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Aberrant promoter methylation of CDH13 and MGMT genes is associated with clinicopathological characteristics of primary non small cell lung carcinoma

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Abstract

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CONFLICT OF INTEREST STATEMENT:

None declared.

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Introduction—Systemic methylation changes may be a diagnostic marker for tumor development or prognosis. Here, we investigate the relationship between gene methylation in lung tumors relative to normal lung tissue, and whether DNA methylation changes can be detected in paired blood samples.

Material and methods—Sixty five patients were enrolled in a surgical case series of non-small cell lung cancer (NSCLC) at a single institution. Using bisulfite pyrosequencing, CpG methylation was quantified at five genes (*RASSF1A*, *CDH13*, *MGMT*, *ESR1* and *DAPK*) in lung tumor, pathologically normal lung tissue, and circulating blood from enrolled cases.

Results—The analyses of methylation in tumors compared to normal lung tissue identified higher methylation of *CDH13*, *RASSF1A*, and *DAPK* genes, while *ESR1* and *MGMT* methylation did not differ significantly between these tissue types. We then examined whether the three aberrantly methylated genes could be detected in blood. The difference in methylation observed in tumors was not reflected in methylation status of matching blood samples, indicating a low feasibility of detecting lung cancer by analyzing these genes in a blood-based test. Lastly we probed whether tumor methylation was associated with clinical and demographic characteristics. Histology and gender were associated with methylation at the *CDH13* gene, while stage was associated with methylation at *MGMT*.

Conclusion—Our results show higher methylation of *RASSF1A*, *CDH13*, and *DAPK* genes in lung tumors compared to normal lung. The lack of reflection of these methylation changes in blood samples from patients with NSCLC indicate their poorly suitability for a screening test.

Keywords

methylation; non-small cell lung cancer; *CDH13*; *MGMT*; clinicopathological characteristics

INTRODUCTION

Lung cancer is one of the most common causes of cancer-related deaths worldwide (1). Despite advances in diagnostic methods and treatment, prognosis remains poor.

Epigenetic control of gene expression plays an important role in carcinogenesis. Aberrant methylation of CpGdinucleotides is a commonly observed epigenetic modification in human cancer (2) and it appears to be even more frequent than genetic mutations in cancer. While tumors are characterized by genome-wide hypomethylation, hypermethylation in the promoter regions of tumor suppressor genes has been a proposed mechanism of transcriptional silencing equivalent to a mutational event (2).

DNA methylation changes in lung cancer are well established, and translation of this knowledge towards screening has the potential for significant health improvement. With the discovery of new disease screening markers it may be possible to achieve early diagnosis, more informed choices for cancer treatment and identify markers associated with prognosis and response to therapy. It has been proposed that in the near future, it may be possible to screen patients for lung cancer using DNA methylation signatures, to measure patient responses to treatment, to identify patients at increased risk, or to monitor interventions designed to reduce cancer incidence (3, 4). To achieve these aims it will be necessary to demonstrate the specificity of epigenetic change to cancerous cells, and to evaluate the sensitivity of detecting aberrations in easily accessible tissue (i.e. circulating blood).

A number of genes have been reported as aberrantly methylated in lung tumors. We have examined the literature to identify a panel of genes most frequently identified as hypermethylated in lung tumors, favoring those for which possible association between hypermethylation and clinicopathologic features was reported. This lead to investigation of a

panel of five genes (*RASSF1A*, *CDH13*, *MGMT*, *ESR1* and *DAPK*) using bisulfite pyrosequencing) to determine whether these methylation changes are specific to lung tumors, and test whether these changes are detectable in patients' blood samples. We further analyzed possible associations between DNA methylation and clinicopathologic features.

