Original Article

Is hypermethylation of SOX1 gene an independent prognostic marker in surgically resected non-small cell lung cancer?

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ABSTRACT

Background: Promoter hypermethylation of tumor suppressor genes presents promising markers for lung cancer diagnosis and prognosis. The purpose of this study was to determine the association between the promoter hypermethylation of multiple genes and 5-year survival rate in patients with Non-small cell lung cancer (NSCLC).

Materials and Methods: Primary tumor samples (n = 65), corresponding nonmalignant lung tissues (n = 65), and circulating blood were obtained from NSCLC patients who underwent curative surgery. Promoter methylation status in seven genes (RASSF1A, CDH13, MGMT, ESR1, DAPK, SOX1, and HOXA9) was quantified by using bisulfite pyrosequencing. Five-year survival data were obtained by a clinician. Cox proportional hazards models were used to analyze the associations between gene methylation status and overall patient survival.

Results: The 5-year survival of the patients with SOX1 aberrant tumor methylation was found to be statistically significantly shorter than for those patients without aberrant tumor methylation (P = 0.01). This effect was independent of TNM stage. No significant survival differences were associated with aberrant methylation in other genes tested in either of the two tissue types.

Conclusion: Our study shows that SOX1 promoter hypermethylation in NSCLC tumors is significantly associated with inferior survival, showing promise as a useful prognostic biomarker in patients with NSCLC.

KEY WORDS: 5-year survival rate, methylation, non-small cell lung cancer, SOX1

INTRODUCTION

For decades, lung cancer remains one of the most common causes of cancer-related death worldwide, associated with over 1.3 million deaths per year.[1] While the treatment options for non-small cell lung cancer (NSCLC) continues to evolve, the prognosis is still very poor.

The poor prognosis is associated with late disease diagnosis and the small number of effective drugs. Good prognostic biomarkers for lung cancer patients are still not available-even patients with Stage I NSCLC who undergo surgical resection have high risk of dying from recurrent disease, with a 5-year relapse rate of 35%–50%.[2]

It is well established that epigenetic control of gene expression plays an important role in carcinogenesis. Aberrant methylation of CpG dinucleotides is a commonly observed epigenetic modification in human cancer; Hypermethylation in the promoters of tumor suppressor genes is the mechanism of transcriptional silencing equivalent to mutations.[3] Epigenetic changes appear to be

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even more frequently found in human cancer than genetic mutations. These methylation changes have been extensively studied in lung cancer, with some of them have been pointed as potential biomarkers with clinical relevance for diagnosis, prognosis, and response to therapy.[4]

The establishment of new prognostic markers holds promise for making more informed choices for cancer treatment and more precise prediction of therapeutic response,[4,5] even though our study design described below did not support evaluation of prediction of therapeutic response. To achieve these aims, it will be necessary to demonstrate the specificity of epigenetic changes to lung cancerous cells, and to evaluate the sensitivity of detecting aberrations in easily accessible tissue.[6] This approach requires investigation of specific gene methylation patterns that might be helpful for identification of NSCLC prognostic markers. While a number of genes have been reported as aberrantly methylated in lung tumors,[6,7] we have focused on the seven genes frequently hypermethylated in lung cancer (RASSF1A, CDH13, MGMT, ESR1, HOXA9, SOX1, and DAPK), for which we have previously shown that their methylation is associated with clinicopathological features of NSCLC tumors.[6] Once the 5-year survival data collection has been completed, it opened the opportunity for us to investigate whether there is a correlation between promoter hypermethylation of these genes and 5-year survival rate in NSCLC patients after a surgical resection.

MATERIALS AND METHODS

In 2009, patients with resectable non-small cell lung cancer (NSCLC) were approached for participation in a study at the Institute for lung diseases, Clinical Center of Serbia, University of Belgrade. The study received the approval of the center's ethics committee, and all the participants provided informed consent. Sixty-five patients undergoing surgical resection for non-small cell lung cancer consented to tissue collection and a small amount of tumor specimen, normal lung tissue distant to the tumor, and blood samples were collected during surgery and immediately snap frozen for research purposes. None of the patients have received neoadjuvant chemotherapy or radiotherapy.

