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Methylation Level of *ASC/TMS1* and *MYD88* Genes in Healthy Larynx and Laryngeal Carcinoma Tissue

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Abstract

Background: There are no biomarkers for diagnosis, prognosis and treatment of patients with laryngeal carcinoma. Methylation changes of *ASC/TMS1* and *MYD88* genes, in healthy and cancer tissue, might be related with development and progression of cancer. The study explored is there a difference in gene's methylation in healthy and tumor tissue and does it correlate with protein expression.

Results: The total of 36 patients were enrolled in the study. Methylation of bisulphite converted DNA was quantified by pyrosequencing in fresh frozen cancer and adjacent non-malignant tissues. The overall methylation of *MYD88* gene is significantly higher in healthy tissue and this finding correlates with protein expression and the overall methylation of *ASC/TMS1* gene is unchanged but the protein expression of *ASC/TMS1* is significantly higher in cancer.

Conclusion: The methylation status of the *ASC/TMS1* and *MYD88* genes are promising prognostic biomarker candidates and may lead to earlier detection of laryngeal cancer.

Keywords: Larynx; Carcinoma; Innate immunity; Genes; Methylation

Introduction

Laryngeal Cancer (LC) is a second most common cancer of Head and Neck Cancer (HNC) group which develops from laryngeal squamous cell epithelium. It comprises 40% of all HNC and 1%-2% of all human malignancies [1]. The most important risk factors in LC development are tobacco smoking and excessive alcohol consumption comprising very important synergistic effect [2,3]. In past 20 years there is a slight decrease in the overall incidence of LC due to global anti-smoking campaign especially in USA and European countries. Unfortunately it has no significant influence on overall patient survival which is not markedly improved as there is still a great number of local and regional recurrences, advanced metastases and secondary primary tumors [4]. Patient prognosis depends mostly on staging status at presentation [5-7]. Last few decades it became obvious that chronic inflammation predisposes to different forms of cancer. Pathogens as bacteria and viruses activate innate immune mechanisms which enables their elimination from the organism [8]. Some pathogens can change innate immune mechanisms and lead to the chronic inflammation. Chronic inflammation has protumorigenic effect and enhances the risk of tumorigenesis [9]. Important examples are *H. pylori* infection and development of gastric carcinoma [10], Hepatitis B and C virus infection and hepatocellular carcinoma [11], Human papilloma virus in development of oropharyngeal carcinoma [12,13]. The profile of expression of different molecules engaged in activation and modulation of immune response and inflammation, their enhanced accumulation and decreased activation determine the balance between protumorigenic and antitumorigenic microenvironment [8]. Cytokines control the inflammation through activation of different signal pathways by inducing protumorigenic immunity (IL-12, TRAIL, IFNγ), tumor development (IL-6, IL-17, IL-23), tumor growth and survival (TRAIL, FasL, TNF-α, EGFR, TGF-β). Generally, we can say that carcinoma is result of the change in signal pathways which participate in the cell proliferation regulation. Key factors in relationship between chronic inflammation and carcinogenesis are transcription factors (NF-κB i STAT3) and proinflammation cytokines (IL-1β, IL-6, IL-23 i TNF-α) [14].

Innate immunity mechanism is the first line of organism defense and it is regulated by germline-encoded Pattern Recognition Receptors (PRRs) expressed by cells, including macrophages, monocytes, dendritic cells, neutrophils and epithelial cells. The most studied PRRs are membrane-bound Toll-Like Receptors (TLRs) which recognize Pathogen-Associated Molecular Patterns (PAMPs) and intracellular NOD-Like Receptors (NLRs) which recognize PAMPs as well as Danger-Associated Molecular Patterns (DAMPs).

Activation of those receptors lead to complex signal pathway activation with resulting transcriptional factors NF-κB i AP-1 activation and synthesis and producing of proinflammatory cytokines and chemokines. Activation of NLRs results in inflammasomes formation which enables inactive to active caspase transformation and pro-IL-1β maturation. NLRs and TLRs pathways are connected so activation of TLRs pathway enhance producing of pro-IL-1β which mature by NLRs signaling pathway activation [15].

There are two genes, *ASC/TMS1* (Apoptosis-associated speck like protein containing a caspase recruiting domain/Target of methylation

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induced silencing-1) gene and *MYD88* (Myeloid differentiation primary response 88) gene, which both encode adaptor molecules with important roles in signal pathways activated by organism response to inflammation or injury.

Adaptor molecule ASC is involved in signal pathway characterized by formation of molecular platforms inflammasomes which activate NLRs and control maturation and secretion of proinflammatory cytokines Interleukin-1β (IL-1β) and Interleukin-18 (IL-18).

From all NLRs NLRP3 is currently the most fully characterized in creation of NLRP3 inflammasomes. NLRP3 inflammasomes consists of NLRP3 scaffold, the ASC adaptor molecule and caspase-1. It is activated upon exposure to specific ligands like pathogens, toxins, crystals, PAMPs and DAMPs. Upon NLRP3 activation, NLRP3 oligomerization leads to the PYD domain clustering homotypic interaction with the PYD domain of adaptor molecule ASC, and interaction of the CARD domain of adaptor molecule ASC with CARD domain of the procaspase-1. Procaspase-1 permits auto cleavage and formation of the active caspase-1 which leads to the maturation and secretion of Interleukin-1β (IL-1β) [8,15,16].

From the literature it is known that the mechanisms involved in NLRP3 activation are not well established and still intensely debated but mostly acceptable are three main models. First model explain that extracellular ATP stimulates opening of ATP-dependent ion channel of the cell membrane and in that way pore formation in the cell membrane allows direct NLRP3 inflammasomes activation by NLRP3 cytosol agonists. Second model propose as activators particulate structures (urates, silica, asbestos) which activate phagocytes and by lysosomal damage release lysosomal contents consequently activating NLRP3 inflammasome formation. According to third model all NLRP3 agonists trigger the generation of Reactive Oxygen Species (ROS) and this pathway activate NLRP3 inflammasome formation [17].

On the other hand, adaptor molecule *MYD88* has a key role in TLRs signaling pathway and enables signal transduction cascade with final activation of transcription factor NF-κB and secretion of proinflammatory cytokines like Interleukin-6 (IL-6), Tumor Necrosis Factor Alpha (TNF-α) and Interleukin-12 (IL-12). The signal transduction begins by biding adaptor protein MYD88 with intracellular TLR domain, activation of kinase IRAK-4, 2, and 1 (IL-4,2,1 receptor associated kinase) and TRAF6 (receptor associated factor 6) phosphorylation, which subsequently activate TAK1 (TGF-β activated kinase 1). TAK1 phosphorylates IKKβ leading to degradation of IκB inhibitory protein dissociating it from NF-κB, thus allowing NF-κB to translocate to the nucleus and finally activate transcription [18].

