Original Article

Importance of Tumour Suppressor Gene Methylation in Sinonasal Carcinomas

(MS-MLPA / DNA methylation / sinonasal carcinoma / epigenetics / HPV)

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Abstract. Epigenetic changes are considered to be a frequent event during tumour development. Hypermethylation of promoter CpG islands represents an alternative mechanism for inactivation of tumour suppressor genes, DNA repair genes, cell cycle regulators and transcription factors. The aim of this study was to investigate promoter methylation of specific genes in samples of sinonasal carcinoma by comparison with normal sinonasal tissue. To search for epigenetic events we used methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) to compare the methylation status of 64 tissue samples of sinonasal carcinomas with 19 control samples. We also compared the human papilloma virus (HPV) status with DNA methylation. Using a 20% cut-off for methylation, we observed significantly higher methylation in *RASSF1, CDH13, ESR1* and *TP73* genes in the sinonasal cancer group compared with the control group. HPV positivity was found in 15/64 (23.4 %) of all samples in the carcinoma group and in no sample in the control group. No correlation was found between DNA methylation and HPV status. In conclusion, our study showed that there are significant differences in promoter methylation in the *RASSF1*, *ESR 1, TP73* and *CDH13* genes between sinonasal carcinoma and normal sinonasal tissue, suggesting the importance of epigenetic changes in these genes in carcinogenesis of the sinonasal area. These findings could be used as prognostic factors and may have implications for future individualised therapies based on epigenetic changes.

Received October 22, 2015. Accepted March 30, 2016.

This study was supported by the Ministry of Health of the Czech Republic – conceptual development of research organization MH CZ – DRO (FNHK) 00179906, by the Charles University Research Development Schemes, Czech Republic PRVOUK P37/11 and P27/LF1/1, and by the project of the Ministry of Health of the Czech Republic – conceptual development of research organization Biobanking and Biomolecular Resources Research Infrastructure BBMRI LM2010004.

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Abbreviations: bp – base pairs, DNMT – DNA methyltransferase, EDTA – ethylenediamine tetraacetic acid, ERα – oestrogen receptor α, FFPE – formalin-fixed, paraffin-embedded, HPV – human papilloma virus, ISH – *in situ* hybridization, LEC – lymphoepithelial carcinoma, MS-MLPA – methylation-specific multiplex ligation-dependent probe amplification, PCR – polymerase chain reaction, RT-PCR – reverse transcription and polymerase chain reaction, SCC – squamous cell carcinoma, SCNEC – small cell neuroendocrine carcinoma, SNC – sinonasal carcinoma, SNUC – sinonasal undifferentiated carcinoma, TNM – tumour, nodes, metastasis.

Introduction

Malignant tumours of the sinonasal tract are generally uncommon tumours of the head and neck area that account for approximately 3 % to 5 % of all upper respiratory tract malignancies (Haerle et al., 2013). In 2011, 51 new cases in men and 28 in women were diagnosed in the Czech Republic, giving incidence rates of 1.0/100,000 and 0.5/100,000 for males and females, respectively (Zvolský, 2014). These figures probably reflect the general status in Europe and worldwide (Turner and Reh, 2002; Syrjänen and Syrjänen, 2013). Diagnosis and treatment of these tumours pose several problems due to their very low incidence, histological diversity, production of non-specific symptoms in the early stages

that can simulate an inflammatory process and, because they have a variable prognosis depending on their histology, location and staging (López et al., 2013). There are established risk factors based on exposure to wood dust and various chemical substances such as nickel, formaldehyde, diisopropyl sulphate and dichloroethyl sulphide (Barnes et al., 2005). In general, sinonasal carcinomas (SNC) are radiosensitive; therefore, adjuvant or neoadjuvant radiation treatment may be indicated in advanced disease. Multidisciplinary surgical and medical oncologic approaches, which include ablation and reconstruction, have enhanced the survival outcome over the past few decades (Haerle et al., 2013).

Cancer has been considered as a disease driven by progressive genetic alterations, such as mutations involving oncogenes or tumour suppressor genes, as well as chromosomal abnormalities. However, more recently, it has been demonstrated that cancer is also driven by epigenetic alterations (Barton et al., 2008). Many different genes have been identified as being hypermethylated and silenced during tumour development. Because SNC are a group of aggressive tumours, it is very important to know their molecular parameters to establish diagnostic strategies and individualised therapies.

Epigenetics can be described as stable alteration in the gene expression potential that takes place during development and cell proliferation, without any changes in the gene sequence. DNA methylation is one of the most common epigenetic events taking place in the mammalian genome. This change, though heritable, is reversible, making it a possible therapeutic target. DNA methylation is a covalent chemical modification mediated by DNA cytosine methyltransferases, resulting in addition of a methyl group at the carbon-5 position of the cytosine ring in the CpG context (Das and Singal, 2004).

In the present study we used methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA), which allows simultaneous assessment of aberrant promoter methylation of a large set of genes. MS-MLPA requires only small quantities of short DNA fragments, making it very suitable for analysis of DNA isolated from formalin-fixed and paraffin-embedded (FFPE) tissue samples (Nygren et al., 2005). We also decided to compare the human papilloma virus (HPV) status with DNA methylation, because it seems that HPV-positive tumours have stronger association with promoter methylation compared with HPV-negative tumours. This could be explained by overexpression and increased activation of DNA methyltransferases DNMT1 and DNMT3b, which are both key proteins in the regulation of DNA methylation (van Kempen et al., 2014).

Material and Methods

Formalin-fixed, paraffin-embedded tissue samples of sinonasal carcinomas and normal sinonasal tissue were obtained from 83 patients: 64 patients with sinonasal carcinoma, and 19 patients with a non-malignant diagnosis. Only tumours primarily originating from the nasal cavity, maxillary sinuses and ethmoid complex were included. No tumours were found in the frontal or sphenoid sinuses. The samples of normal tissue (10 mucosal specimens from the nasal cavity and 9 from the maxillary sinus) were obtained from patients treated for a non-malignant diagnosis such as chronic rhinitis and sinusitis. The paraffin blocks were retrieved from the archives of the Fingerland Department of Pathology, University Hospital Hradec Králové; the Department of Pathology, General University Hospital, Prague, Czech Republic; and the Department of Pathology, University Hospital Olomouc, Czech Republic. All malignant epithelial tumours of the sinonasal tract were diagnosed between August 1995 and August 2014. All slides were reviewed by an experienced head and neck pathologist (J. L.) and the carcinomas were classified according to the current WHO classification (Barnes et al., 2005). The study was approved by the Ethics Committee of University Hospital Hradec Králové. The need for informed consent was waived by the review board in view of the retrospective nature of the study and long archival period of the FFPE tissue samples involved.