MATERIAL AND METHODS

Primary tumor samples (n=65), corresponding nonmalignant lung tissues (n=65) and matching blood samples (n=51) were obtained from NSCLC patients who had been treated in 2009 with curative resectional surgery at the Institute for lung diseases, Clinical Center of Serbia, University of Belgrade. This study was approved by the institution's ethics committee, and an informed consent was obtained from all study participants. Five mL of blood was collected using EDTA vacutainers and stored at -20 C until processing. DNA was extracted from whole blood samples by using the method described by Higuchi (5). Briefly, blood cells and platelets were lysed by adding equal volume of lysis buffer (0.32 M sucrose, 10 mM Tris-HCl pH 7.5, 5mM MgCl₂ and 1% Triton X-100). The lysate was centrifuged at 3,000 rpm for 10 min. The supernatant was removed and the pellet was again subjected to lysis and centrifugation. After supernatant removal, the pellet was suspended in 3 mL buffer (10 mM Tris-HCl, 0.4 M NaCl, 2 mM EDTA, and 25 µL proteinase K) followed by addition of SDS (0.7% final concentration). After overnight incubation at 37C, 1 mL of 6M NaCl was added, and the proteins were pelleted by centrifugation. Supernatant containing DNA was transferred into fresh tubes and centrifuged at 4000 rpm for 10 min. Supernatant was transferred and into new microcentrifuge tubes and equal volume of isopropanol was added. DNA becomes visible and is transferred, washed in 1 mL 70% ethanol and air dried, then resuspended in distilled water and analyzed for quality by electrophoresis on agarose gel and quantified by using Nanodrop spectrophotometer (ThermoScientific Inc., Wilmington, DE). The isolation of DNA from fresh frozen tumors was performed as previously described (3). The subsequent laboratory research was carried out at the Masonic Cancer Center, University of Minnesota, USA. The detection of DNA methylation is based on a treatment of genomic DNA with sodium bisulfite, which converts unmethylated cytosines to uracil, while methylated cytosines stay unaltered. Bisulfite modification of genomic DNA was performed according to the manufacturer's protocol (Zymo Research, Irvine, CA). A strand-specific polymerase chain reaction product is then generated to provide a suitable DNA template for pyrosequencing (Table 1). Primer sequences and PCR conditions for the *RASSF1A*, *CDH13*, *MGMT* and *DAPK* genes have been described previously (6–9). To facilitate data comparison with other publications, the sequences of the primers, as well as the sequences analyzed within each gene are shown in Table 1. 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).

Statistical analysis

Descriptive statistics were calculated for each CpG site as well as the average over multiple CpG at each gene. Medians and interquartile ranges (IQR) were calculated for continuous variables since the data was not normally distributed. Box plots were created to visually assess the distribution of values at each CpG site. Due to the non-normality of methylation values, differences in methylation between tissue types were assessed using the paired Wilcoxon signed-rank test on the average methylation values over all CpG sites within a gene region. Using individual CpG sites or the average CpG site produced similar results.

Tumor hypermethylation was assigned for those samples that had methylation values more than three standard deviations away from the mean of the normal tissue values for average

methylation over CpG sites within the gene. . We compared methylation in blood samples between patients with and without hypermethylation in the tumor sample using the Wilcoxon rank-sum test. The association between tumor hypermethylation and patient and tumor characteristics was assessed using Fisher's exact test. Variables assessed included estimated patient age(<55, 55–59, 60–64, >65), gender (male, female), smoking (current, former/never), pack years (<40, 40–70, >70), histology (squamous cell carcinoma, adenocarcinoma, and other), grade (<2, 2, >2) and stage (I, II, III, IV). Patient age was estimated since only year of birth was available; July 1st of the corresponding year was used to estimate age. All analysis was performed using SAS 9.2 (SAS Institute, Inc, Cary, NC).

RESULTS

Patient characteristics are presented in Table 2. There were more male than female patients. Most patients (81.3%) were current smokers and 15.6% were former smokers. Specific histological subtypes included 20 (30.8%) adenocarcinomas, 35 (53.8%) squamous cell carcinomas and 10 (15.4%) other subtypes of NSCLC (4 large cell carcinomas, 3 giant-cell carcinomas, 1 atypical carcinoids, 1 mucinous carcinoma and 1 carcinoma sarkomatoid).

The distribution of methylation across CpG sites for each gene is presented in Figure 1. Methylation percentages were fairly similar across CpG sites for each gene studied. Tumor samples showed more variation in methylation, with a substantial number of outliers for each CpG site. Blood samples tended to have consistently low methylation levels for all CpG sites.

Methylation at three of the five genes (*DAPK*, *RASSF1A* and *CDH13*) was significantly higher in tumor compared to normal lung tissue (Table 3). We next investigated whether methylation changes at these three genes could be detected in circulating blood samples. Cases were classified as either positive or negative for methylation at each gene in their tumor. There was no significant association between average promoter methylation of *DAPK*, *RASSF1A*, *MGMT* and *CDH13* in DNA isolated from blood and tumor methylation status (Table 4) However, there was a significant association between methylation in blood for those with hypermethylation in tumors for the *ESR1* gene..