Demographic information was abstracted from medical records and additional information, was obtained through patient interviews. Patient follow-up was conducted by a research nurse or dedicated physician in order to obtain the data on 5-year survival. Biospecimens collection included primary tumor samples ($n = 65$), corresponding nonmalignant lung tissues ($n = 65$), and matching blood samples ($n = 51$). Five milliliters of blood was collected using EDTA vacutainers and stored at $-$ 20°C until processing. DNA was extracted from whole-blood samples by using a well-established method.[7] Similarly, DNA extraction from fresh frozen tumors using standard methods,[8] which we previously summarized.[6] The quality of DNA was analyzed by electrophoresis on agarose gel, while Nanodrop spectrophotometer (ThermoScientific Inc., Wilmington, DE, USA) was used for DNA quantification. The methylation analyses were carried out at the University of Minnesota Masonic Cancer Center using a well-established method of bisulfide sequencing. Briefly, genomic DNA is initially treated with sodium bisulfite, which converts unmethylated cytosines to uracil, while methylated cytosines stay unaltered. This bisulfite modification step was performed according to the manufacturer's protocol (Zymo Research, Irvine, CA, USA). Upon completion of bisulfite modification of DNA, a strand-specific polymerase chain reaction (PCR) product is generated to provide a suitable DNA template for pyrosequencing. Primer sequences and PCR conditions have been described elsewhere[9-11] and show that multiple products were sequence per each gene analyzed. Amplicons were resolved by agarose electrophoresis to confirm proper amplification and quality of product. Percent methylation for each CpG as well as average methylation across CpG's was calculated for each promoter using PyroMark software (Qiagen, Germantown, MD 20874, Maryland, USA).

Statistical analysis

Statistical analyses included calculations for each CpG site, as well as average over multiple CpG sites for each gene. Methylation values were not normally distributed, thus Wilcoxon signed-rank test on average methylation over all CpG sites within a gene were used to assess differences in methylation between tumor and normal tissue. When the analysis was repeated using individual CpG sites instead of average for each gene, the obtained results were similar. Compared to the normal tissue, tumor was considered hypermethylated when the average methylation across different CpG sites for a specific gene was more than 3 standard deviations higher than the corresponding average methylation observed in the normal tissue, as previously described.[6] Methylation in blood samples was compared between patients with hypermethylated tumors and those without by using Wilcoxon rank-sum test.

Cox proportional hazard regression was used to evaluate clinicopathologic factors (age, gender, smoking, histology type, histology grade, and TNM stage) as predictors of 5-year survival. Multivariate Cox proportional hazard regression was used to evaluate methylation in specific genes as predictors of 5-year survival. TNM was the covariate used in these models. Kaplan–Meier method (log rank test) was used to evaluate differences in overall survival for the groups, defined as having values above and below the cutoff, for methylation levels of each gene. The optimal cutoff values were previously estimated from the data, as described by Dardis.[12] R package version 0.5.5, which included Miscellaneous Functions for Survival Data package (survMisc) was used for these analyses.[12]

RESULTS

Patient characteristics are shown in Table 1. The majority were male (76.1%), with long-term smoking history (>40 pack years).

At the time of the surgery, 81.3% of the patients were current smokers, while the rest were former smokers. All patients were older than 55 years, with almost half being over 65 years old at the time of the surgery. The predominant histology type was squamous cell carcinoma (56%), followed by adenocarcinoma. The remaining histologies included giant-cell carcinoma ($n = 3$), atypical carcinoids ($n = 1$), mucinous carcinoma ($n = 1$), and carcinoma sarcomatoid ($n = 1$), while large cell carcinoma was not included in this study.

Average methylation across all CpG sites within a gene was significantly higher in tumor tissue compared to normal adjacent tissue for all genes evaluated in this study [Table 2]. The only exception was ERS1, where the use of the second primer did not reach statistical significance ($P = 0.07$). When blood samples were compared between the patients with tumor hypermethylation for a specific gene and those without hypermethylation, no statistically significant difference was observed for any of the genes examined. Therefore, these results show that elevated methylation levels observed in genes SOX1, RASSF1A, HOXA9, CDH13, and DAPK in NSCLC were cancer specific, while the lack of reflection of these methylation changes in patients blood indicate their poorly suitability for a screening test.

Five-year survival was not significantly associated with age, gender, smoking history, tumor histology or grade. The only statistically significant predictor of survival was TNM stage ($P = 0.011$), as shown in Table 3. Thus, TNM stage was the only covariate used to adjust Cox-proportional hazard models which evaluated the effect of tumor methylation level for specific genes on 5-year survival. For SOX1 gene, the model identified a cutoff value for tumor methylation at 29.11, which was higher that the average tumor methylation value of 26.44 [shown in Table 2]. This cutoff value that clearly distinguished two groups of patients in terms of 5-year survival: those with tumor methylation above the cutoff value [marked as Poz on Figure 1a showing survival curves] had poorer survival compared to those marked Neg, which had tumor methylation values below the cutoff. Conversely, when two groups were compared in Cox proportional hazard models adjusted for TNM stage, hazard ratio = 2.371 was observed with 95% confidence interval of 1.001–5.647, which was statistically significant ($P = 0.05$).