Each patient had recorded the gender, age at the time of diagnosis, smoking history (non-smoker vs. ex-smoker vs. current smoker), occupation (risky vs. non-risky), and tumour localization, including the nasal cavity, maxillary sinus, and ethmoid complex, laterality and pathological TNM. During the follow-up period (until October 2014), local recurrence, regional recurrence, distant recurrence, death, and tumour-related death staging were recorded. When radical surgery was not performed, clinical TNM staging was used instead. Treatment modalities were radical surgery, radiotherapy, and chemotherapy in various combinations.

The tumour types included squamous cell carcinoma (SCC) (conventional, verrucous, papillary, basaloid, spindle cell, acantholytic, adenosquamous), lymphoepithelial carcinoma (LEC), sinonasal undifferentiated carcinoma (SNUC), adenocarcinoma (intestinal-type, non-intestinal-type, salivary gland-type), and neuroendocrine tumours (typical carcinoid, atypical carcinoid, small cell neuroendocrine carcinoma (SCNEC)). Vascular invasion, perineural spread, status of resection margins (in the case of radical surgery), and microscopic findings in the surrounding mucosa were also noted.

DNA for methylation analysis was extracted from the FFPE tissue samples using a Qiagen (Hilden, Germany) DNA extraction kit.

The HPV status was analysed using HPV DNA *in situ* hybridization (ISH), HPV E6/E7 mRNA ISH, HPV DNA polymerase chain reaction (PCR) and typing, and HPV E6/E7 mRNA reverse transcription and polymerase chain reaction (RT-PCR) as previously described (Laco et al., 2015). For the purposes of statistical analysis, a case was considered HPV-positive if it was positive for HPV DNA ISH/PCR and/or HPV E6/E7 mRNA ISH/PCR. For details of HPV analyses, see Laco et al. (2015).

Methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA)

The present study used the MS-MLPA probe set ME001 (MRC-Holland, Amsterdam, The Netherlands), which can simultaneously check for aberrant methylation in 24 tumour suppressor genes (Table 1). Probe sequences, gene loci and chromosome locations can be found at http://www.mlpa.com. Individual genes were evaluated by two probes which recognized different *Hha*1 restriction sites in their regions. The experimental procedure was carried out according to the manufacturer's instructions, with minor modifications.

In short, DNA (100 ng) was dissolved up to 5 µl in TE-buffer (10 mM Tris·Cl; 0.5 mM EDTA; pH 9.0), denatured and subsequently cooled down to 25 °C. After adding the probe mix, the probes were allowed to hybridize (overnight at 60 °C). Subsequently, the samples were divided into two groups: in one half, the samples were directly ligated, while for the other half, ligation was combined with the *Hha*I digestion enzyme. This digestion resulted in ligation of the methylated sequences only. PCR was performed with all the samples using a standard thermal cycler (GeneAmp 9700, Applied Biosystems, Foster City, CA), with 35 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 1 min, with a final extension of 20 min at 72 °C. Aliquots of 0.6 µl of the PCR reaction were combined with 0.2 µl LIZ-labelled internal size standard (Applied Biosystems) and 9.0 µl deionized formamide. After denaturation, fragments were separated and quantified by electrophoresis in an ABI 3130 capillary sequencer and analysed using GeneMapper4.0 (both Applied Biosystems). Peak identification and values corresponding to peak size in base pairs (bp), and peak areas were used for further data processing. The methylation dosage ratio was obtained by the following calculation: $Dm = (P_x/P_{ctrl})Dig/ (P_x/P_{ctrl})Undig$, where Dm is the methylation dosage ratio, P_x is the peak area of a given probe, P_{ctrl} is the sum of the peak areas of all control probes, Dig stands for *Hha*I-digested sample and Undig for undigested sample. Dm can vary between 0 and 1.0 (corresponding to 0–100 % of methylated DNA). Based on previous experiments, a promoter was considered to be methylated if the dosage ratio was

Table 1. Genes included in the MS-MLPA KIT ME001 tumour suppressor

Gene	Name	Probes	Chromosomal location
TIMP3	TIMP metallopeptidase inhibitor 3	02255-L03752	22q12.3
APC	Adenomatous polyposis coli	01905-L01968	5q22.2
CDKN2A	Cyclin-dependent kinase inhibitor 2A	01524-L01744	9p21.3
MLH1a	MutL homologue 1a	01686-L01266	3p22.2
ATM	Ataxia telangiectasia mutated	04044-L03849	11q22.3
RARB	Retinoic acid receptor, β	04040-L01698	3p24.2
CDKN2B	Cyclin-dependent kinase inhibitor 2B	00607-L00591	9p21.3
HIC1	Hypermethylated in cancer 1	03804-L00949	17p13.3
CHFR	Checkpoint with forkhead and ring finger domains, E3 ubiquitin protein ligase	03813-L03753	12q24.33
BRCA1	Breast cancer 1	05162-L04543	17q21.31
CASP8	Caspase 8, apoptosis-related cysteine peptidase	02761-L02210	2q33.1
CDKN1B	Cyclin-dependent kinase inhibitor 1B	07949-L07730	12p13.1
KLLN	Killin, p53-regulated DNA replication inhibitor	02203-L08261	10q23.3
BRCA2	Breast cancer 2	04042-L03755	13q12.3
CD44	CD44 molecule (Indian blood group)	03817-L01731	11p13
RASSF1a	Ras association (RalGDS/AF-6) domain family member 1	02248-L01734	3p21.31
DAPK1	Death-associated protein kinase 1	01677-L01257	9q21.33
VHL	Von Hippel-Lindau tumour suppressor	03810-L01211	3p25.3
ESR1	Oestrogen receptor 1	02202-L01700	6q25.1
RASSF1b	Ras association (RalGDS/AF-6) domain family member 1	03807-L02159	3p21.31
TP73	Tumour protein p73	04050-L01263	1p36.32
FHIT	Fragile histidine triad	02201-L01699	3p14.2
IGSF4	Cell adhesion molecule 1	03819-L03848	11q23.3
CDH13	Cadherin 13, H-cadherin	07946-L07727	16q23.3
GSTP1	Glutathione S-transferase pi 1	01638-L01176	11q13.2
MLH1b	MutL homologue 1b	02260-L01747	3p22.2

≥ 0.20, which corresponds to 20 % of methylated DNA (Pavicic et al., 2011; Chmelařová et al., 2013).