Finally, we evaluated the clinical and demographic correlates of gene methylation (Table 5). Histology and gender were both associated with hypermethylation at *CDH13*, which was observed more frequently in women and patients with adenocarcinoma. In addition, stage was associated with hypermethylation at *MGMT*; patients with stage four disease were more likely to have hypermethylation at *MGMT*.

DISCUSSION

In this European-based case series of non small cell lung cancers we have evaluated the specificity of DNA methylation changes for lung tumors and investigated whether these changes are detectable in patients' blood. In our 5-gene panel we observed that three genes were significantly hypermethylated in lung tumors compared to matched normal tissue, however, these changes could not be detected in patients' blood samples. In contrast, one gene not found to be statistically significantly different in tumor compared to normal samples did have higher methylation levels in blood from individuals with hypermethylation in their tumor sample. This association should be confirmed in a larger study.

Several studies have investigated the clinical correlates of DNA methylation in selected genes in lung cancer (2, 3, 6, 10). Interestingly, the bulk of this literature used methylation-specific polymerase chain reaction (PCR) as the method for quantification of promoter methylation levels (2, 3, 6, 7, 11, 12, 18, 22). We are not aware of any study that directly

compared this method with the bisulfite pyrosequencing method we used to analyze the same methylation sites, thus it is not possible to address whether any discrepancies of our study compared to previous studies can be attributed to the differences in the methods used. However, the bisulfite pyrosequencing is regarded a sensitive method that allows the detection of low copy numbers (i.e. the presence of circulating tumor DNA in blood).

These five genes were selected as they have been previously reported to be hypermethylated in lung tumors (6, 11–15). Our results were not entirely consistent with these findings, as we failed to observe hypermethylation of *ESR1* and *MGMT* in NSCLC compared to adjacent normal lung tissue. The discrepancies may be attributed to differences in analysis methods used and interpretation of hypermethylation status, as well as different CpG sites investigated. This emphasizes the importance of reporting which methylation sites are being analyzed in defining methylation status, and it has been suggested that such reports would facilitate the comparison of gene-specific methylation data obtained in different studies (10). Another possible explanation for the inconsistencies in these finding may be the very low levels of methylation observed in the promoters of these two genes in our tumor series, obviating the detection of differences in methylation in adjacent normal lung tissues.

It is well established that double-stranded DNA fragments can be detected in considerable quantities in blood (serum or plasma) of cancer patients, due to the lysis of tumor cells (16). Thus, it is possible to detect the tumor-specific DNA alterations in patients' peripheral blood, and it has been demonstrated that they can serve as biomarkers for NSCLC detection in a Chinese population (17). Specifically, Zhang et al. identified a panel of 5 genes as markers for early diagnosis of NSCLC, these included RASS1A and CDH13. We therefore investigated whether hypermethylation of three genes observed in tumors would also be present in blood, but were unable to detect elevated DNA methylation in blood. This may be due to consistently low methylation levels across all CpG sites characteristic for our blood samples. It remains plausible that the selection of different methylation sites in promoter regions of the three genes would yield stronger methylation signals in blood samples, but given that we used average methylation values over all CpG sites within the gene, this is unlikely. Alternatively, the level of tumor DNA present in circulation may have been low, resulting in low gene specific methylation signal. The limitation of our study is that it did not include healthy controls. Thus, it remains plausible that differences in methylation in blood samples from healthy controls compared to blood samples from the NSCLC patients would reveal a valuable target for NSCLC screening. Withstanding this limitation, our results therefore suggest that these are not appropriate targets for early screening of NSCLC in our population.

We also investigated the correlation between aberrant methylation and clinicopathological characteristics. Our results demonstrate that methylation of certain genes may be associated with some clinicopathological characteristics of patients. Comparison of methylation of multiple CpG sites in the promoter and histopathology revealed that methylation of CDH13 ($p < 0.001$) was higher in adenocarcinoma. This is in contrast with previous a report that the prevalence of hypermethylation was indistinguishable between major histological subtypes of lung cancer (15). This discrepancy might be due to differences in exposure and different techniques used for analysis of DNA methylation (10). On the other hand, our study was consistent with the reported differences in the methylation patterns of squamous cell carcinoma and adenocarcinoma (18). This histology finding is in line with the report of Toyooka and colleagues (20).

