through December 2004, 381 patients were enrolled in the study, of which 260 were HNN; all had a minimum known 2 year clinical follow-up time. Tissue samples from 120 HNN patients were used for this analysis. Median time to follow-up time was 77.7 (range 17–105) months. Characteristics of the 120 patients included in the methylation analysis and characteristics of the entire group of 260 HNN patients are presented in Table 3. To ensure that the 107 non-recurred patients selected for methylation analysis were representative of the entire cohort of non-recurred patients, we compared baseline characteristics of the 107 patients who had methylation analysis to the remaining 137 non-recurred patients who had surgery as of 12/31/2004. There were no significant differences between the groups. Breast cancer recurrence was noted either as a local (within the breast), regional (in the regional node basin), or distant (metastatic).

Clinical outcomes

In the 260 HNN patients who had surgery through December of 2004, there were 16 recurrences (6.2%, 95% CI [3.3%, 9.0%]). In the 120 subjects undergoing methylation analysis, there were 13 patients with recurrence (11%), which is fewer than predicted. Using the computational algorithm www.adjuvantonline.com, we would expect a recurrence rate between 8 and 15% depending on ER/PR status (Appendix 1). As compared to patients without recurrence, a higher frequency of patients with recurrence were non-white (46.2% vs. 19.6%, respectively; $P = 0.07$), had high grade primary tumors (67.7% vs. 22.1%, $P = 0.005$), had a primary breast tumor with lymphovascular invasion (23.1% vs. 3.8%; $P = 0.03$), and had a higher trend of receiving adjuvant chemotherapy (53.8% vs. 29.4%, $P = 0.11$). Additionally, those who recurred were less likely to have estrogen receptor positive breast cancer (46.2% vs. 76.5%, $P = 0.04$) and were less likely to have received hormonal therapy (30.7% vs. 59.8%, $P = 0.07$). More sentinel nodes were dissected from the patients without recurrence than those with recurrence (median difference = 1, $P = 0.05$). Of the 13 patients who recurred, there were 4 local, 6 regional, and 11 distant recurrences. The median time to the first recurrence was 36 (range 8.4–74.7) months. Results of baseline clinical comparisons by recurrence status for the 120 patients with methylation data and all 260 HNN patients who underwent sentinel node resection appear in Table 3.

Changes in promoter methylation

Promoter hypermethylation of at least one marker was identified in 77 out of 79 primary breast tumors using the following panel of genes: AP2, RASSF1A, P16, cyclinD, CDH1, SOCS, HIN-1, GSTP and FHIT. The most frequently hypermethylated gene detected was RASSF1A occurring in 81% (64/79) of primary tumors; this was followed by SOCS = 76% (60/79), FHIT = 58% (46/79), cyclin-D = 52% (41/79), HIN-1 = 44% (35/79), p16 = 44% (35/79), GSTP = 42% (33/79), AP2 = 24% (18/79) and CDH1 = 6% (5/79). Normal breast tissue controls from autopsy specimens showed no methylation of any gene (Table 4 and Supplemental Fig. 2). Three percent (2/79) of primary tumors had no genes methylated, and 4% (3/79) had one gene methylated, 57% (42/74) had 2–4 genes methylated and 43% (32/74) had methylation of more than four genes methylated. The median number of genes methylated for each primary tumor was four and this was independent of clinical recurrence status. Using only two or three genes, over 93% of the tumors could be identified by 22 different gene combinations (Supplemental Table 1).

Methylation status was tested in a small number of histologically positive sentinel lymph nodes (HPSN) with the same gene panel to validate the feasibility of the nested technique on sentinel lymph nodes. Thirty-two HPSN from this same prospective study were tested with

the following results: RASSF1 = 65% (20/31), FHIT = 48% (14/29), cyclin-D = 11% (3/28), HIN-1 = 89% (25/28), p16 = 72% (23/32), GSTP = 31% (9/29), AP2 = 72% (21/29) and CDH1 = 36% (8/22) (Table 4). These frequencies are similar to those for stage 1 breast tumors and suggest that, indeed, cancer specific methylation can be detected in HPSN.