It is well known that some epigenetic mechanisms, like DNA methylation, can change gene expression by methylation of CpG islands in the regulatory regions of the genome. As those islands, rich with CG dinucleotides (>55%) are situated in regulatory gene regions they affect the transcription of genes. If CpG islands are unmethylated genes are expressed and if they are hypermethylated the expression of the genes is repressed (gene silencing). The inflammation induce DNA Methyltransferase (DNMT) dependent DNA methylation which subsequently leads to silencing of the genes associated with carcinogenesis [19].

In this study, we hypothesized that the changes in the methylation status of promoter regions of *ASC/TMS1* and *MYD88* genes, responsible for activation and regulation of inflammation in healthy and laryngeal cancer tissue, might be related with development and progression of the laryngeal cancer. The aim of our study is to investigate is there a difference in the methylation status of this genes in healthy and tumor tissue and does it correlate with protein expression.

In recent literature the methylation status of this genes has not yet been analyzed in laryngeal cancer and the pyrosequencing method used to determinate methylation status of this genes has not been used in other cancers studies.

Materials and Methods

In this study, during routine surgical procedure in the treatment of laryngeal cancer, we have collected 36 samples of cancer tissue with related histologically healthy/not tumor involved tissue. All samples of cancer and healthy/not tumor involved tissue were analyzed histopathologically. Cancer tissue was analyzed to determine the value of cancer density and only cancer tissue with >70% of tumor cells was used for further analysis. Adjacent healthy/not tumor involved tissue was analyzed to confirm lack of malignant cells. We started research after applying and receiving approval from Ethics Committee of University Hospital Center Zagreb and all procedures followed were in the accordance with the ethical standards of the Committee. The study was performed in the Department of Otorhinolaryngology and Head and Neck Surgery University Hospital Center Zagreb and all molecular researches were done in the Department for Molecular Medicine, Laboratory for Advanced Genomics Ruđer Bošković Institute in Zagreb. All patients signed informed consent prior to a surgical intervention.

Analysis of DNA methylation by pyrosequencing

The methylation status of both genes promoters was analyzed by pyrosequencing method which as quantitative analysis of DNA CpG methylation enables precise expression of percentage of methylated DNA in the analyzed sample tissue. This method offers a robust, versatile platform with rapid quantitative results without the onerous time commitment, high costs and technical difficulty of alternative methods.

First step was isolation of genomic DNA of interest by using commercially available DNA isolation kit (Qiagen, Hilden, Germany; DNeasy Blood and Tissue Kit). Genomic DNA of interest was submitted to bisulfite conversion in which treating genomic DNA with sodium bisulfite selectively converts cytosine to uracil; however 5-methylcytosine was protected from deamination and the CG sequence was preserved in downstream reactions. Unmethylated cytosine through conversion by deamination into uracil was finally converted into thymine with epigenetic change in DNA sequence. Bisulfite conversion was performed by using commercially available kit (Qiagen, Hilden, Germany; Epitect® 96 bisulfite kit). DNA sequence for Polymerase Chain Reaction (PCR) primers were acquired from free genetic sequence database GenBank (National Center for Biotechnology Information, NCBI). Identification of *MYD88* gene in the database was NG_016964 and of *ASC/TMS1* gene was AF184072.1. Primer designing started by determination of promoter DNA sequence of interest of *ASC/TMS1* gene and *MYD88* gene. After detecting Region Of Interest (ROI) of both genes primers were designed. Primers for PCR of bisulfite converted DNA and pyrosequencing were designed using PyroMark Assay Design Software 2-0 (Qiagen, Hilden; Germany). Following pyrosequencing, pyrograms were analyzed by Pyro Q-CpG™ program (Qiagen, Hilden; Germany). This program can automatically determine individual methylation frequencies for all CpG islands as well as to evaluate the assays quality. Pyrograms which did not satisfy by quality were excluded from the study.

The results of the pyrosequencing of DNA sequence showed

percentage of DNA methylation rate in analyzed sequence by using paired *t*-test (p-values<0.05 were statistically significant) in GraphPad prism 5.0 program.

Extraction of total proteins from laryngeal cancer tissue and healthy/not tumor involved tissue

Proteins from cancer tissue and healthy/not tumor involved tissue were extracted by Passive Lysis 5x Buffer (Promega, Fitchburg, Wisconsin, USA). By Western Blotting procedure particular proteins were identified from the mixture of protein extracted from the samples. By SDS-PAGE (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis) proteins were separated according their electrophoretic mobility. Following separation, proteins were transferred from gel to nitrocellulose membrane using Trans Blot Turbo™ Transfer System (BIO-RAD, Hercules, California, USA). Finally, protein of interest were labeled by primary and secondary antibodies and the signal was detected by the method of chemiluminescence using reagent Clarity™ Western ECL Blotting Substrates (BIO-RAD, Hercules, California, USA). In order to quantify detected signal we used free ImageJ 1.49v software. The values of tested proteins (MyD88 and ASC/TMS1) were normalized with the values of control protein (vinkulin) from the adjacent gel. Normalized values were statistically analyzed in GraphPad Prism 5.0 program (GraphPad Software, San Diego, USA) by using 2-way Analysis of Variance (2way ANOVA test) and paired *t*–test. P-values less than 0,05 were regarded as statistically significant.

Results

The total of 36 patients participated in this study; 33 male patients and 3 female patients. All of them had Squamous Cell Carcinoma (SCC) of the larynx with different grades of differentiation from grade I to grade III. As the most important variable for overall survival of those patients beside differentiation grade is tumor localization, tumor size, and existence or absence of tumor metastasis and in all patients the disease staging was defined using UICC TNM (Union for International Cancer Control Tumor Node Metastasis) classification.

Results of analysis of *MYD88* gene methylation status in healthy/not tumor involved and tumor tissue

To accomplish differential methylation analysis of *MYD88* gene in healthy/not tumor involved and tumor tissue, the methylation status of CpG islands of gene promoter region was performed (Table 1).