Quantitative profiling in a study by Vaissiere et al (15) revealed correlation of RASSF1A hypermethylation with gender (with males showing higher levels of methylation), as well as Buckingham (10) (with females had lower methylation levels in RASSF1A). In our study

we found no association of *RASSF1A* hypermethylation and gender. The discrepancies may reflect different methods of analysis and interpretation of hypermethylation status as well as different CpG sites investigated (10,21).

Although aging has been associated with methylation of certain genes (18), we did not find correlation between the overall fraction of tumor methylation in *RASSF1A*, *CDH13*, *MGMT*, *ESR1* and *DAPK* genes and age. The lack of the association may be attributed to the narrow age range among our study participants (the youngest and the oldest age groups were only 10 years apart).

Further, we found no association of hypermethylation at any of our studied genes and smoking status, thus further studies are needed to elucidate the role of smoking in the epigenetic inactivation of specific genes in smokers (15, 22).

In conclusion, we confirm that hypermethylation of *RASSF1A*, *CDH13*, and *DAPK* plays a role in NSCLC pathogenesis, but also showed that these genes are not suitable markers for early detection of NSCLC in Central European Caucasians enrolled in our study. Thus other potential epigenetic biomarkers of NSCLC remain to be examined in order to identify those that might be utilized as a lung cancer screening tool. Our data indicate that some of the more commonly investigated epigenetic changes in lung cancer may not be well suited for this purpose, and that additional studies on larger gene panels are needed. In addition, our study revealed some interesting associations of hypermethylation in specific genes with clinicopathological features. However, these findings should be considered exploratory, due to size limitation, and remain to be validated in larger cohorts.

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Clinical Practice Points

DNA methylation changes in lung cancer are well established, and translation of this knowledge towards screening has the potential for significant health improvement. With the discovery of new disease screening markers it may be possible to achieve early diagnosis, more informed choices for cancer treatment and identify markers associated with prognosis and response to therapy.

A number of genes have been reported as aberrantly methylated in lung tumors. Here, we have investigated a panel of five genes (RASSF1A, CDH13, MGMT, ESR1 and DAPK) using bisulfite pyrosequencing to determine whether these methylation changes are specific to lung tumors, and test whether these changes are detectable in patients' blood samples. We also analyzed possible associations between DNA methylation and clinicopathologic features.

We observed that three genes were significantly hypermethylated in lung tumors compared to matched normal tissue, however, these changes could not be detected in patients' blood samples. We confirmed that hypermethylation of RASSF1A, CDH13, and DAPK play a role in NSCLC pathogenesis, but also showed that these genes are not suitable markers for early detection of NSCLC in Central European Caucasians enrolled in our study. Thus other potential epigenetic biomarkers of NSCLC remain to be examined in order to identify those that might be utilized as a lung cancer screening tool.

In addition, our study revealed some interesting associations of hypermethylation in specific genes with clinicopathological features which remain to be validated in larger cohorts.

Figure 1. Graphical representation (boxplots) comparing the DNA methylation levels in tumours (T), normal lung tissue (N) and blood samples (B). Results are obtained by the analysis of individual genes and CpG sites.

Table 1

Promoter methylation analysis in genes *ESR1*, *RASSF1A*, *CDH13*, *MGMT* and *DAPK* by pyrosequencing: shown are the amplification primers (forward and reverse), sequencing primer(s) used and the sequence that was analyzed.

	ESR1	RASSF1A	CDH13	MGMT	DAPK
Forward primer	TTTTTGGGTATTTTATAGTAG ATT	Biotin- ATAGGTTTTTGTATAATGAGT	TTGGGAAGTTGGTTGGTTG	TTGGTAAATTAAAGGTATAGA GTTTT	GAGGGTAGTTTAGTAAGTTG TTATAG
Reverse primer	Biotin- CAAAAAACAACCTTCCCTAAAC T	CTACACCCAAAATTTGCCATTAC	Biotin- ACAAACCCCTCTTCCCTACCT	Biotin- AAACAATCTACRCATCCT	Biotin- CCTCCAACCTACCCCTACCAAA
Sequencing primer	GGTTATTTTATAGTAGATTTT	ATCTAAATCTCTAAAAAAAC	GGAAAAATATGTTTAGTGTAG	GGAAAGTTGGGAAGG	TTTAGTAATGTGTATAGGT
Analyzed sequence	YGTGYGTTTTYGTGTTTTTTGGTY GTG	RCTAAAAATCRAAAACCCRCCT ATAACCCCRCCCRACCCRCRC TTACTAACRCCCAAAACCAAC RAAAACACRAACCCCAACCAAC C	TYGYGTGTATGAATGAAAY GTYGTYGGYGTTTTTA	YGTYGTGTTGGTTTGTATYGGT YGAAGGGTTA	GGGGYGTGYGYGTTTYGGGY GGAYGTATTTGGTTTITTYGGTY GGYGTGGGTGTGGGGYGAG TGGGTGTGTGYGGGGGTGTGY GYGGT
Sequencing primer 2	AATTTAGTTTTTATTAGTA				
Analyzed sequence 2	GYGAYGATAAGTAA				