A total of 227 HNSN were examined for methylation at these nine genes from 120 patients. The frequency of methylation of HNSN (Table 4), is comparable to the HPSN as well as corresponding primary breast tumors. The frequency of methylation of the HNSN is determined as methylated if >50% of the nodes sampled for one patient were methylated (50% collapsed). Percent methylation of sentinel nodes from patients with recurrence (RM) over percent methylation of sentinel nodes from patients without recurrence (NRM) is also shown. This ratio is limited given the small number of recurrences. Frequencies are reported for all 120 samples and also by recurrence status.

Correlation of methylation in the primary tumor and sentinel lymph nodes was evaluated looking at percent agreement. Percentage of unexpected methylation pattern was calculated by identifying those cases where the node was methylated and the primary tumor was unmethylated. The percentage of agreement included all of the other methylated status situations. Percentage of expected methylation pattern (Table 5) ranged from 74 to 100% for all genes except AP2, which had a lower percentage at 51%. AP2 appears to have a high frequency of methylation in the sentinel nodes (59%) and has a low frequency of methylation in the primary tumor (24%).

Promoter methylation and clinical recurrence of breast cancer

The primary objective of this study was to identify a panel(s) of genes associated with an increased or decreased risk of breast cancer recurrence when methylated in HNSN. Gene methylation status was evaluated for 120 breast cancer patients with 13 having a clinical recurrence. The total number of methylation profiles recorded was 306; 227 in nodes and 79 in tumors. For sample size considerations, this yielded 20 “events” out of 227 nodes and 25 “events” out of 306 nodes and tumors.

A logistic regression analysis was performed to test if the methylation in each gene was associated with recurrence status, and each gene was analyzed separately for the tumor dataset or for variations of each node datasets (defined by node data with either no cutoff or 50% cutoff) (Supplemental Table 2). There was an increase in the risk of breast cancer recurrence if p16 or AP2 was methylated in the primary tumor: OR = 5.4 (95% CI [0.58, 50.9]) and OR = 5.9 (95% CI [0.9, 38.5]), respectively. Methylation of GSTP in the primary tumor was also associated with an increased risk of recurrence: OR = 2.2 (95% CI [0.35, 14.0]).

Analysis of non-summarized node methylation values with logistic regression did not show significant associations between recurrence and methylation of any gene, except for Cyclin D which was associated with a lower risk of recurrence (OR = 0.58, 95% CI [0.39, 0.86], $P = 0.004$). AP2 and p16 were consistent with this trend with borderline p values: OR = 0.66 (95% CI [0.43, 1.01]; $P = 0.03$) and OR = 0.66 (95% CI [0.44, 0.99], $P = 0.02$), respectively, favoring a lower chance of recurrence with nodal methylation. This association of lower recurrence with methylation of AP2 or p16 genes in the sentinel node was also observed in the node data analysis with a 50% cutoff, although P values were not statistically significant. Evaluation of the total number of genes methylated was performed for both tumor and as node status (50% cutoff). For all patients, independent of recurrence status, there was a median of four methylated genes in the primary breast tumor. In those patients with recurrence, there were fewer genes methylated in the nodes (median = 2) than in those patients without recurrence (median = 3).

Although frequent methylation was appreciated in the sentinel nodes, there was a trend toward decreased methylation in the sentinel lymph nodes of those patients with recurrence. A subset analysis was performed in those patients with recurrence who underwent sentinel node dissection at a separate time from removal of primary breast tumor ($n = 8$) versus those who had sentinel node dissection at the time of primary tumor resection ($n = 5$). The rationale for this subset analysis was that a second surgery to remove the sentinel lymph node at a distinct time from the primary tumor (which was the case in 8 of 13 recurrences) could change the likelihood of molecular evidence of disease since manipulation of the primary tumor with mechanical “spread” of tumor cells to the node would be less likely to occur and could explain a lower frequency of methylation events. No significant trend was appreciated with this subset analysis. Table 6 shows the percent methylation of all sentinel lymph nodes independently as well as by recurrence status.