By comparison, the cutoff value of 9.01 identified for RASSF1A gene (second primer), was lower than the average tumor methylation observed for this gene [10.44, shown in Table 2]. The Kaplan–Meier survival curves [Figure 1b], did not show any favorable survival of the group with less methylated tumors (those below the 9.01 cutoff).

DISCUSSION

We used bisufite pyrosequencing method including 7-gene panel to compare methylation in patient-matched tumor and

Table 1. Descriptive data, *n*=46

		Frequency (%)
Grade	G1	17 (36.9)
	G2	18 (39.1)
	G3	7 (15.2)
	G4	4 (8.7)
Histology	Adenocarcinoma	13 (28.3)
	Squamous cell carcinoma	26 (56.5)
	Other	7 (15.2)
Stage	1	7 (15.2)
	2	22 (47.8)
	3	12 (26.1)
	4	5 (10.8)
Gender	Male	35 (76.1)
	Female	11 (23.91)
Pack years	<40	9 (19.6)
	Between 40 and 70	33 (71.2)
	>70	0
	Missing info	4 (8.6)
Estimated age at the operation	<55	0
	55-59	15 (32.6)
	60-64	9 (19.5)
	>=65	22 (47.8)

Table 2. Mean CpG methylation and standard deviation at all analyzed genes

	*P**	Normal	Tumor	Blood
CDH13	0.004147	6.91±4.32	11.26±11.73	6.2±2.16
ESR1, first primer	0.03165	3.9±1.28	5.34±7.27	2.76±1.01
ESR1, second primer	0.06499	3.01±0.84	4.78±8.69	2.13±0.78
MGMT	0.007723	1.81±1.76	6.33±13.52	1.32±0.39
DAPK	0.0006633	2.47±0.82	4.19±3.3	2.55±0.86
RASSF1A, first primer	0.0001317	1.39±0.55	11.4±17.81	1.17±0.18
RASSF1A, second primer	2.144e-05	2.17±1.71	10.44±15.83	1.39±0.39
SOX1	3.541e-11	9.78±5.95	26.44±18.88	9.22±3.25
HOXA9	< 2.2e-16	17.82±8.47	36.17±17.08	3.39±1.58

*p value from one-way ANOVA

Table 3: Association between five-year survival and clinical-pathological patients characteristics

		HR	95%CI	*P*
age		1.00314	(0.9533-1.056)	0.904
smoking		1.0002369	(0.9993-1.001)	0.619
gender **		0.6453	(0.2802-1.486)	0.304
Histology (type)***	adenocarcinoma	1.04552	(0.3570-3.062)	0.2045
	squamocellular Ca	0.51200	(0.1771-1.480)	
Histology (grade)	G2	1.26145	(0.5012-3.175)	0.7315
	G3	1.08075	(0.4300-2.716)	
	G4	1.52548	(0.6167-3.774)	
TNM stage*****	II	4.3053	(1.3045-14.209)	0.01057
	III	0.6060	(0.2321-1.582)	
	IV	0.7135	(0.3531-1.442)	