According to nucleotide sequence of DNA in *MYD88* gene 3 Regions of Interest (ROI) were defined. In region of interest 1 (ROI_1) we detected 4 CpG islands, in Region of Interest 2 (ROI_2) 3 CpG islands, and in Region of Interest 3 (ROI_3) 3 CpG islands. In promoter region of *MYD88* gene in ROI_1 in CpG1 island we found that the average methylation status is slightly higher in healthy/not tumor involved tissue than in cancer tissue without statistically significant difference (p>0.05; p=0.276). In CpG2 island we found that the average methylation status is statistically significantly lower in cancer tissue than in healthy/not tumor involved tissue with statistical significance of p=0.0267 (p<0.05). In CpG3 and CpG4 islands of promoter region of *MYD88* gene there was no statistically significant difference in methylation status between cancer and healthy/not tumor involved tissue of the larynx with p-values p=0.9803 (p>0.05) and p=0.2048 (p>0.05), respectively. In ROI_2 the methylation status of CpG5 island and CpG6 island showed there was no statistically significant difference between cancer and healthy/not tumor involved tissue of the larynx with p-values p=0.2302 (p>0.05) and p=0.1870 (p>0.05), respectively. In the same ROI_2 in the promoter region of *MYD88* gene in CpG7 island we found statistically significant difference in the methylation status between cancer and healthy/not tumor involved tissue. We found that the methylation status of CpG7 in promoter region of *MYD88* gene was statistically significantly lower in cancer tissue in relation to healthy/not tumor involved tissue according to p-value, p=0.0174 (p<0.05). In the ROI_3 in CpG8 and CpG9 islands of promoter region of *MYD88* gene there was no statistically significant difference in the methylation status between cancer and healthy/not tumor involved tissue with p-values, p=0.1740 (p>0.05) and p=0.1255 (p>0.05), respectively. In the CpG10 island of promoter region of *MYD88* gene we found that cancer tissue is statistically significantly hypo methylated in relation to healthy/not tumor involved tissue of the larynx with p-value p=0.0407 (p<0.05).

The purpose of this analysis was to define overall methylation status of the promoter region of the *MYD88* gene. The results of this analysis showed there was statistically significant difference in overall methylation status between cancers and healthy/not tumor involved tissue in the promoter region of *MYD88* gene. The cancer tissue was statistically significantly hypomethylated in relation to healthy/ not tumor involved tissue of the larynx with p-value p=0.0061. In overall difference in the methylation status significant contribution was obtained in 3 CpG islands: CpG2(p=0.0267), CpG7(p=0.0174), and CpG10(p=0.0407) (Figure 1).

Results of analysis of *ASC/TMS1* gene methylation status in healthy/not tumor involved and tumor tissue

To accomplish differential methylation analysis of *ASC/TMS1* gene in healthy/not tumor involved and tumor tissue, the methylation status of CpG islands of gene promoter region was performed (Table 2).

According to nucleotide sequence of DNA in gene only 1 Region of Interest (ROI) was defined. In this ROI_1 3 CpG islands were detected. The methylation status of CpG1 island in the promoter region of *ASC/TMS1* gene showed there was no statistically significant difference in the methylation status between healthy/not tumor involved and cancer tissue of the larynx according to p-value p=0.0797 (p>0.05). In the CpG2 and CpG3 islands of the promoter region of the *ASC/TMS1* gene there was no statistically significant difference between methylation status of the cancer and healthy/not tumor involved tissue of the larynx with p-values p=0.2665 (p>0.05), and p=0.3526 (p>0.05), respectively.

The results of the analysis of the overall methylation status of CpG islands in the promoter region of *ASC/TMS1* gene showed no statistically significant difference between cancer and healthy/not tumor involved tissue of the larynx p=0.1562 (p>0.05) (Figure 2).

As promoter region methylation is one of the mechanism of gene expression control the purpose of the analysis on the protein level was to investigate if there is a correlation of promoter gene methylation and protein expression.

Results of analysis of MyD88 protein expression in healthy/not tumor involved and tumor tissue

To accomplish MyD88 protein expression we used Western blot method applied on 21 samples of healthy/not tumor involved and tumor tissue. The results were statistically analyzed and we found statistically significant difference in MyD88 protein expression between healthy/not tumor involved and tumor tissue of the larynx with p<0.0001 (Figure 3).