Further analysis was performed to evaluate clinical treatment(s) given to those patients who had breast cancer recurrence. Among the 13 patients with breast cancer recurrence, four received neither hormonal therapy nor adjuvant chemotherapy. Among these four patients, one had a local recurrence only. Among the nine patients who received either or both therapies, three had local recurrences only. The odds ratio for the association between methylation in the node tissue and recurrence of cancer was similar for those that had distant and local recurrence versus those with only distant recurrence (Supplemental Table 3). Frequency of methylation in sentinel nodes from untreated patients versus all treated patients was also evaluated. No difference was appreciated in frequency of methylation of the sentinel nodes in treated patients or untreated patients (Supplemental Table 4).

Discussion

Epigenetic silencing of tumor suppressor genes plays an important role in pathogenesis of many types of cancer, including breast cancer. We studied the methylation status of nine genes in tissue samples from HNSN breast cancer patients to determine if methylation frequency or patterns predicted cancer recurrence. The frequency of methylation of genes tested in this analysis was similar to the established literature [26, 27, 33–42]. Methylation of single genes and panels of genes in the primary tumor was associated with an increased risk of breast cancer recurrence. Recurrence risk increased 2 to 6-fold with methylation of any of the following genes: p16, AP2, or GSTP (Supplemental Table 2).

Correlation of methylation was evaluated looking at agreement of the methylation status of the primary tumor and corresponding sentinel node(s). Percentage of expected methylation pattern ranged from 74 to 100% for all genes except AP2, which had a higher frequency of methylation in the sentinel nodes than in the tumor (59% vs. 24%, respectively). This may be a result of the heterogeneity of tissues and the processing of material for evaluation. Alternatively, AP2 methylation may reflect a local inflammatory reaction in HNSN rather than a tumorigenic response.

Logistic regression models were utilized to identify a panel of genes whose methylation might reflect an increased or decreased risk of recurrence. For sentinel lymph nodes, no gene panels were associated with an increased risk of recurrence. However, several gene panels were identified that may be useful for primary tumor methylation and increased risk of recurrence, with variable gene combinations using AP2, p16, RASSF1, GSTP, HIN1 and SOCS. Validation of this molecular technique on larger populations is necessary to confirm these data.

Methylation analysis of HNSN was performed to determine if prediction of breast cancer recurrence was possible with molecular detection of evidence of lymph node metastasis.

Frequent methylation of HNSN was common in both patients with and without breast cancer recurrence. No increase or pattern of methylation in HNSN hallmarked tumor recurrence. Contrary to our initial hypothesis, there was a trend for decreased methylation of HNSN in those patients with recurrence. This may be explained, in part, because patients with recurrence had fewer nodes resected, resulting in sampling error due to under-staging. Sampling of additional nodes may have revealed promoter methylation or histologic evidence that these patients had HPSN rather than HNSN.

The proportion of HNSN with detectable methylation was higher than expected for several genes but did not correlate with recurrence, as would be expected for micrometastatic disease. The process of sentinel node identification may account for this, whereby injection of dye/radiotracer into the breast for sentinel node identification caused movement of tumor cells or DNA into the node which is detected by molecular approaches, but not detected histologically.

Additional analyses were performed to investigate whether systemic adjuvant therapy could account for the lack of association between sentinel node methylation and subsequent recurrences. Frequency of methylation of the sentinel nodes did not correlate with breast cancer recurrence either in patients who received systemic therapy or those who did not. Thus, clinical treatment did not impact methylation outcome.

This study was designed to find clinically meaningful differences in sentinel node methylation frequency in breast cancer patients with recurrence. This study had 80% power to detect an absolute difference of 40% in the methylation of sentinel nodes in patients who recurred versus those who did not, with a 5% Type I error. The largest absolute difference we observed in a single marker was a 26% difference in the methylation of AP2, (i.e., 66% of nodes from non-recurring breast cancer patients methylated and 40% of the nodes from recurring patients methylated). Thus, our study suggests there are no large (>40%) differences in the methylation frequency or pattern in the sentinel nodes from early breast cancer lesions that predict recurrence.

