

EZH2 and *SMYD3* expression in papillary thyroid cancer

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Abstract. Recent studies have revealed the significant role of *SMYD3* and *EZH2* genes in the development and aggressiveness of numerous types of malignant tumor. Therefore, the present study aimed to investigate the expression of *SMYD3* and *EZH2* in papillary thyroid cancer, and to determine the correlation between the expression of these genes and clinical characteristics. Resected thyroid tissue samples from 62 patients with papillary thyroid cancer were investigated. Thyroid tissue derived from the healthy regions of removed nodular goiters from 30 patients served as the control group. Reverse transcription-quantitative PCR analysis was employed to detect relative mRNA expression levels. Primer sequences and TaqMan[®] hydrolysis probe positions for *EZH2* and *SMYD3* were determined using the Roche Universal ProbeLibrary Assay Design Center version 2.50. *EZH2* expression was detected in all thyroid cancer samples and in 83.3% of benign lesions. Notably, *EZH2* was revealed to be upregulated in thyroid cancer tissues compared with control tissues ($P=0.0002$). *EZH2* expression was positively correlated with tumor stage ($P<0.0001$; $r=0.504$), and multiple comparison analysis revealed that the highest expression of *EZH2* was detected in samples staged pT4 ($P=0.0001$). *SMYD3* expression was detected in all thyroid cancer samples and in 96.7% of healthy thyroid tissues; notably, the expression levels were similar in both groups. In addition, there was no correlation between *SMYD3* expression and the aggressiveness of papillary thyroid cancer. In conclusion, overexpression of the *EZH2* gene may be associated with the development of papillary

thyroid cancer and *EZH2* may be a potential therapeutic target in papillary thyroid cancer.

Introduction

Modifications to histone amino-terminals are significant for the regulation of chromatin structure, interaction with chromatin-associated proteins, transcription and DNA replication (1-3). Histone lysine methylases (KMTs) are responsible for modifications in amino-acid residues of the exposed N-terminal domain of histones by methylation of lysines. As a result, the gene is activated or repressed (2,3). Methyltransferases of mixed lineage leukemia (MLL) and the SET and MYND domain-containing protein (SMYD) family are involved in trimethylation of lysine 4 at histone 3 (3). Expression of SMYD family members is significantly altered in various human diseases (3,4). Their participation has been studied in cancer, embryonic heart development and inflammatory processes (4). The SMYD family consists of five members with a different structure from the other KMTs: The SET domain is split into two segments by an MYND domain, followed by a cysteine-rich post SET domain (1,3,5,6). SMYD3 was the first member of the SMYD family for which catalytic significance was also demonstrated for domains other than classical SET (3,7). SMYD3 was recently shown also to catalyze methylation of lysine 5 of histone 4 (3,8). Specific SMYD3 binding elements in the target DNA (5'-CCCTCC-3' or 5'-GGAGGG-3') are present in gene promoter regions below *SMYD3*, such as Nks2.8, WHT10B, and HTERT (1,9-11). SMYD3 activates transcription of several oncogenes (e.g. *C-Met*, *JUND* and *Wnt10B*), cell cycle regulating genes (e.g. *CDK2* and β DNA topoisomerase) and genes responsible for signal transduction (e.g. *RAB40C* and *GNAQ2*). However, SMYD3 inhibits the expression of some tumor suppressor genes (e.g. *RIZ1*) by epigenetic regulation (1,9,12,13). Increased *SMYD3* expression is significant for cell viability, adhesion, migration and invasion (1,14). It correlates with poor prognosis in various types of cancer. The nucleus placed protein-coding gene *SMYD3* is a selective transcription enhancer of oncogenes and the process of cell proliferation in liver and colon cancers. The mechanism is based on interaction with RNA Pol II and H3K4me3 and, in the case of liver tumors, is strongly associated with poor prognosis. Additionally, SMYD3 has an impact on Ras/ERK signaling in lung and pancreatic cancers by methylation of MAP3K2 kinase (3). A significant correlation has been found

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between the genetic variant with the variable number tandem repeat (VNTR) in the *SMYD3* gene and the development of breast cancer in Jordanian women (15). Overexpression of *SMYD3* also correlates with a more aggressive phenotype of prostate cancer (16). The role of the *SMYD3* gene is also being studied in cholangiocarcinoma, esophageal squamous cell carcinoma, cervical, ovarian, bladder, gastric cancers, chronic lymphocytic leukemia and glioma (17-26). No studies are being undertaken to evaluate *SMYD3* expression in papillary thyroid cancers, while one previous study confirmed its overexpression in medullary thyroid cancers (24).