The results of methylation status of promoter region of MYD88 gene by pyrosequencing of healthy/not tumor involved and tumor tissue																						
Healthy/not tumor involved laryngeal tissue												Laryngeal tumor tissue										
Patient	CpG1	CpG2	CpG3	CpG4	CpG5	CpG6	CpG7	CpG8	CpG9	CpG10	Mean meth (%)	CpG1	CpG2	CpG3	CpG4	CpG5	CpG6	CpG7	CpG8	CpG9	CpG10	Mean meth (%)
ORL_1	13	16.5	5	12	2.865	2	4.77	9.79	0	0	6.5925	13.54	26.365	11.165	14.53	2	3.5	4	4.58	0	0	7.968
ORL_2	17.02	19.5	4.895	10.45	4	6	4.5	6.355	4.095	5.82	8.2635	15.92	13.835	10.015	12.115	3	4	3	4.155	0	0	6.604
ORL_3	11.375	7.565	5.64	10.11	4.53	4.67	5.485	6.99	0	3.015	5.938	8.94	7.38	4.12	8.465	2.5	2	4	8.065	3.46	0	4.893
ORL_4	12.615	15.045	8.195	8.84	3.96	6.44	1.275	7.13	0	0	6.35	4.605	3.67	5.5	11.545	2.38	3.445	4	3.665	0	0	3.881
ORL_5	4.135	14.265	5.92	12.765	3.72	4.175	3.835	10.96	5.595	9.885	7.5255	11.105	12.675	5.98	14.39	3	4	5.5	1.91	0	1.9	6.046
ORL_6	11.37	11.31	5.74	15.305	2.815	3.34	4.575	8.53	1.295	5.745	7.0025	3.965	5.87	4.84	8.765	1.5	3	3	7.535	0	0	3.8475
ORL_7	9.33	9.915	7.105	15.645	4.135	5.34	8	10.695	0	9.335	7.95	4.43	8.535	2.93	14.645	3	0	4	4.54	0	0	4.208
ORL_8	10	15.775	5.195	15.75	2.74	3.9	5.965	11.145	3.655	6.335	8.046	21.145	23.035	6.385	17.505	3	4.375	0	3.95	0	7.125	8.652
ORL_9	8.165	10.1	2.735	10.22	3.28	3.18	4.64	3.82	3.02	0	4.916	7.94	16.35	4.17	9.89	2	3	3	4.62	0	1.76	5.273
ORL_10	9.48	20.885	8.07	17.365	3	4.5	4.5	2.68	0	2.985	7.3465	20.44	15.685	5.885	14.845	3.28	3.82	5	1.475	0	1.955	7.2385
ORL_11	11.78	12.625	2.79	11.515	2.785	3.055	6	4.54	0	2.545	5.7635	4.81	8.7	6.535	14.49	2.54	3	3	4.99	0	6.49	5.4555
ORL_12	14.425	24.205	7.975	15.045	2	6	3	0	0	0	7.265	7.44	11.295	11.47	9.75	2.335	1	5	8.77	0	7.57	6.463
ORL_13	9.675	10.845	3.805	14.375	3	2.5	5	6.37	0	3.27	5.884	9.06	14.35	5.415	16.985	3	4	4	7.955	5.455	0.78	7.1
ORL_15	10.545	11.505	5.72	10.835	3	3.41	4	4.82	0	4.74	5.8575	10.61	13.74	2.825	15.235	2.775	4.02	4.785	7.355	1.42	4.775	6.754
ORL_16	9.815	6.92	9.55	7.62	2	3	4	4.225	0	0	4.713	9.36	8.96	6.67	13.5	2.96	4.5	3.8	7.685	0	4.01	6.1445
ORL_17	10.585	15.7	8.955	11.725	2.5	4	5	6.115	0	4.08	6.866	8.16	10.45	11.41	13.185	0	3.5	0	9.005	1.11	0	5.682
ORL_14	20.365	23.53	15.84	36.305	14.875	15.95	17.985	12.215	1.72	8.945	16.773	15.665	13.625	13.715	27.82	11.04	12.89	13.935	9.275	2.795	8.415	12.9175
ORL_18	16.815	17.795	14.8	42.89	11.735	12.86	16.43	12.655	4.23	6.645	15.6855	10.59	7.8	9.28	22.845	12.65	15.13	14.92	11.475	4.425	5.15	11.4265
ORL_21	17.75	18.6	14.965	41	9.215	12.67	17.64	19.565	7.51	7.815	16.673	10.72	8.62	9.53	21.64	15.105	14.87	15.595	7.945	1.64	9.405	11.507
ORL_25	20.575	24.79	15.655	38.04	13.08	16.65	19.61	17.3	4.45	10.245	18.0395	17.78	22.46	12.555	31.465	14.04	13.255	16.895	11.34	1.275	6.175	14.724
ORL_28	21.86	21.86	15.275	36.865	12	11.18	15.81	12.835	4.29	6.47	15.8445	12.61	16.515	8.38	27.02	8.48	20.765	14.155	12.52	5.425	4.06	12.993
ORL_29	14.97	18.275	11.885	39.03	16.07	13.465	22.2	15.16	6.555	11.25	16.886	13.82	13.15	12.67	27.78	13.01	11.83	14.05	9.92	2.3	4.39	12.292
ORL_30	14.225	18.95	12.035	41.865	15.775	18.08	10.47	17.475	6.235	12.255	16.7365	14.06	14.22	11.605	27.11	6.19	10.56	8.17	10.4	3.525	5.16	11.1
ORL_31	9.91	16.06	7.6	26.75	16.655	13.56	18.59	15.885	5.665	9.07	13.9745	15.53	16.77	12.615	36.825	5.35	7.31	11.165	14.49	2.59	9.405	13.205
ORL_32	18.72	19.4	16.99	32.225	8.725	14.9	19.005	10.17	1.985	7.62	14.974	18.335	20.03	15.145	29.985	11.835	9.625	13.77	10.04	3.61	5.52	13.7895
ORL_34	11.045	11.4	9.84	26.095	4.985	10.985	14.25	9.355	1.225	4.44	10.362	11.485	15.455	10	29.975	6.305	12.76	8.79	22.58	0	7.6	12.495
ORL_35	14.995	21.52	8.75	26.215	9.38	4.5	12.83	9.35	1.405	6.56	11.5505	12.675	13.145	11.93	32.315	14.095	6.765	15.79	7.32	3.465	5.52	12.302
ORL_36	9.635	15.86	7.9	26.215	10.665	10.995	11.79	9.57	3.26	6.525	11.2415	13.48	14.665	10.455	34.295	15.11	11.465	20.07	10.81	1.17	6.71	13.823

Table 1: The results of methylation status of promoter region of *MYD88* gene by pyrosequencing of healthy/not tumor involved and tumor tissue.

The results of methylation status of promoter region of ASC/TMS1 gene by pyrosequencing of healthy/not tumor involved and tumor tissue								
Healthy/not tumor involved laryngeal tissue					Laryngeal tumor tissue			
Patient	CpG1	CpG2	CpG3	Mean meth (%)	CpG1	CpG2	CpG3	Mean meth (%)
ORL_1	7.775	4.9	10.275	7.65	6.805	9.96	14.275	10.346667
ORL_2	7.365	18.65	10.65	12.221667	3.86	9.655	3.84	5.785
ORL_3	5.935	14.16	2.665	7.5866667	3.465	11.585	2.49	5.8466667
ORL_4	3.465	2.865	11.335	5.8883333	4.71	4.32	4.32	4.45
ORL_5	7.64	4.165	3.035	4.9466667	3.865	6.5	10.71	7.025
ORL_6	3.9	14.44	3.79	7.3766667	3.075	10.265	2.535	5.2916667
ORL_7	5.85	12.22	2.855	6.975	1.85	8.91	0.97	3.91
ORL_8	3.54	14.785	3.125	7.15	3.145	13.065	3.555	6.5883333
ORL_9	4.6	13.245	2.96	6.935	3.365	9.485	2.87	5.24
ORL_10	6.64	11.215	14	10.618333	4.79	3.04	7.015	4.9483333
ORL_11	5.355	5.46	7.385	6.0666667	3.515	3.68	6.835	4.6766667
ORL_12	5.685	8.545	9.03	7.7533333	2.93	7.015	0	3.315
ORL_13	3.47	5.735	9.83	6.345	3.705	4.365	5.875	4.6483333
ORL_15	2.9	10.275	2.775	5.3166667	6.125	11.545	2.08	6.5833333
ORL_14	4.08	13.515	3.18	6.925	4.07	8.215	1.885	4.7233333
ORL_16	2.645	5.27	11.32	6.4116667	5.615	2.84	15.885	8.1133333
ORL_17	4.965	5.22	8.665	6.2833333	4.85	6.73	8.58	6.72
ORL_18	5.345	14.115	3.94	7.8	3.43	12.23	1.525	5.7283333
ORL_21	5.51	14	4.205	7.905	3.285	8.275	1.23	4.2633333
ORL_25	4.525	15.52	4.11	8.0516667	3.15	10.255	1.44	4.9483333
ORL_28	6.495	15.365	3.34	8.4	1.005	8.74	2.445	4.0633333
ORL_29	4.615	15.075	4.44	8.0433333	2.71	6.83	1.965	3.835
ORL_30	6.19	17.16	5.115	9.4883333	6.585	9.955	8.435	8.325
ORL_31	5.94	20.525	4.53	10.331667	0	14.535	0	4.845
ORL_32	3.965	11.41	2.295	5.89	3.295	8.925	2.845	5.0216667
ORL_34	2.77	7.99	0.715	3.825	3.965	10.54	2.09	5.5316667
ORL_35	3.405	8.79	1.765	4.6533333	4.145	11.585	2.7	6.1433333
ORL_36	2.545	10.7	1.85	5.0316667	5.535	14.4	2.265	7.4

Table 2: The results of methylation status of promoter region of *ASC/TMS1* gene by pyrosequencing of healthy/not tumor involved and tumor tissue.