Some genes (i.e., AP2 and ECAD) had a higher rate of methylation in the HNSN than in the corresponding primary breast tumor. Since, silencing of AP2 and ECAD are associated with metastatic disease [43, 44], a higher frequency of methylation might be expected at the nodal site than at the primary tumor. Conversely, some genes (i.e., RASSF1, Cyclin-D and SOCS) had significantly less methylation in the nodes than in the primary tumor. These genes have very different patterns in the node as compared to the primary tumor and may be the most likely candidate genes for future studies.

To determine whether the method of sentinel node identification or manipulation of primary tumor at the time of sentinel node resection influenced methylation patterns [45–47], sub-analyses were performed on patients who had a primary breast tumor resected at the time of sentinel node resection versus patients whose primary had been previously resected. No difference was observed between the groups (data not shown).

Our study has several limitations. The combination of low recurrence rate (6%) in the prospective study and the fewer number of nodes sampled in patients with recurrence are limitations of this study. Furthermore, maturation of the clinical outcome of this patient population may yield promising correlative work that is not yet appreciated with the current analysis. With longer follow-up data, a molecular profile of patients at risk for late recurrence (>5 years) may come to light.

Conclusion

Methylation of sentinel nodes is a frequent event in HNSN breast cancer patients. Frequency of methylation in the primary tumor correlated with clinical recurrence, yet the difference in this study was not statistically significant. Conversely, there was a trend towards an association between less sentinel lymph node methylation and higher risk of recurrence. Hypermethylation in sentinel nodes may have limited importance in predicting risk of recurrence within the first several years of diagnosis; however, further study with longer clinical follow-up is required.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Table 1

MSP primers (external) and conditions for annealing temperature

Gene	External flank up	External flank down	Size (bp)	Anneal temp.
AP2	AGTATTTTGTGTTTATTTAGAGAGTAGTTTATTTGGG	AAAAAAAAATCAAACCTCAAACCTATAACC	188	56
p16	GGGTTGGTTGGTTATTAGAGGGT	RACCRTAACCAACCAATCAACC	140	56
RASSF1	GTTTAGTTTGGATTGTGGGGGAG	CCCRCAACTCAATAAACTCAAACCTC	144	56
CDH1	GTGTTTTYGGGGTTTATTTGGTTGT	TACRACTCCAAAAACCCATAACTAACC	186	56
Cyclin D	YGAGGAAGYGGGTTTTTTTG	AACAAAAACCATCCTATTCTAAAAACTC	141	56
GSTp	GGGATTTTAGGGYGTTCCTTTTG	ACCTCCRAACCTTATAAAAAATAATCCC	159	56
SOCS	GATTGTTTTTYGAGTTGTTGGAGTATT	AAATTAAAAAAAATACRAACCAAATTCTC	161	56
HIN-1	GAGYGGGTAGGGTTTTTTTAGGAG	CTCACCRAAACTACAAAACAAAACCAC	184	56
FHIT	TTTAGTTGTTAATATTTTGAAGGTAGGG	AAAATACTCRAAACAAAACCCACC	164	56