CpG universal methylated and unmethylated DNA (Zymoresearch, Irvine, CA) were used in every run as controls.

Statistical analysis

Basic descriptive statistics were adopted for the analysis: median, mean, and 95% confidence interval for continuous data, and absolute and relative frequencies for categorical data. The relationship between gene methylation and other independent factors was analysed using the χ^2 test, Fisher's exact test, or logistic regression analysis. Kaplan-Meier and log-rank tests were used for survival analysis; Cox regression analysis was used to determine the influence of gene methylation upon survival. We considered $P < 0.05$ to be statistically significant. All statistical analyses were performed using the NCSS 8 statistical software program (NCSS, Keysville, UT).

Results

Promoter methylation using a 20% cut-off

In the present study we used the MS-MLPA probe set ME001 (MRC-Holland) to analyse samples from 64 patients with sinonasal carcinoma and 19 control samples. Using a 20% cut-off for methylation we observed statistically significantly higher methylation in the *RASSF1* ($P = 0.01$), *ESR 1* ($P = 0.03$), *TP73* ($P = 0.01$) and *CDH13* ($P < 0.001$) genes of patients with sinonasal carcinoma compared with the control group. For the *ATM, CDKN1B* and *GSTP1* genes, the methylation rate did not exceed the 20% threshold; the other genes also showed relevant differences in methylation between samples with sinonasal carcinoma and control samples (Fig.1).

Correlation with clinicopathological features

Clinicopathological data are listed below and summarized in Table 2. Due to a few missing clinical data, sums in the entire study sample or partial sums do not always add up to the total number of patients.

The median age of patients at the time of diagnosis was 62.5 years (range 23–83 years) in the carcinoma group and 56.5 years (range 24–74 years) in the control group. The carcinoma group consisted of 43 males and 21 females and the control group of 10 males and 9 females.

Of those in the carcinoma group with a known history, 26 patients were non-smokers, 11 ex-smokers, and 17 were current smokers. In only 7/54 (13 %) patients, occupational exposure to wood dust or other air pollutants/irritants was recorded (2× joiner, 1× wood industry worker, 1× miller, 1× locksmith, 2× rubber industry worker).

Concerning the entire study sample, most of the tumours arose in the nasal cavity, but SCCs were slightly more common in the maxillary sinus. The majority of the patients were diagnosed with advanced tumours and four patients had lung metastases (cM1) at the time of diagnosis.

The treatment modalities included radical surgery with radiotherapy (22×), radical surgery with chemo-radiotherapy (16×), radical surgery only (12×), radiotherapy only (5×), and chemo-radiotherapy (5×).

Microscopic typing of the tumours resulted in 44 SCCs, 13 adenocarcinomas, three neuroendocrine tumours, three SNUCs, and one case of LEC. Vascular invasion was found in 9/64 (14.1 %) tumours and perineural spread in 5/64 (7.8 %).

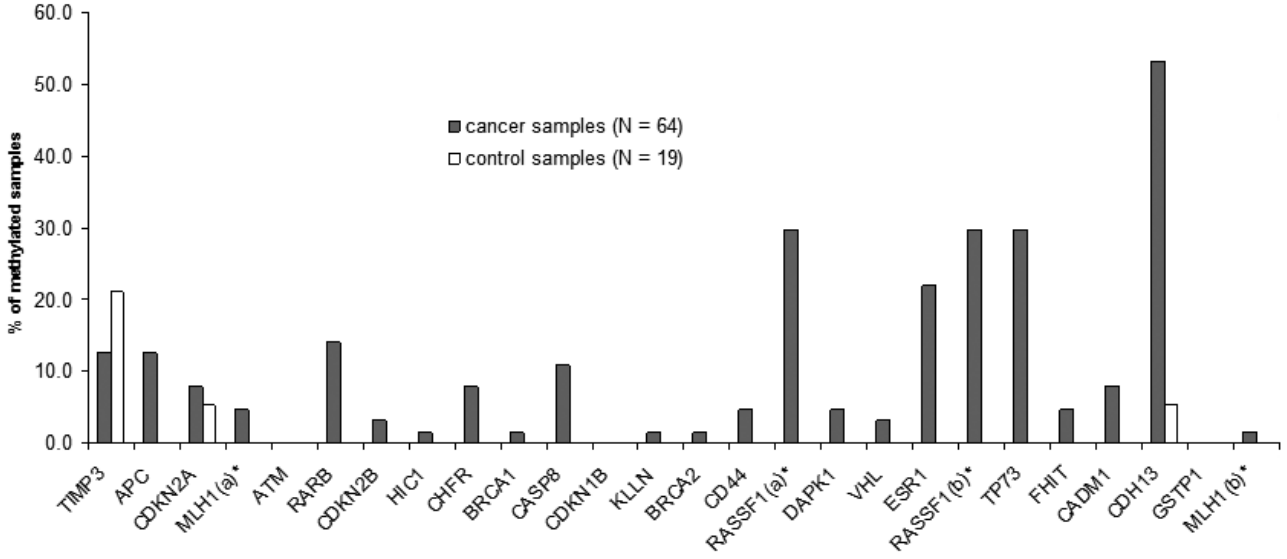

Fig. 1. Methylation of specific genes in cancer samples and control samples
Comparison of methylation frequencies (cut-off value 20 %) of the 24 analysed genes in cancer and control samples. *Two CpG loci (a and b) were analysed.