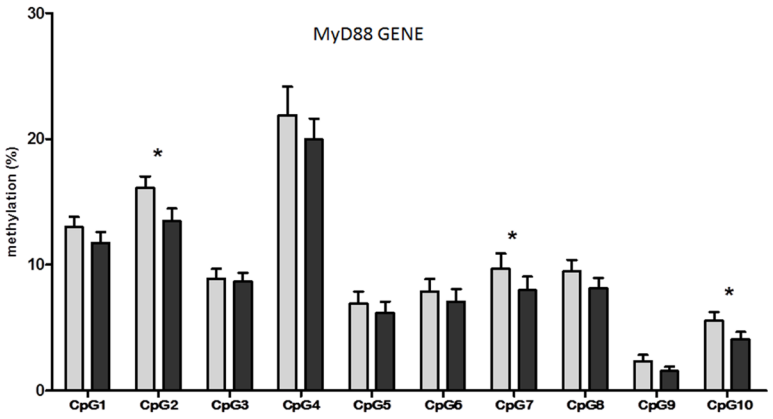

Figure 1: The graph (GraphPad Prism 5.0) showing overall methylation status of CpG islands promoter region of *MYD88* gene in healthy/not tumor involved and tumor tissue. Paired *t*-test; $p \leq 0.05$. Results are shown as mean ± SEM. **Note:** (▭) Healthy, (▬) Tumour, *CpG islands in gene promotor region in which we found statistically significant difference in the methylation status between cancer and healthy/not tumor involved tissue.

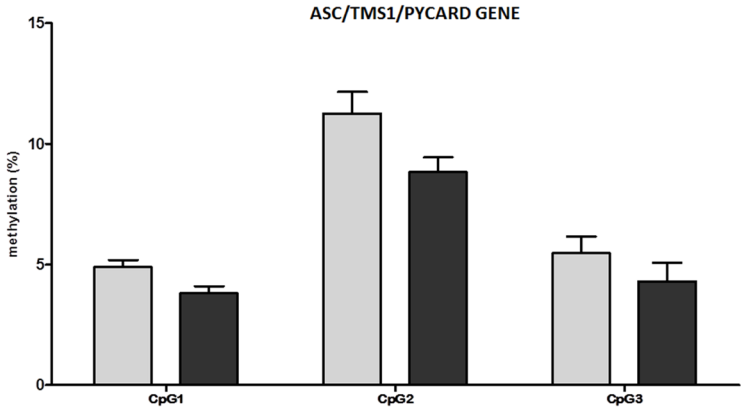

Figure 2: The graph (GraphPad Prism 5.0) showing overall methylation status of CpG islands promoter region of *ASC/TMS1* gene in healthy/not tumor involved and tumor tissue. Paired *t*-test; $p \leq 0.05$. Results are shown as mean ± SEM. **Note:** (▭) Healthy, (▬) Tumour.

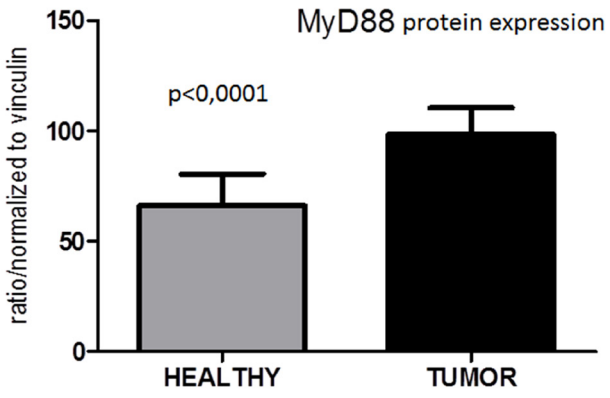

Figure 3: The graph (GraphPad Prism 5.0) showing densitometric quantification of protein MYD88 bends. There is statistically significant difference in MyD88 protein expression between healthy/not tumor involved and tumor tissue. The values are shown as mean value ± SEM. **Note:** (▭) Healthy, (▬) Tumour.

Results of analysis of ASC/TMS1 protein expression in healthy/not tumor involved and tumor tissue

ASC/TMS1 protein expression was analyzed by Western blot method applied on 23 samples of healthy/not tumor involved and tumor tissue. The results were statistically analyzed and we found statistically significant difference in MyD88 protein expression between healthy/not tumor involved and tumor tissue of the larynx with p=0.0157 (Figure 4).

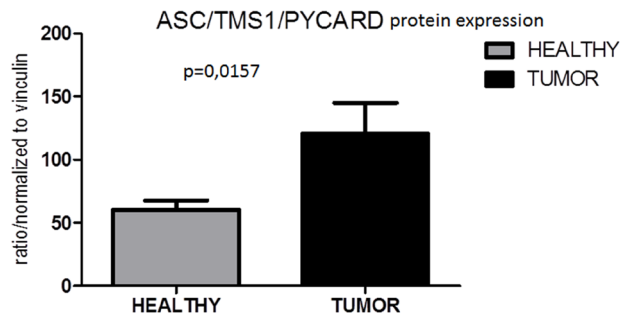

Figure 4: The graph (GraphPad Prism 5.0) showing densitometric quantification of protein ASC/TMS1 bends. There is statistically significant difference in ASC/TMS1 protein expression between healthy/not tumor involved and tumor tissue. The values are shown as mean value ± SEM. **Note:** (▭) Healthy, (▬) Tumour.