Table 2

MSP primers (internal) and conditions for annealing temperature

Gene	MS	MAS	M. P. size (bp)	
AP2	GAGGGGCATATTCGTTTACGTC	AATAATCGAACCGACGTCGCG	105	
p16	TTATTAGAGGGTGGGGCGGATCGC	GAAAACTCCATACTACTCCCGCCG	91	
RASSF1	GGGTTCGTTTTGTGGTTTCGTTC	TAACCCGATTAAACCCGTACTTCG	76	
CDH1	TGTAGTTACGTATTTATTTTAGTGCGTC	CGAATACGATCGAATCGAACCG	112	
Cyclin D	GGAATCGTTGGGAGTTTGTTC	GAAACCCGTAAAACGACGCG	71	
GSTp	TTCGGGGTGTAGCGTCGTC	GCCCCAATACTAAATCAGACG	91	
SOCS	TGTGGAGTATTACGTGGCGGC	CGACACAACCTCTACAACGACCG	88	
HIN-1	GTTTCGTGGTTTTGTTCGGGTAGTC	GCAAAACCCCAAAAAACGACG	86	
FHIT	GCGGGTTGGGTTTTACGC	CGACGCCGACCCCACTAAA	61	
Gene	US	UAS	U. P. size (bp)	Anneal temp. (°C)
AP2	GTAGTTTTATTTGGGTGTGAGATTGAG	ACACAAATAATCAAACCAACATCACA	135	62
p16	GTTGGTTATTAGAGGGTGGGGTGGATTGT	AACCAAAAACCTCCATACTACTCCCCACCA	123	64
RASSF1	GGGGTTTGTTTTGTGGTTTTGTTT	AACATAACCCAATTAAACCCATACTTCA	81	64
CDH1	TGGTTGTAGTTATGTATTTATTTTAGTGGTGT	ACACCAAATACAATCAAATCAAACCAAA	120	64
Cyclin D	GGGAATTGTTGGGAGTTTGTTC	AACTCTTCAAAACCCATAAAACAACACA	80	60
GSTp	GATGTTTGGGGTGTAGTGGTTGTT	CCACCCAATACTAAATCACAACA	97	60
SOCS	GGGTGTGGGTTTGGGTTTTATGT	CCATAACAACACCAACCCCACTAAA	100	60
HIN-1	GAAGTTTGTGGTTTTGTTCGGGTAGTT	CACACAAAACCCCAAAAAACAACA	92	64
FHIT	GGGTGTGGGTTTGGGTTTTATGT	CCATAACAACACCAACCCCACTAAA	72	60

Baseline characteristics of patients, by recurrence for patients with methylation analysis and for all patients who had negative sentinel node dissection on or before 12/31/2004

Table 3

Characteristic	Patients with methylation analysis		All patients, surgery before 12/31/04	
	Recurred N = 13	Non-recurred N = 107	Recurred N = 16	Non-recurred N = 244
Mean Age (SD)	56.5 (13.1)	56.5 (13.1)	56.0 (12.8)	58.3 (11.1)
Sex—No. (%)				
Male	0 (0.0)	2 (1.9)	0 (0.0)	3 (1.2)
Female	13 (100.0)	105 (98.1)	16 (100.0)	241 (98.8)
Race—No. (%)				
White	7 (53.8)	86 (80.4)	8 (50.0)	205 (84.0)
Non-white	6 (46.2)	21 (19.6)	8 (50.0)	39 (16.0)
Tumor size	1.45 (0.95)	1.32 (0.70)	1.62 (0.96)	1.30 (0.72)
Post menopausal—No. (%)	10 (76.9)	77 (76.2)	12 (75.0)	174 (71.3)
Elston grade—No. (%)				
I	0 (0.0)	21 (20.4)	0 (0.0)	50 (20.5)
II	5 (33.3)	60 (57.7)	6 (37.5)	131 (53.7)
III	8 (67.7)	23 (22.1)	10 (62.5)	57 (23.4)
Histology—No. (%)				
DUC	11 (84.6)	72 (67.2)	14 (87.5)	165 (67.6)
LOB	0 (0.0)	8 (7.5)	0 (4.2)	24 (9.8)
Mix	2 (15.4)	21 (19.6)	2 (12.5)	40 (16.4)
Other	0 (0.0)	6 (5.6)	0 (0.0)	15 (4.1)
Lymphovascular invasion—No. (%)	3 (23.1)	4 (3.8)	3 (18.8)	10 (4.1)
ER Positive—No. (%)	6 (46.2)	78 (76.5)	8 (50.0)	180 (73.8)
PR Positive—No. (%)	7 (53.8)	69 (67.6)	8 (50.0)	166 (68.0)
Adjuvant chemo—No. (%)	7 (53.8)	30 (29.4)	10 (62.5)	75 (30.7)
Hormonal therapy—No. (%)	4 (30.7)	64 (59.8)	5 (31.3)	145 (59.4)
Local radiation—No. (%)	8 (61.5)	73 (68.2)	9 (56.3)	163 (66.8)