Table 2

Subject demographic, tumor histology, grade, stage and smoking status data

Variable	Levels	Frequency (%)
Grade	<2	21 (33.3)
	2	23 (36.5)
	>2	19 (30.2)
	Missing	2
Histology	Squamous cell carcinoma	35 (53.8)
	Adenocarcinoma	20 (30.8)
	Other	10 (15.4)
Stage	1	16 (25.0)
	2	25 (39.1)
	3	19 (29.7)
	4	4 (6.3)
	Missing	1
Sex	Female	21 (32.3)
	Male	44 (67.7)
Smoking Status	Current	53 (81.3)
	Former	10 (15.6)
	None	2 (3.1)
	Missing	1
Pack years	< 40	18 (28.6)
	40–70	33 (52.4)
	>70	12 (19.0)
Estimated Age	<55	14 (21.5)
	55–59	17 (26.2)
	60–64	15 (23.1)
	>=65	19 (29.2)
	Mean (SD)	60.00 (6.86)

Table 3

Average difference in CpG methylation at five tumor suppressor genes in tumor tissue and pathologically normal lung samples removed at surgical resection

Gene	Methylation change (Methylation _{tumor} – Methylation _{normal lung})		
	N	Median (IQR ^a)	p-value ^b
CDH13	64	2.43 (–1.41, 6.9)	<0.001
RASSF1A	62	0.91 (–0.15, 11.7)	<0.001
ESR1	65	0.28 (–0.67, 1.46)	0.07
MGMT	64	0.12 (–0.27, 0.95)	0.08
DAPK	47	0.87 (–0.97, 2.26)	0.03

^aIQR = Interquartile range

^bWilcoxon signed-rank test of within-person difference in methylation (tumor vs normal lung)

Table 4
DNA methylation in circulating blood samples stratified by tumor methylation status

Gene	Cut-off value for hypermethylation	Average CpG methylation in circulating blood				p-value ^b
		Hypermethylation in tumor		No hypermethylation in tumor		
		N	Median (IQR) ^a	N	Median (IQR) ^a	
CDH13	18.52	9	6.56 (5.42–7.67)	41	6.22 (5.30–6.89)	0.26
RASSF1A	6.93	17	1.23 (1.18–1.45)	31	1.26 (1.16–1.45)	0.78
ESR1	6.92	4	3.37 (2.88–3.57)	46	2.36 (1.99–2.67)	0.02
MGMT	6.68	5	1.17 (1.15–1.24)	45	1.22 (1.09–1.47)	0.74
DAPK	5.20	8	2.74 (2.33–3.23)	25	2.52 (2.07–3.11)	0.95