Discussion

Head and Neck Squamous Cell Carcinoma (HNSCC) which comprise laryngeal carcinoma is frequent cancer with unfortunately increasing rate of incidence [20]. According to our knowledge based on diagnosis and treatment of this cancer we still need development of molecular targets, signaling molecular pathways and reliable diagnostic modality in discovering early disease state. In most European countries according to the data of World Health Organization (WHO) the incidence of laryngeal cancer is decreasing in last two decades [21]. A slight decrease in the overall incidence of HNSCC comprising laryngeal cancer has been detected mostly due to global anti-smoking and anti-alcohol consumption campaign [22]. The relationship between chronic inflammation, innate immunity and carcinogenesis is not new and it is widely recognized but most of the molecular mechanisms involved in this relationship are still not revealed. Oncogenes associated with different molecular patterns and functions led to proinflammatory response and inflammation following infection is associated with carcinogenesis [21]. Chronic inflammation triggers increase the risk of cancer formation and development like infection (*Helicobacter pylori* for gastric cancer and gastric mucosal lymphoma, Human papilloma virus for cervical and oropharyngeal cancer, Hepatitis B/C virus for hepatocellular cancer), immune deregulation and autoimmune diseases and unknown origin infections [23]. The key mechanisms include transcription factors activation by the activation of the receptors of innate immune system resulting in proinflammatory cytokines secretion. Accordingly, we can say that molecular pathways related to cancer and inflammation are crucial factors in carcinogenesis and genetic instability of important molecules in this process are related to cancer development. It is the reason why molecular pathways related to cancer and inflammation present the aim of research of new strategies for cancer treatment and prevention. The innate immunity system is very important part of immune system and comprises mechanisms to defend organism from dangerous signals like pathogenic microbes or cellular stress signals. The innate immunity response is based on activation of an array of germline-encoded Pattern-Recognition Receptors (PRRs) and by ligand activation of those receptors the cells which expressed receptors are activated. It is well known that innate immunity receptors play a role in cancerogenesis and metastatic cancer as chronic inflammation is induced by innate immunity system and stimulates occurrence and development of cancer [24]. The important role in activation and regulation of activation of innate immunity receptors have adaptor molecules of signal pathways. In our study we investigated the correlation of methylation status and expression of *MYD88* gene and *ASC/TMS1* gene, adaptor molecules, in TLR and NLR signal pathways and we wanted to search if the methylation status of this genes can be used as potential prognostic biomarker. We hypothesized that changed methylation status of promoter regions of genes involved in activation and regulation of the inflammation, in healthy/not tumor involved and cancer tissue of the larynx, can be related with occurrence and progression of laryngeal cancer. We think that results of our research represent important contribution in investigation of adaptor molecules methylation status as yet in the literature there is no data considering *MYD88* gene methylation status in cancer. Also, methodology used in this research is innovative as we used in our analysis of the methylation status the pyrosequencing method which is the "golden standard" method in methylation status determination. All results of *ASC/TMS1* gene methylation status in recent literature were defined only by methylation-specific PCR.

The results of this study confirmed that there is significant difference in the methylation status of the promoter region of CpG islands of *MYD88* gene between healthy/not tumor involved and cancer tissue of the larynx. Precisely, the results showed that the cancer tissue of the larynx is statistically significantly hypomethylated according to the healthy/not tumor involved tissue. Moreover, results of this research indicated that the overall rate of methylation status of the promoter region of *MYD88* gene is statistically significantly reduced in cancer tissue according to healthy/not tumor involved tissue. Furthermore, MyD88 protein expression level, explored to define the correlation between methylation status and protein expression, strongly confirmed the correlation between methylation and expression. According to the results, reduced methylation of the promoter region of *MYD88* gene in DNA isolated from cancer tissue corresponds to higher expression of this protein in cancer tissue as opposite to healthy/not tumor involved tissue higher methylation corresponds to reduced expression of MyD88 protein. In recent literature only Lu et al. studied the expression of MyD88 adaptor molecule and clinical impact of this protein expression to laryngeal cancer. According to the author, there is 68.6% of positive expression of MyD88 protein in laryngeal cancer tissue which is significantly higher in relation to healthy surrounding tissue where expression is 11.8%. Positive expression correlates with stage of the disease and occurrence of lymph node metastasis which is inversely proportional to 5 year survival rate. *MyD88* can be important factor in development of laryngeal cancer as in future target cancer therapy may improve prognosis of patients with this cancer [25]. Rakoff-Nahoum, et al. showed that adaptor molecule *MyD88* has a critical role in spontaneous tumor development in mice with heterozygous mutation in gene APC (Adenomatous Polyposis Coli). Adaptor molecule *MyD88*-dependent signaling pathway controls expression of main modifying genes in intestinal tumorigenesis and has a crucial role in both spontaneous and carcinogen-induced tumors [26]. Similar results were shown by Naugler, et al. on the model of Hepatocellular Carcinoma (HCC) in mouse. The author showed that Diethylnitrosamine (DEN)