Table 2. Clinicopathological data of patients and presence of methylation

Clinical and Pathological Characteristics														
Sample No.	Gender	Age	Smoking	Occupation	cT	cN	cM	Typing	Grading	Invasion	Treatment	Recurrence	HPV status	
1	M	23	NS	NR	cT1	cN0	cM0	ACC	n/a	PN	S	L,D	neg.	
2	F	70	NS	NR	cTX	cN0	cM0	SCC	G2	no	S,R,C	L	neg.	
3	M	68	NS	risk	cT1	cN0	cM0	ITAC	G3	no	S,R	L	neg.	
4	M	57	S	risk	cTX	cN0	cM0	SCNEC	G1	no	S,R	L,Re	neg.	
5	F	74	NS	NR	cT4a	cN0	cM0	SCC	G2	no	R	n/a	neg.	
6	M	78	NS	NR	cT1	cN0	cM0	SCC	G2	no	S	L	neg.	
7	M	57	S	NR	cT1	cN0	cM0	NITAC	LG	no	S,R	no	neg.	
8	M	83	(S)	risk	cT2	cN0	cM0	ITAC	G1	no	S,R	no	neg.	
9	M	49	S	NR	cT4a	cN0	cM0	SCC	G1	no	S,R,C	L	neg.	
10	F	36	NS	NR	cT4a	cN0	cM0	ACC	n/a	no	S,R,C	L	neg.	
11	F	32	NS	NR	cT2	cN0	cM0	NITAC	LG	no	S,R	L	neg.	
12	F	81	NS	NR	cT1	cN0	cM0	SCC	G2	no	S,R	L	neg.	
13	M	64	NS	NR	cT3	cN0	cM0	SCC	G2	no	R,C	L	neg.	
14	M	65	NS	NR	cT3	cN0	cM0	ITAC	G2	no	S,R,C	D	neg.	
15	M	30	NS	NR	cT4a	cN0	cM0	SCC	G2	V	S,R	L	neg.	
16	M	51	S	NR	cT4b	cN0	cM0	SCC	G2	no	S,R	no	neg.	
17	M	53	NS	NR	cT3	cN2c	cM0	SCC	G2	no	S,R	no	neg.	
18	M	74	S	n/a	cT4a	cN2b	cM0	SCC	G2	no	R,C	L,Re,D	neg.	
19	M	76	NS	NR	cT4a	cN0	cM0	SCC	G2	no	S,R	L	neg.	
20	F	76	NS	NR	cT4a	cN0	cM0	SCC	G2	no	S,R	L	neg.	
21	F	54	NS	NR	cT4a	cN0	cM0	SCC	G2	no	S,R	L	pos.	
22	M	76	(S)	NR	cT2	cN0	cM0	SCC	G2	no	S,R	no	pos.	
23	M	27	NS	NR	cT4a	cN0	cM0	SCC	G3	no	S,R,C	L	pos.	
24	M	60	S	risk	cT4b	cN2b	cM0	SCC	G2	no	S,R	no	neg.	
25	F	58	NS	NR	cT2	cN0	cM0	SCC	G2	no	S,R,C	Re	neg.	
26	F	56	NS	NR	cT4a	cN0	cM0	SCC	G2	no	R	no	neg.	
27	M	65	(S)	NR	cT2	cN0	cM0	SCC	G3	V	S,R,C	D	pos.	
28	F	63	NS	NR	cT4a	cN0	cM0	SCC	G2	no	S	L,Re	neg.	
29	F	82	(S)	NR	cT4a	cN2b	cM0	SCC	G1	no	S,R	no	neg.	
30	M	82	(S)	NR	cT4b	cN2c	cM0	SCC	G3	no	R	no	neg.	
31	M	55	NS	NR	cT4a	cN2b	cM0	SCNEC	n/a	V	S,R,C	L,Re,D	pos.	
32	F	72	S	NR	cT4b	cN0	cM0	SCC	G1	no	S,R	no	neg.	
33	M	71	(S)	NR	cT4b	cN0	cM0	SCC	G2	no	R,C	no	pos.	
34	M	56	NS	NR	cT1	cN0	cM0	SCC	G1	no	S	no	pos.	
35	F	71	NS	risk	cT1	cN0	cM0	SCC	G1	no	S	no	neg.	
36	F	60	(S)	NR	cT3	cN0	cM0	ACC	n/a	V,PN	S	no	neg.	
37	F	38	(S)	NR	cT4b	cN2c	cM0	SCNEC	G3	no	R,C	L	neg.	
38	F	56	S	NR	cT4b	cN0	cM0	SCC	G3	no	R	no	pos.	
39	M	68	NS	risk	cT1	cN0	cM0	ITAC	G2	no	S,R,C	L	neg.	
40	F	70	S	NR	cTX	cN0	cM0	SCC	G2	no	S,R	no	neg.	
41	M	31	n/a	n/a	cTX	cN0	cM0	ACC	n/a	no	S	n/a	neg.	
42	M	57	S	NR	cTX	cN0	cM0	SCC	G3	no	S	no	pos.	
43	M	55	S	NR	cTX	cN0	cM0	ACC	n/a	V,PN	S,R	no	neg.	
44	M	51	(S)	NR	cTX	cN0	cM0	SNUC	n/a	no	R	no	neg.	
45	F	46	n/a	n/a	cTX	cN0	cM0	NITAC	LG	no	n/a	n/a	neg.	
46	M	65	NS	NR	cTX	cN0	cM0	SCC	G2	no	S	no	neg.	
47	M	64	n/a	n/a	cTX	cN0	cM0	SCC	G2	no	S	n/a	pos.	
48	M	65	(S)	NR	cT2	cN1	cM0	SCC	G3	V	S,R	no	pos.	
49	M	62	n/a	n/a	cT4a	cN0	cM0	SCC	G2	no	S	n/a	neg.	
50	F	66	n/a	n/a	cTX	cN0	cM0	SNUC	n/a	V	n/a	n/a	neg.	
51	M	75	n/a	n/a	cTX	cN0	cM0	SCC	G2	no	R,C	n/a	pos.	
52	M	67	n/a	n/a	cTX	cN0	cM0	SCC	G2	no	n/a	L	neg.	
53	F	61	NS	NR	cTX	cN0	cM0	ACC	n/a	PN	S,R,C	L	neg.	
54	M	65	n/a	n/a	cTX	cNX	cMX	SCC	G2	no	n/a	n/a	neg.	
55	M	46	S	NR	cT4a	cN0	cM1	SCC	G2	no	S,R,C	no	neg.	
56	M	55	n/a	NR	cT2	cN0	cM0	SCC	G3	no	S,R	no	neg.	
57	M	71	S	NR	cT3	cN2b	cM0	SCC	G3	no	S,R,C	no	neg.	
58	F	81	NS	NR	cT3	cN0	cM0	SCC	G2	no	S,R	no	neg.	
59	M	46	S	risk	cT3	cN0	cM0	SCC	n/a	V,PN	S,R,C	no	neg.	
60	M	60	n/a	n/a	cTX	cN0	cM1	SCC	n/a	no	S,R,C	D	pos.	
61	M	68	(S)	NR	cT4a	cN3	cM1	SNUC	n/a	V	S	no	neg.	
62	M	68	S	NR	cT4a	cN0	cM0	SCC	G3	no	S,R	no	pos.	
63	M	49	S	NR	cT4a	cN1	cM0	SCC	G3	no	S,R,C	no	pos.	
64	M	62	S	NR	cT3	cN1	cM1	LEC	n/a	no	S,R,C	D	neg.	