Characteristic	Patients with methylation analysis			All patients, surgery before 12/31/04		
	Recurred N = 13	Non-recurred N = 107	P value*	Recurred N = 16	Non-recurred N = 244	P value
Number of nodes dissected (median)	1	2	0.05	1	2	0.08
Tumor present at surgery—No. (%)	12 (92.3)	86 (80.4)		15 (93.8)	176 (72.1)	
Median follow-up time (days)	1,067	2,475		1,034	2004.5	

* P value for t tests for differences in means for continuous variables between recurred and non-recurred patients; P value for Fisher's exact test for differences in distributions of categorical variables between recurred and non-recurred patients

Table 4
Frequency of methylation in primary breast tumors and positive and negative sentinel lymph nodes

	SOCS	P16	RASSF1	FHIT	CYCLIND	HIN1	GSTP	AP2	CDHI
Tumor (N = 79)	76% (60/79)	44% (35/79)	81% (64/79)	58% (46/79)	52% (41/79)	45% (35/79)	42% (33/79)	24% (18/79)	6% (5/79)
Positive nodes (N = 32)	N/A	72% (23/32)	65% (20/31)	48% (14/29)	11% (3/28)	89% (25/28)	31% (9/29)	72% (21/29)	35% (8/22)
Negative nodes (N = 227), 50% collapsed	63%	57%	4%	6%	12%	51%	38%	59%	31%
Negative nodes N = 227 (%RM/%NRM)	62/59	38/53	0/4	54/56	0/11	54/48	23/36	38/55	15/29

Last row is percent methylation in recurrent nodes (RM) over percent methylation over non-recurrent nodes (NRM), with a non-significant trend for more methylation in the NRM. N/A, not assessed; N, number of samples

Methylation status of primary tumor and sentinel node. In all genes except for AP2, the expected methylation pattern was seen over 74% of the time

Table 5

Methylation status of tumor	Node status (N)	AP2	CYCLIND	ECAD	FHIT	GSTP	HIN1	p16	RASSF1	SOCS
TU	NM	39	2	19	16	16	20	18	0	8
TM	NU	7	35	3	19	17	13	10	63	21
TM	NM	11	6	2	27	16	22	26	1	39
TU	NU	22	36	55	17	30	24	26	15	11
Percentage of expected methylation pattern (%)		51%	97%	76%	80%	80%	74%	79%	100%	90%
Percentage of unexpected methylation pattern (%)		49%	3%	24%	20%	20%	25%	23%	0%	10%

TU, unmethylated tumor; TM, methylated tumor; NU, unmethylated node; NM, methylated node; N, number of samples

Table 6
Frequency distribution of number of samples for which a specific gene was methylated

	All nodes		Nodes with no matching tumor		Nodes with matching tumor	
	Recur N = 13	Non-recur N = 107	Recur N = 8	Non-recur N = 33	Recur N = 5	Non-recur N = 74
AP2	5 (0.38)	59 (0.55) ^a	1 (0.13)	13 (0.39)	4 (0.80)	46 (0.62) ^a
p16	5 (0.38)	57 (0.53)	2 (0.25)	16 (0.48)	3 (0.60)	41 (0.55)
ECAD	2 (0.15)	31 (0.29)	2 (0.25)	10 (0.30)	0 (0.00)	21 (0.28)
RASSF1	0 (0.00)	4 (0.04)	0 (0.00)	3 (0.09)	0 (0.00)	1 (0.01)
GSTP	3 (0.23)	38 (0.36) ^b	0 (0.00)	9 (0.27)	3 (0.60)	29 (0.39) ^b
CYCLIND	0 (0.00)	12 (0.11) ^a	0 (0.00)	4 (0.12)	0 (0.00)	8 (0.11) ^a
FHIT	7 (0.54)	60 (0.56) ^a	4 (0.50)	20 (0.61)	3 (0.60)	40 (0.54) ^a
HIN1	7 (0.54)	51 (0.48)	5 (0.63)	11 (0.33)	2 (0.40)	40 (0.54)
SSOCS	8 (0.62)	63 (0.59)	6 (0.75)	18 (0.56)	2 (0.40)	45 (0.61)

Node data was collapsed at 50%. N, number of samples and are also in (%)

^aOne sample had an unknown methylation status

^bTwo samples had unknown methylation status