induced Hepatocellular Carcinoma (HCC) is associated with increased serum Interleukin-6 (IL-6) concentration whose production is largely dependent on *MyD88* signaling pathway [27]. Chefetz, et al. demonstrated that during surgical debunking or chemotherapy in ovarian cancer, cellular debris released during the process of injury and cell death may activate the TLR2-*MyD88*-NFκB pathway in a specific cancer cell population, the EOC stem cells, leading to tumor repair and hence disease recurrence [28]. Higgins, et al. analyzed TLR-driven inflammation in a mouse model for stage IV breast cancer. The author found that High Mobility Group Box 1 protein (HMGB1) was produced by 4T1 in a *Myd88*-dependent manner and that neutralizing extracellular HMGB1 resulted in 50% decrease in growth of the tumor cells [29]. Unlike other authors, Kinowaki, et al. reported that tumor progression is associated with reduced expression of *MyD88* in Hepatocellular Carcinoma (HCC) [30]. Pyrosequencing results with the aim of determining the methylation status of promoter region of *MYD88* gene showed the difference in overall level of methylation status between healthy/not tumor involved tissue and cancer tissue. The overall difference level is statistically significant and three CpG islands highly contribute to this difference; CpG2, CpG7, and CpG10. The other tested CpG islands did not expressed the difference in the overall methylation level between healthy/not tumor involved and cancer tissue. Our results are significant as for the first time according to the literature the methylation status of promoter region of *MYD88* gene between healthy/not tumor involved and cancer tissue was analyzed. The result revealed statistically significant difference in the methylation status, *MYD88* gene is hypomethylated in cancer tissue, and the results correlates with protein expression. Our results also showed there is a pattern of methylation of promoter region of *MYD88* gene as only CpG2, CpG7, and CpG10 islands contributed to the result. If there is tissue specific methylation status of CpG islands for the same gene, than, in the future, according to this model, we can be able to differentiate second primary lung cancer from solitary pulmonary metastasis in laryngeal cancer. It can be a diagnostic problem as patients suffering from laryngeal cancer have a great risk to develop primary lung cancer due to extensive tobacco smoking, or to develop solitary pulmonary metastasis which can sometimes comprise difficulties in obtaining satisfactory biopsy. The results of the methylation status of promotor region of the *ASC/TMS1* gene showed no statistically significant difference in the methylation status of different CpG islands between healthy/not tumor involved and cancer tissue. In addition, there is no significant difference in overall methylation status between healthy/not tumor involved and cancer tissue. However, according to our results there is significant difference in expression of *ASC/TMS1* gene between healthy/not tumor involved and cancer tissue, expression of protein ASC is significantly higher in cancer tissue. Adaptor molecule *ASC/TMS1* plays important role in proinflammatory signaling pathway and cancer related inflammation. Many authors analyzed *ASC/TMS1* gene expression in diver's cancers but the methylation status of this gene with correlation of the expression between healthy/not tumor involved and laryngeal cancer tissue was not yet published. Salaminen, et al. examined the mechanisms involved in the epigenetic regulation of *ASC/TMS1* as well as their significance in the coordination of apoptosis and inflammasomes functions [31]. Hong, et al. demonstrated that restoration of ASC expression in human colorectal cancer DLD-1 cells, in which *ASC/TMS1* gene is silenced by aberrant methylation, potentiated cell death mediated by DNA damaging agent [32]. According to Virmani, et al. methylation of *ASC/TMS1* gene correlated inversely with expression in the pathogenesis of small cell and non-small lung and breast cancers [33]. Xiaofang, et al. analyzed methylation status of tumor suppressor genes, including *ASC/TMS1*, in cholangiocarcinoma. The authors revealed that the methylation of DAPK, p14ARF, and ASC/TMS in cholangiocarcinoma is a common event. Furthermore, p53 mutation combined with DAPK, p14ARF, and/or ASC methylation correlates with malignancy and poor prognosis [34]. Unlike former authors Tamandani, et al. described the methylation status of *TMS1/ASC* and *CASP8* genes in cervical cancer. In this study the authors concluded that the frequency of *TMS1/ASC* and *CASP8* genes methylation in cervical cancer is rare (<6%), and plays no critical role in development of cervical cancer [35]. Mirza, et al. observed significant correlation between promoter methylation of *ASC/TMS1* gene and loss of protein expression in breast cancer confirming that promoter methylation is an important mechanism for transcriptional silencing of these genes in breast cancer [36]. Deswaerte, et al. revealed a protumorigenic role in gastric cancer for the key inflammasomes adaptor *ASC/TMS1* and its effector cytokine IL18. Genetic ablation of *ASC/TMS1* in the spontaneous mouse model of intestinal-type gastric cancer suppressed tumorigenesis by augmenting caspase-8-like apoptosis in the gastric epithelium, independently from effects on myeloid cells and mucosal inflammation. The authors concluded that inflammasomes activation that elevates IL18 helps drive gastric cancer by protecting cancer cells against apoptosis, with potential implications for new therapeutic strategies in this setting [37]. Machida, et al. studied proapoptotic *ASC/TMS1* gene in lung cancer and used the findings to develop a sputum marker. The authors concluded that hypermethylation of *ASC/TMS1* gene is a potential marker for late-stage lung cancer and, in sputum, could predict prognosis in patients resected for early-stage disease [38].

Results of the analysis of the methylation status of *ASC/TMS1* gene in our study are different related to results from published articles. According to our results there is no statistically significant difference in the methylation status between healthy/not tumor involved and cancer tissue. This can be explained by the fact that in this study the methylation status was determined using pyrosequencing method which is considerably more precise than commonly used methods, like methylation-specific PCR.

In addition, in this study there is no correlation between methylation status of *ASC/TMS1* gene and protein expression which can indicate that protein expression undergoes alternative post-transcriptional mechanisms of regulation.

Conclusion

The main contribution of the present study is in determination of the methylation status of *MYD88* gene and *ASC/TMS1* gene by pyrosequencing method which is still considered to be the "golden standard" in methylation status analysis. Furthermore, we found statistically significant difference in the methylation status of *MYD88* gene between healthy/not tumor involved and laryngeal cancer tissue. We also showed that regarding gene/protein *MYD88* there is a positive correlation between methylation status and gene expression-hypermethylation correlates with reduced protein expression. It is worth mentioning that regarding gene/protein *ASC/TMS1* there is no positive correlation of methylation status and protein expression. In fact, our results showed there is no statistically significant difference in gene methylation status but there is significantly higher protein expression in laryngeal cancer tissue. The pattern of methylation which comprises the tissue specific methylation status indicates potential biomarkers in differential diagnosis of Head and Neck and lung squamous cell carcinoma.

Declarations

Ethics approval and consent to participate

This study was approved by Ethics Committee of University Hospital Center Zagreb and all procedures followed were in the accordance with the ethical standards of the Committee. All patients signed informed consent prior to a surgical intervention.

Consent for publication

All participants in this study signed informed consent to participate in the study and to publish their data.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

All persons designated as authors have participated sufficiently in the work to take public responsibility for the content of the manuscript. Associate professor Lana Kovač Bilić and professor Mario Bilić are mainly responsable for conception and design of this study. They have both participated in all surgical procedures and literature examination. All analysis and data interpretation were done by Jelena Knežević, PhD and biologist Maja Šutić from Department for Molecular Medicine, Laboratory for Advanced Genomics Ruđer Bošković Institute. Important intellectual content and final approval of the version was done by associate professor Krsto Dawidowsky and professor Srećko Branica.

Acknowledgement

Not applicable

Conflict of interest

All authors declare that they have no conflict of interest.