From left to right: M – male, F – female; S – smoker, NS – non-smoker, (S) – former smoker; NR – non-risk; ACC – adenoid cystic carcinoma, therapy, C – chemotherapy; L – local, Re – regional, D – distant; neg. – negative, pos. – positive; M – methylation; n/a – data not available

	Methylation of selected genes																						
	TIMP3	APC	CDKN2A	MLH1a	RARB	CDKN2B	HIC1	CHFR	BRCA1	CASP8	KLLN	BRCA2	CD44	RASSF1a	DAPK1	VHL	ESR1	RASSF1b	TP73	FHIT	CADM1	CDH13	MLH1b
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															M								
					M			M										M					

ITAC – intestinal adenocarcinoma, NITAC – non-intestinal adenocarcinoma, V – vascular, PN – perineural; S – radical surgery, R – radio-

Table 3. Clinicopathological characteristics vs. methylation of selected genes

Clinicopathological characteristic		Quantity	%	% of methylated samples						
				RASSF1a	ESR1	RASSF1b	TP73	CDH13	TIMP3	CASP8
Gender (N = 64)	female	21	32.8	38.1	14.3	33.3	14.3	38.1	4.8	14.3
	male	43	67.2	25.6	23.3	27.9	34.9	58.1	16.3	9.3
Age (N = 64)	≤ 45	7	10.9	42.9	14.3	57.1	42.9	28.6	14.3	14.3
	> 45	57	89.1	28.1	21.1	26.3	26.3	54.4	12.3	10.5
Smoking status (N = 54)*	non-smoker	26	48.1	30.8	23.1	26.9	23.1	42.3	7.7	7.7
	smoker	17	31.5	23.5	5.9	23.5	11.8	41.2	17.6	5.9
	former smoker	11	20.4	45.5	27.3	45.5	27.3	54.5	18.2	18.2
Occupation (N = 54)*	no risk	47	87.0	31.9	14.9	29.8	19.1	42.6	14.9	8.5
	risk	7	13.0	28.6	42.9	28.6	28.6	57.1	0.0	14.3
TNM clasiffication: cT (N = 48)*	cT1	8	16.7	12.5	37.5	12.5	25.0	50.0	0.0	12.5
	cT2	7	14.6	42.9	14.3	28.6	28.6	71.4	0.0	0.0
	cT3	8	16.7	25.0	12.5	37.5	12.5	25.0	12.5	0.0
	cT4	25	52.1	24.0	16.0	24.0	20.0	40.0	16.0	12.0
TNM clasiffication: cN (N = 63)*	cN0	51	81.0	33.3	23.5	29.4	31.4	52.9	11.8	9.8
	cN1	3	4.8	33.3	0.0	66.7	0.0	33.3	33.3	0.0
	cN2	8	12.7	12.5	0.0	25.0	0.0	37.5	0.0	25.0
	cN3	1	1.6	0.0	100.0	0.0	100.0	100.0	100.0	0.0
TNM clasiffication: cM (N = 63)*	cM0	59	93.7	32.2	20.3	30.5	25.4	50.8	11.9	11.9
	cM1	4	6.3	0.0	25.0	25.0	50.0	50.0	25.0	0.0
Typing (N = 64)	SCC	44	68.8	29.5	15.9	25.0	27.3	47.7	6.8	4.5
	AC	13	20.3	15.4	23.1	23.1	30.8	61.5	15.4	0.0
	SNUC	3	4.7	66.7	100.0	66.7	66.7	100.0	100.0	66.7
	SCNEC	3	4.7	66.7	0.0	66.7	0.0	33.3	0.0	100.0
	LEC	1	1.6	0.0	0.0	100.0	0.0	0.0	0.0	0.0
Grading (N = 51)*	G1	10	19.6	10.0	10.0	10.0	10.0	30.0	0.0	10.0
	G2	29	56.9	31.0	24.1	24.1	31.0	58.6	10.3	6.9
	G3	12	23.5	33.3	16.7	33.3	25.0	50.0	8.3	8.3
Invasion (N = 64)	vascular	9	14.1	55.6	33.3	44.4	44.4	55.6	44.4**	22.2
	perineural	5	7.8	40.0	0.0	40.0	0.0	0.0**	20.0	0.0
	no	53	82.8	26.4	18.9	28.3	26.4	52.8	7.5	9.4
HPV status (N = 64)	positive	15	23.4	40.0	13.3	33.3	26.7	60.0	6.7	6.7
	negative	49	76.6	26.5	22.4	28.6	28.6	49.0	14.3	12.2
Recurrence (N = 56)*	local	22	39.3	31.8	18.2	27.3	22.7	45.5	4.5	18.2**
	regional	5	8.9	20.0	0.0	20.0	0.0	40.0	0.0	40.0**
	distant	7	12.5	14.3	0.0	14.3	28.6	71.4	14.3	14.3
	no	29	51.8	27.6	20.7	27.6	20.7	44.8	17.2	3.4

AC – adenocarcinoma
*Due to a few missing clinical data, partial sums do not always add up to the total number of patients. **P < 0.05

The follow-up period ranged from one to 241 months (median 18 months). Local recurrence was found in 22/56 (39.3 %) tumours, 5/56 (8.9 %) recurred regionally, and 7/56 (12.5 %) patients developed distant metastases in the lungs. During the follow-up period, 31/59 (52.5 %) patients died, of whom 15/56 (26.8 %) due to the tumour.