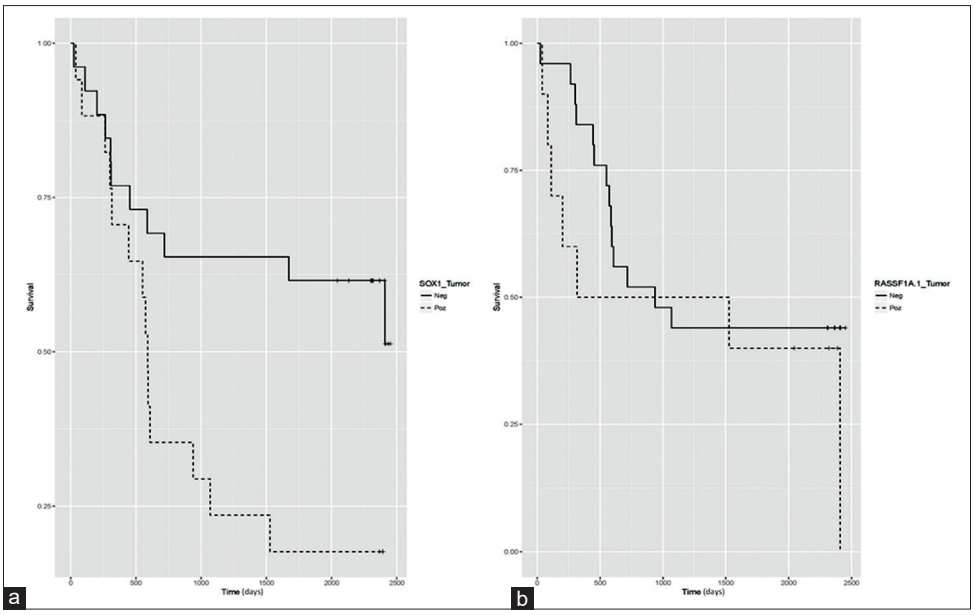

Figure 1: Kaplan–Meier survival curve, according to tumor methylation status for SOX1 (a) and RASSF1A (b) gene. Positive (Poz) and negative (Neg) survival curves depict subjects with tumors with methylation above (Poz) and those below (Neg) respective cutoff values for SOX1 and RASSF1A

nontumor tissue. We observed hypermethylation in tumor tissue for all the genes examined, while the methylation in normal tissue (adjacent to tumor) was similar to the one observed in matched patient's blood, rending blood not a suitable indicator of methylation in tumor tissue. We also evaluated whether the observed tumor hypermethylation was associated with 5-year survival of NSCLC in this patient population. Patients with hypermethylation in SOX1 gene in tumor tissue, which was above the average value of methylation across all tumors, had a significantly poorer survival.

SOX1 hypermethylation in NSCLC has been previously reported.[13] These authors have used methylation array method and identified SOX1 as the top five most hypermethylated genes, with over 5-fold larger methylation in tumor compared to normal tissue and confirmed these findings in two independent cohorts. Similarly, Zhao *et al*.[14] reported SOX1, as one of the several hypermethylated genes in stage I NSCLC. These represent rare reports of SOX1 methylation in NSCLC and none of these examined the relationship between SOX1 hypermethlation and survival. SOX1 antibodies have been reported as a serological marker of a specific small cell carcinoma type, but no relationship between this marker and survival has been observed.[15] In addition to lung cancer, SOX1 hypermethylation has been reported in several different malignancies, including ovarian,[16] esophageal,[17] prostate cancer,[18] and bladder cancer.[19]

SOX1 (sex-determining region Y [SRY] related high-mobility group bo × 1) encodes a transcription factor belongs to a SRY box gene family of genes, which plays an important role in embryonal development.[20] SOX1 is evolutionary conserved displaying similar protein structure in many species and has been implicated as one of the key regulators of neurodevelopment.[21] Its role in cancer has only recently begun to emerge through the reports of genetic silencing in a number of tumor tissues, as discussed above. The plausible mechanism underlying the role of SOX1 in carcinogenesis was proposed by Li and Li,[22] by suggesting that epigenetic silencing of SOX1 in NSCLC was associated with cell migration. Namely, the authors showed that restoration of SOX1 inhibited cell migration by regulating actin cytoskeletal remodeling in NSCLC.[22]

Several limitations of our study need to be noted. All participants in this study were either former or current smokers, thus tissue sample adjacent to tumor, which was considered normal tissue sample, may have been affected by long-term exposure to tobacco carcinogens. To limit the effect of cancer field, the normal tissue sample was attempted to be obtained distant from the resected tumor mass. However, in smokers, all of lung tissue would be exposed to tobacco carcinogens, thus we acknowledge that this approach may not have been impactful. The methylation observed in normal lung tissue was very similar to that in the matched blood sample, while being significantly lower compared to that observed in the tumor tissue, thus it is unlikely that this limitation impacted our results. On the other hand, the approach of comparing methylation in tumor to normal lung tissue has the potential to reflect the changes that occur along the specter of neoplastic transformation and thus increase the utility of such biomarkers for screening.[13] Small sample size and the use of samples from a single center warrant that our results need to be confirmed in our, preferably larger studies.

To our knowledge, this is the first report of association between SOX1 methylation and NSCLC survival, while there are only a handful of publications associating SOX1 tumor methylation and poorer survival in other malignancies, as noted above. In conclusion, hypermethylation of SOX1 gene in NSCLC correlates with poor prognosis of patients. It may serve as novel epigenetic-based diagnostic biomarker with further clinical impact for risk stratification of NSCLC patients. Caution is warranted due to small sample size and further studies are needed to confirm the specificity of this marker to NSCLC and therefore whether this could be utilized as a prognostic biomarker of NSCLC survival.

Authorship

All authors contributed to this manuscript.

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Conflicts of interest

There are no conflicts of interest.

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