References

1. Hazelden LA, Newman MJ, Shuey S, Waldfogel JM, Brown VT (2019) Evaluation of the head and neck cancer patient population and the incidence of hospitalization at an academic medical center. J Oncol PharmPract 25:333-338.
2. Jaber MA (2010) Tobacco smoking and alcohol consumption as risk factors for site-specific intraoral epithelial dysplasia. J Investig Clin Dent 1:101-107.
3. Leon ME, Peruga A, McNeill A, Kralikova E, Guha N, et al. (2015) European code against cancer, 4th Edition: Tobacco and cancer. Cancer Epidemiol 39:20-33.
4. Vigneswaran N, Williams MD (2014) Epidemiologic trends in head and neck cancer and aids in diagnosis. Oral Maxillofac Surg Clin North Am 2:123-141.
5. Brandstorp-Boesen J, Sørum Falk R, Boysen M, Brøndbo K (2017) Impact of stage, management and recurrence on survival rates in laryngeal cancer. PLoS One 12:e0179371.
6. García J, López M, López L, Bagué S , Granell E, et al. (2017) Validation of the pathological classification of lymph node metastasis for head and neck tumors according to the 8th edition of the TNM classification of malignant tumors. Oral Oncol 70:29-33.
7. Lambiel S, Dulguerov P (2017) Changes in the new TNM classification in head and neck oncology. Rev Med Suisse 13:1684-1689.
8. Grivennikov SI, Greten FR, Karin M (2010) Immunity, inflammation, and cancer. Cell 140:883-899.
9. Leonardi GC, Accardi G, Monastero R, Nicoletti F, Libra M (2018) Ageing: From inflammation to cancer. Immun Ageing 15:1.
10. Sitarz R, Skierucha M, Mielko J, Offerhaus GJA, Maciejewski R, et al. (2018) Gastric cancer: Epidemiology, prevention, classification, and treatment. Cancer Manag Res 10:239-248.
11. Moustafa S, Karakasiliotis I, Mavromara P (2018) Hepatitis C virus core+1/ARFP modulates Cyclin D1/pRb pathway and promotes carcinogenesis. J Virol 92:e02036.
12. Adelstein DJ, Ridge JA, Gillison ML, Chaturvedi AK, D'Souza G, et al. (2009) Head and neck squamous cell cancer and the human papillomavirus: Summary of a national cancer institute state of the science meeting, november 9-10, 2008, Washington, D.C. Head Neck 31:1393-1422.
13. Nowińska K, Ciesielska U, Podhorska-Okołów M, Dzięgiel P (2017) The role of human papillomavirus in oncogenic transformation and its contribution to the etiology of precancerous lesions and cancer of the larynx: A review. Adv Clin Exp Med 26:539-547.
14. Leemans CR, Braakhuis BJM, Brakenhoff RH (2011) The molecular biology of head and neck cancer. Nat Rev Cancer 11:9-22.
15. Takeuchi O, Akira S (2010) Pattern recognition receptors and inflammation. Cell 140:805-820.
16. Mantovani A, Allavena P, Sica A, Balkwill F (2008) Cancer-related inflammation. Nature 454:436-444.
17. Schroder K, Tschopp J (2010) The Inflammasomes. Cell 140:821-832.
18. Wang JQ, Jeelall YS, Ferguson LL, Horikawa K (2014) Toll-like receptors and cancer: *MYD88* mutation and inflammation. Front Immunol 5:367.
19. Herman JG, Baylin SB (2003) Gene silencing in cancer in association with promoter hypermethylation. N Engl J Med 349:2042-2054.
20. Dansky Ullmann C, Harlan LC, Shavers VL, Stevens JL (2012) A population-based study of therapy and survival for patients with head and neck cancer treated in the community. Cancer 118:4452-4461.
21. Chatenoud L, Garavello W, Pagan E, Bertuccio P, Gallus S, et al. (2016) Laryngeal cancer mortality trends in European countries. Int J Cancer 138:833-842.
22. Cui J, Chen Y, Wang HY, Wang R (2014) Mechanisms and pathways of innate immune activation and regulation in health and cancer. Hum Vaccin Immunother 10:3270-3285.
23. Aggarwal BB, Shishodia S, Sandur SK, Pandey MK, Sethi G (2006) Inflammation and cancer: How hot is the link? Biochem Pharmacol 72:1605-1621.
24. Wu DD, Song J, Bartel S, Krauss-Etschmann S, Rots MG, et al. (2018) The potential for targeted rewriting of epigenetic marks in COPD as a new therapeutic approach. Pharmacol Ther 182:1-14.

25. Lu B, Di W, Lian R, Lu Z, Yu W (2015) The expression and clinical significance of *MyD88* in laryngeal cancer. J of Clin Otorh 29:1477-1479.

26. Rakoff-Nahoum S, Medzhitov R (2007) Regulation of spontaneous intestinal tumorigenesis through the adaptor protein *MyD88*. Science 317:124-127.

27. Naugler WE, Sakurai T, Kim S, Maeda S, Kim K, et al. (2007) Gender disparity in liver cancer due to sex differences in *MyD88*-dependent IL-6 production. Science 317:121-124.

28. Chefetz I, Alvero AB, Holmberg JC, Lebowitz N, Craveiro V, et al. (2013) TLR2 enhances ovarian cancer stem cell self-renewal and promotes tumor repair and recurrence. Cell Cycle 12:511-21.

29. Higgins MJ, Serrano A, Boateng KY, Parsons VA, Phuong T, et al. (2016) A multifaceted role for *Myd88*-dependent signaling in progression of murine mammary carcinoma. Breast Cancer Basic Clin Res 10:157-167.

30. Kinowaki K, Soejima Y, Kumagai A, Kondo F, Sano K, et al. (2017) Clinical and pathological significance of myeloid differentiation factor 88 expression in human hepatocellular carcinoma tissues. Pathol Int 67:256-263.

31. Salminen A, Kauppinen A, Hiltunen M, Kaarniranta K (2014) Epigenetic regulation of *ASC/TMS1* expression: potential role in apoptosis and inflammasome function. Cell Mol Life Sci 71:1855-1864.

32. Hong S, Hwang I, Lee YS, Park S, Lee WK, et al. (2013) Restoration of ASC expression sensitizes colorectal cancer cells to genotoxic stress-induced caspase-independent cell death. Cancer Lett 331:183-191.

33. Virmani A, Rathi A, Sugio K, Sathyanarayana UG, Toyooka S, et al. (2003) Aberrant methylation of TMS1 in small cell, non-small cell lung cancer and breast cancer. Int J Cancer 20;106:198-204.

34. Xiaofang L, Kun T, Shaoping Y, Zaiqiu W, Hailong S (2012) Correlation between promoter methylation of p14ARF, *TMS1/ASC*, and DAPK, and p53 mutation with prognosis in cholangiocarcinoma. World J Surg Oncol 10:5.

35. Kordi Tamandani DM, Sobti RC, Shekari M, Huria A (2009) CpG island methylation of *TMS1/ASC* and *CASP8* genes in cervical cancer. Eur J Med Res 14:71-75.

36. Mirza S, Sharma G, Prasad CP, Parshad R, Srivastava A, et al. (2007) Promoter hypermethylation of TMS1, BRCA1, ERalpha and PRB in serum and tumor DNA of invasive ductal breast carcinoma patients. Life Sci 81:280-287.

37. Deswaerte V, Nguyen PM, West A, Browning AF, Yu L, et al. (2018) Inflammasomes adaptor ASC suppresses apoptosis of gastric cancer cells by an IL-18 mediated inflammation-independent mechanism. Cancer Res 78:1293-1307.

38. Machida EO, Brock MV, Hooker CM, Nakayama J, Ishida A, et al. (2006) Hypermethylation of *ASC/TMS1* is a sputum marker for late-stage lung cancer. Cancer Res 66:6210-6218.