HPV positivity was found in 15/64 (23.4 %) of all samples in the carcinoma group and in no sample in the control group.

The methylation results from the carcinoma specimens were compared with clinicopathological characteristics mentioned above (see Tables 2, 3). The *TIMP3* gene showed significantly higher methylation in patients with angioinvasion (P = 0.01). In SCC, methylation of *TIMP3* was associated with the age of the patients, being present mainly in younger patients (P = 0.01). The *CASP8* gene showed significantly higher methylation in patients with local recurrence (P = 0.05) and also in patients with regional recurrence (P = 0.01). The *CDH13* gene showed significantly lower methylation in patients with perineural invasion (P = 0.02). The presence of methylation of the *CDH13* gene was also associated with lower survival (Fig. 2). No correlation was found between DNA methylation and tumour type, stage, histological grade, smoking history or HPV status.

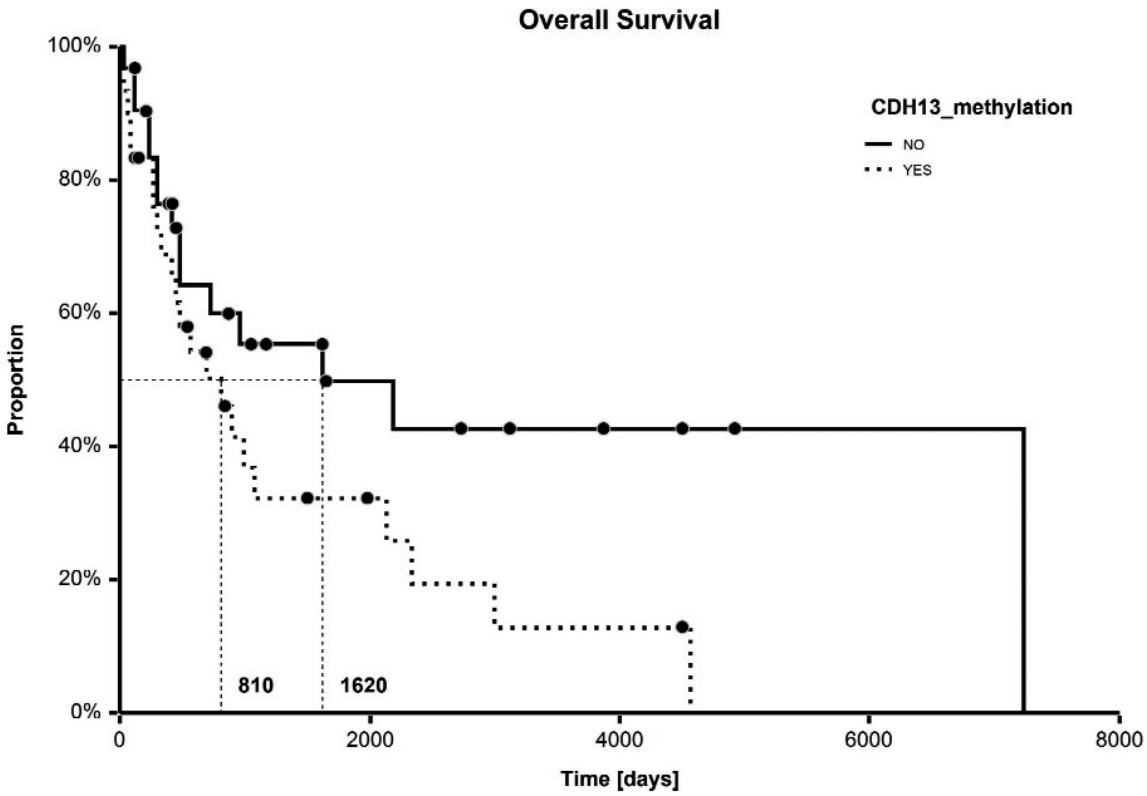

Fig. 2. Methylation of the *CDH13* gene as clinical outcome for patients with sinonasal carcinoma
Kaplan-Meier curve survival analysis indicates that tumours with the methylated *CDH13* gene showed poorer survival than those with the unmethylated *CDH13* gene (P = 0.057).

Discussion

Similarly to other cancers, malignant tumours of the sinonasal area have been shown to be a complex disease driven by different factors. Genetic aberrances such as variations in gene expression and mutation in cancer-related genes have been identified, but these do not fully explain carcinogenesis in the sinonasal area. Epigenetic changes are now being examined. In particular, aberrant DNA methylation is thought to play an important role in head and neck cancer. To date, numerous genes have been found to undergo hypermethylation. These methylation patterns persist and usually increase during the disease progression, which makes them a suitable tool to obtain early detection (Yang et al., 2015) predictive or prognostic information (Demokan and Dalay, 2011). The genes that are susceptible are the genes involved in cell cycle regulation, genes associated with DNA repair, apoptosis, drug resistance, detoxification, angiogenesis and metastasis (Das and Singal, 2004). The purpose of this study was to investigate promoter methylation of a set of common tumour suppressor genes in 64 tissue samples of sinonasal carcinoma and 19 control samples using MS-MLPA.

The *ATM, CDKN1B* and *GSTP1* genes never showed methylation above the 20% threshold, suggesting that promoter methylation of selected CpG loci of these tumour suppressors may not play an important role in carcinogenesis of the sinonasal area. On the other hand, *TIMP3, APC, CDKN2A, MLH1a, RARB, CDKN2B, HIC1, CHFR, BRCA1, CASP8, KLLN, BRCA2, CD44, RASSF1a, DAPK1, VHL, ESR1, RASSF1b, TP73, FHIT, CADM1, CDH13* and *MLH1b* did show promoter methylation to a varying extent above the 20% threshold. Methylation of some of these genes (particularly *TIMP3, CASP8* and *CDH13*) correlated with clinicopathological features such as age, perineural invasion, angiogenesis, regional and local recurrence, and survival, indicating that they could be used as prognostic markers in the future.