^aIQR = Interquartile range
^bp-values from Wilcoxon rank-sum test

Table 5

Patient and tumor characteristics and tumor suppressor gene methylation

Variable	CDH13 Hypermethylation		RASFA1 Hypermethylation		MGMT Hypermethylation		DAPK Hypermethylation		ESR1 Hypermethylation	
	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No
Sex										
Male	5 (11.4)	39 (88.6)	15 (34.9)	28 (65.1)	6 (13.6)	38 (86.4)	8 (20.5)	31 (79.5)	3 (6.8)	41 (93.2)
Female	6 (28.6)	15 (71.4)	8 (38.1)	13 (61.9)	2 (9.5)	19 (90.5)	4 (26.7)	11 (73.3)	3 (14.3)	18 (85.7)
p-value ^a	0.15		0.99		0.99		0.72		0.38	
Histology										
Squamous cell carcinoma	1 (2.9)	34 (97.1)	12 (35.3)	22 (64.7)	6 (17.1)	29 (82.9)	7 (24.1)	22 (75.9)	2 (5.7)	33 (94.3)
Adenocarcinoma	8 (40.0)	12 (60.0)	9 (45.0)	11 (55.0)	2 (10.0)	18 (90.0)	4 (22.2)	14 (77.8)	4 (20.0)	16 (80.0)
Other	2 (20.0)	8 (80.0)	2 (20.0)	8 (80.0)	0 (0.0)	10 (100.0)	1 (14.3)	6 (85.7)	0 (0.0)	10 (100.0)
p-value	<0.001		0.40		0.40		0.99		0.18	
Tumor Grade										
<2	4 (19.0)	17 (81.0)	7 (33.3)	14 (66.7)	4 (19.0)	17 (81.0)	6 (35.3)	11 (64.7)	2 (9.5)	19 (90.5)
2	2 (8.7)	21 (91.3)	8 (36.4)	14 (63.6)	2 (8.7)	21 (91.3)	3 (15.8)	16 (84.2)	3 (13.0)	20 (87.0)
>2	4 (21.1)	15 (78.9)	8 (42.1)	11 (57.9)	2 (10.5)	17 (89.5)	3 (17.6)	14 (82.4)	1 (5.3)	18 (94.7)
p-value	0.47		0.90		0.64		0.36		0.87	
Clinical Stage										
I	1 (6.3)	15 (93.8)	6 (37.5)	10 (62.5)	2 (12.5)	14 (87.5)	3 (23.1)	10 (76.9)	1 (6.3)	15 (93.8)
II	6 (24.0)	19 (76.0)	9 (37.5)	15 (62.5)	5 (20.0)	20 (80.0)	7 (31.8)	15 (68.2)	3 (12.0)	22 (88.0)
III	3 (15.8)	16 (84.2)	7 (36.8)	12 (63.2)	0 (0.0)	19 (100.0)	2 (13.3)	13 (86.7)	2 (10.5)	17 (89.5)
IV	0 (0.0)	4 (100.0)	1 (25.0)	3 (75.0)	1 (25.0)	3 (75.0)	0 (0.0)	3 (100.0)	0 (0.0)	4 (100.0)
p-value	0.47		0.99		0.11		0.52		0.99	
Smoking status										
Former/Never	2 (16.7)	10 (83.3)	4 (33.3)	8 (66.7)	1 (8.3)	11 (91.7)	1 (9.1)	10 (90.9)	1 (8.3)	11 (91.7)
Current	9 (17.3)	43 (82.7)	19 (37.3)	32 (62.7)	7 (13.5)	45 (86.5)	11 (26.2)	31 (73.8)	5 (9.6)	47 (90.4)
p-value	0.99		0.99		0.99		0.42		0.99	
Pack Years smoking										

Variable	CDH13 Hypermethylation		RASSFA1 Hypermethylation		MGMT Hypermethylation		DAPK Hypermethylation		ESR1 Hypermethylation	
	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No
<40	2 (11.1)	16 (88.9)	8 (44.4)	10 (55.6)	2 (11.1)	16 (88.9)	2 (14.3)	12 (85.7)	0 (0.0)	18 (100.0)
40–70	7 (21.2)	26 (78.8)	13 (39.4)	20 (60.6)	5 (15.2)	28 (84.8)	8 (26.7)	22 (73.3)	5 (15.2)	28 (84.8)
>70	1 (8.3)	11 (91.7)	2 (18.2)	9 (81.8)	1 (8.3)	11 (91.7)	2 (25.0)	6 (75.0)	1 (8.3)	11 (91.7)
p-value	0.53		0.34		0.99		0.66		0.40	
Age										
<55	4 (26.7)	11 (73.3)	6 (40.0)	9 (60.0)	3 (20.0)	12 (80.0)	3 (25.0)	9 (75.0)	1 (6.7)	14 (93.3)
55–59	4 (18.2)	18 (81.8)	6 (28.6)	15 (71.4)	3 (13.6)	19 (86.4)	4 (20.0)	16 (80.0)	2 (9.1)	20 (90.9)
60–64	1 (6.7)	14 (93.3)	5 (33.3)	10 (66.7)	1 (6.7)	14 (93.3)	2 (18.2)	9 (81.8)	2 (13.3)	13 (86.7)
65	2 (15.4)	11 (84.6)	6 (46.2)	7 (53.8)	1 (7.7)	12 (92.3)	3 (27.3)	8 (72.7)	1 (7.7)	12 (92.3)
p-value	0.54		0.76		0.73		0.97		0.99	

^a all p-values derived from Wilcoxon rank-sum test