Methylation of *TIMP3* and *CDKN2A* was detected in both control and cancer samples. *TIMP3* methylation was present in more than 20 % of control samples. The presence of methylation can be associated with the nature of control samples. All control samples were obtained from patients with inflammation in sinonasal area. These findings correlate with the findings of Wang et al. (2014), who reported methylation of *TIMP3*, *GSTP1* and *14-3-3σ* in patients with chronic inflammation and in cancer patients.

The *RASSF1* gene encodes a protein similar to the RAS effector proteins. Loss or altered expression of this gene has been associated with the pathogenesis of a variety of cancers, which suggests the tumour suppressor function of this gene (Donninger et al., 2007). The inactivation of this gene was found to be correlated with the

hypermethylation of its CpG-island promoter region (Hesson et al., 2007). The protein was also shown to inhibit accumulation of cyclin D1, and thus induce cell cycle arrest (Donninger et al., 2007). As in other malignancies, we found significantly higher methylation of this gene (P = 0.01) in SNC, indicating that its methylation plays an important role in carcinogenesis of the sinonasal area as well. In oropharyngeal SCC, methylation of the *RASSF1* gene correlated with the HPV status (Dong et al., 2003). In our study we did not find any correlation between the HPV status and methylation of this gene, nor with any another genes. Our study does not confirm the hypothesis that HPV-positive tumours have stronger association with promoter methylation compared with HPV-negative tumours, at least in the case of sinonasal carcinoma.

The *ESR1* gene encodes the oestrogen receptor α, a ligand-activated transcription factor composed of several domains important for hormone binding, DNA binding, and activation of transcription. Oestrogen and its receptors are essential for sexual development and reproductive function, but are also involved in pathological processes including breast (Segal and Dowsett, 2014) and endometrial (Rahman et al., 2013) cancer. *ESR1* was previously shown to be widely methylated in breast cancer and hepatocellular carcinoma, and there was a statistically significant association with lower expression of oestrogen receptor α (ERα) (Geudet et al., 2009; Dai et al., 2014). In our study we also found statistically significantly higher methylation of the *ESR1* gene (P = 0.03) in patients with sinonasal carcinoma compared with the control group, indicating its importance in carcinogenesis of the sinonasal area.

The *TP73* gene encodes a member of the p53 family of transcription factors involved in cellular responses to stress and development (Levrero et al., 2000). This gene is hypermethylated in several types of head cancer, including oral SCC (Khor et al., 2014), oropharyngeal SCC (Noorlag et al., 2014) and nasopharyngeal carcinoma (Wong et al., 2003). Our study showed hypermethylation of the *TP73* gene also in sinonasal carcinoma. This finding confirms the important role of *TP73* methylation in carcinogenesis of head tumours.

The *CDH13* gene (*H-cadherin*) encodes a member of the cadherin superfamily. The protein acts as a negative regulator of axon growth during neural differentiation and also protects vascular endothelial cells from apoptosis due to oxidative stress, and is associated with resistance to atherosclerosis. The gene is hypermethylated in several types of head and neck cancers, including nasopharyngeal carcinoma (Sun et al., 2007) and oesophageal adenocarcinoma (Jin et al., 2008). In our study we found significantly higher methylation in the *CDH13* gene in sinonasal carcinoma compared to healthy controls (P < 0.001). This finding indicates that *CDH13* methylation is an important event in carcinogenesis of the sinonasal area as well. Methylation of this gene was correlated with poor patient survival, and hence it could potentially be used as a prognostic marker in the future.

In conclusion, our study showed that there are significant differences in promoter methylation in *RASSF1*, *ESR 1, TP73* and *CDH13* genes between sinonasal carcinoma and normal sinonasal tissue, suggesting the importance of epigenetic changes in these genes in carcinogenesis of the sinonasal area. These findings could be used as prognostic factors and may have implications for future individualised therapies based on epigenetic changes.

Acknowledgments

The authors are grateful to Ian McColl MD, PhD for assistance with the manuscript.

The authors declare that they have no conflict of interest.

References

Barnes, L., Eveson, J. W., Reichart, P., Sidransky, S. (eds) (2005) *World Health Organization Classification of Tumours. Pathology and Genetics. Head and Neck Tumours*. pp. 9-80, IARC Press, Lyon.

Barton, C. A., Hacker, N. F., Clark, S. J., O'Brien, P. M. (2008) DNA methylation changes in ovarian cancer: implications for early diagnosis, prognosis and treatment. *Gynecol. Oncol.* **109**, 129-139.

Chmelařová, M., Dvořáková, E., Špaček, J., Laco, J., Mžik, M., Palička, V. (2013) Promoter methylation of *GATA4, WIF1, NTRK1* and other selected tumour suppressor genes in ovarian cancer. *Folia Biol. (Praha)* **59**, 87-92.

Dai, B., Geng, L., Yu, Y., Sui, C., Xie, F., Shen, W., Zheng, T., Yang, J. (2014) Methylation patterns of estrogen receptor α promoter correlate with estrogen receptor α expression and clinicopathological factors in hepatocellular carcinoma. *Exp. Biol. Med. (Maywood, N. J.)*. **239**, 883-890.

Das, P. M., Singal, R. (2004) DNA methylation and cancer. *J. Clin. Oncol.* **22**, 4632-4642.

Demokan, S., Dalay, N. (2011) Role of DNA methylation in head and neck cancer. *Clin. Epigenetics* **2**, 123-150.

Dong, S. M., Sun, D. I., Benoit, N. E., Kuzmin, I., Lerman, M. I., Sidransky, D. (2003) Epigenetic inactivation of RASSF1A in head and neck cancer. *Clin. Cancer Res*. **9**, 3635-3640.

Donninger, H., Vos, M. D., Clark, G. J. (2007) The RASSF1A tumor suppressor. *J. Cell Sci.* **120**, 3163-3172.

Gaudet, M. M., Campan, M., Figueroa, J. D., Yang, X. R., Lissowska, J., Peplonska, B., Brinton, L. A., Rimm, D. L., Laird, P. W., Garcia-Closas, M., Sherman, M. E. (2009) DNA hypermethylation of ESR1 and PGR in breast cancer: pathologic and epidemiologic associations. *Cancer Epidemiol. Biomarkers Prev.* **18**, 3036-3043.

Haerle, S. K., Gullane, P. J., Witterick, I. J., Zweifel, C., Gentili, F. (2013) Sinonasal carcinomas: epidemiology, pathology, and management. *Neurosurg. Clin. N. Am*. **24**, 39-49.

Hesson, L. B., Cooper, W. N., Latif, F. (2007) The role of RASSF1A methylation in cancer. *Dis. Markers* **23**, 73-87.

Jin, Z., Cheng, Y., Olaru, A., Kan, T., Yang, J., Paun, B., Ito, T., Hamilton, J. P., David, S., Agarwal, R., Selaru, F. M., Sato, F., Abraham, J. M., Beer, D. G., Mori, Y., Shimada, Y. Meltzer, S. J. (2008) Promoter hypermethylation of CDH13

is a common, early event in human esophageal adenocarcinogenesis and correlates with clinical risk factors. *Int. J. Cancer* **123**, 2331-2336.

van Kempen, P. M., Noorlag, R., Braunius, W. W., Stegeman, I., Willems, S. M., Grolman, W. (2014) Differences in methylation profiles between HPV-positive and HPV-negative oropharynx squamous cell carcinoma: a systematic review. *Epigenetics* **9**, 194-203.

Khor, G. H., Froemming, G. R., Zain, R. B., Abraham, M. T., Thong, K. L. (2014) Screening of differential promoter hypermethylated genes in primary oral squamous cell carcinoma. *Asian Pac. J. Cancer Prev*. **15**, 8957-8961.

Laco, J., Sieglová, K., Vošmiková, H., Dundr, P., Němejcová, K., Michálek, J., Čelakovský, P., Chrobok, V. Mottl, R., Mottlová, A., Tuček, L., Slezák, R., Chmelařová, M, Sirák, I, Vošmik, M., Ryška, A. (2015) The presence of high-risk human papillomavirus (HPV) E6/E7 mRNA transcripts in a subset of sinonasal carcinomas is evidence of involvement of HPV in its etiopathogenesis. *Virchows Arch.* **467**, 405-415

Levrero, M., De Laurenzi, V., Costanzo, A., Gong, J., Wang, J. Y., Melino, G. (2000) The p53/p63/p73 family of transcription factors: overlapping and distinct functions. *J. Cell Sci*. **113,** 1661-1670.

López, F., Llorente, J. L., Costales, M., García-Inclán, C., Pérez-Escuredo, J., Alvarez-Marcos, C., Hermsen, M., Suárez, C. (2013) Molecular characterisation of sinonasal carcinomas and their clinical implications. *Acta Otorrinolaringol. Esp*. **64**, 289-296.

Noorlag, R., van Kempen, P. M., Moelans, C. B., de Jong, R., Blok, L. E., Koole, R., Grolman, W., van Diest, P. J., van Es, R. J., Willems, S. M. (2014) Promoter hypermethylation using 24-gene array in early head and neck cancer: better outcome in oral than in oropharyngeal cancer. *Epigenetics* **9**, 1220-1227.

Nygren, A. O., Ameziane, N., Duarte, H. M., Vijzelaar, R. N., Waisfisz, Q., Hess, C. J., Schouten, J. P., Errami, A. (2005) Methylation-specific MLPA (MS-MLPA): simultaneous detection of CpG methylation and copy number changes of up to 40 sequences. *Nucleic Acids Res.* **33**, e128.

Pavicic, W., Perkiö, E., Kaur, S., Peltomäki, P. (2011) Altered methylation at microRNA-associated CpG islands in hereditary and sporadic carcinomas: a methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA)-based approach. *Mol. Med*. **17**, 726-735.

Rahman, M. T., Nakayama, K., Rahman, M., Ishikawa, M., Katagiri, H., Katagiri, A., Ishibashi, T., Sato, E., Iida, K., Ishikawa, N., Nakayama, N., Miyazaki, K. (2013) ESR1 gene amplification in endometrial carcinomas: a clinicopathological analysis. *Anticancer Res*. **33**, 3775-3781.

Segal, C. V., Dowsett, M. (2014) Estrogen receptor mutations in breast cancer – new focus on an old target. *Clin. Cancer Res*. **20**, 1724-1726.

Sun, D., Zhang, Z., Van do, N., Huang, G., Ernberg, I., Hu, L. (2007) Aberrant methylation of CDH13 gene in nasopharyngeal carcinoma could serve as a potential diagnostic biomarker. *Oral Oncol.* **43**, 82-87.

Syrjänen, K., Syrjänen, S. (2013) Detection of human papillomavirus in sinonasal carcinoma: systematic review and meta-analysis. *Hum. Pathol*. **44**, 983-991.

Turner, J. H., Reh, D. D. (2002) Incidence and survival in patients with sinonasal cancer: a historical analysis of population-based data. *Head Neck* **34**, 877-885.

Wang, Y. J., He, L., Yuan, M., Tsang, W. W., Hao, L., Wang, M., Chow, L. W., Cheung, M. N., Liu, Q., Ng, E. L., Loo, W. T., Chow, C. Y., Bai, L. J., Yang, Z. (2014) Epigenetic changes of TIMP-3, GSTP-1 and 14-3-3 σ genes as indication of status of chronic inflammation and cancer. *Int. J. Biol. Markers* **29**, e208-214.

Wong, T. S., Tang, K. C., Kwong, D. L., Sham, J. S., Wei, W. I., Kwong, Y. L., Yuen, A. P. (2003) Differential gene methylation in undifferentiated nasopharyngeal carcinoma. *Int. J. Oncol.* **22**, 869-874.

Yang, X., Dai, W., Kwong, D. L., Szeto, C. Y., Wong, E. H., Ng, W. T., Lee, A. W., Ngan, R. K., Yau, C. C., Tung, S. Y., Lung, M. L. (2015) Epigenetic markers for noninvasive early detection of nasopharyngeal carcinoma by methylation-sensitive high resolution melting. *Int. J. Cancer* **136**, E127-135

Zvolský, M. (2014) *Incidence of Malignant Neoplasms in the Czech Republic in 2011*. IHIS, Volume **25**, Prague, Czech Republic. (in Czech)