An enhancer of zeste homolog 2 (*EZH2*) is a histone methyltransferase, the catalytic subunit of the Polycomb 2 repression complex responsible for the trimethylation of histone H3 lysine 27 (H3K27me3) (27,28). Research on embryonic *EZH2*-zero (ESC) stem cells has shown residual H3K27me3, termed *EZH1* methyltransferase. This may indicate at least partial compensation of both enzymes (29-31). Genetic loss-of-function studies have demonstrated a role for *EZH2* in the establishment and physiology of several cell types and tissues, such as the skin, heart and mammary gland (29-33). Similar to *SMYD3*, *EZH2* is highly expressed in various types of cancer, which is often also correlated with poor prognosis. The effect of *EZH2* gene expression on carcinogenesis is the promotion of survival, proliferation, transformation of epithelium to mesenchyme, and the invasion and drug resistance of cancers. However, tumor-suppressive effects of *EZH2* have also been identified. *EZH2* has a significant impact on immune cells (27). The overexpression of *EZH2* has been demonstrated in breast, prostate, endometrial, bladder, liver, lung, ovarian cancer, melanoma, glioblastoma and Natural killer/T-cell lymphoma. Gain-of-function mutations are present in Non-Hodgkin's lymphoma and melanoma. Through repression of the *TIMP-3* metastatic suppressor gene, *EZH2* leads to progression and spread in prostate and lung cancers and, through missense mutation in lymphomas, leads to increased function of the mutated protein (28).

These genes are currently undergoing extensive research in various types of cancers, including thyroid cancers. These are seen as the goals for targeted therapeutic strategies in oncology. Both genes have already been studied in medullary thyroid cancer (MTC) (24). In addition, research on the *EZH2* gene has also been carried out on poorly differentiated (PDT) and anaplastic thyroid carcinoma (ATC) (34), and also papillary thyroid cancer (35). Our study aimed to analyze *EZH2* and *SMYD3* gene expression in papillary thyroid cancer (PTC), the most common form of malignancy in this organ, and to correlate this with clinical outcome.

Material and methods

Tissue samples. Samples of resected thyroid tissue from consecutive patients were collected: papillary thyroid cancers and thyroid tissue from thyroids without cancer excised for nodular goiter. All patients underwent primary thyroid surgery. We excluded patients with mixed thyroid cancers. Tissue samples were stored in RNA protective medium at -80°C until reverse transcription-quantitative PCR (RT-qPCR) analysis. The Ethical Committee of Poznan University of Medical Sciences approved the study (approval

no. 228/14) and each patient provided written informed consent.

Nucleic acid extraction and validation. RNA was isolated from tissue specimens using the Direct-zol RNA kit column system for high molecular weight RNA isolation according to the manufacturer's protocol (Zymo Research). In short, ~ 25 mg of tissue was homogenized in liquid nitrogen, and suspended in TriReagent (GenoPlast). After chloroform addition, the samples were centrifuged ($12,000 \times g$, 15 min, 4°C) and the aqueous phase was transferred in the column. The isolation, following the protocol, finished with RNA recovery from silica matrix columns in pre-warmed water. The quality, quantity and purity of RNA were analyzed as described before (36) with the use of a NanoPhotometer[®] NP-80 (IMPLEN). The integrity was evaluated by electrophoretic separation in denaturing conditions.

RT-qPCR. Complementary to RNA DNA (cDNA) was synthesized in a three-step reaction conducted following the Transcriptor Reverse Transcriptase manufacturer's protocol (Roche Diagnostics GmbH) in a total volume of $20 \mu\text{l}$. A mixture of 5 mM oligo(d)T₁₀, RNA (1 μg) and RNase-, DNase- and pyrogen-free water was incubated for 10 min at 65°C . Subsequently, the samples were chilled. Subsequently, 10 U/ μl ribonuclease inhibitor (RNasin, Roche Diagnostics GmbH), 10 U/ μl of transcriptor reverse transcriptase (Roche Diagnostics GmbH), 100 mM deoxyribonucleotide triphosphates (Novayzm) and 1X reaction buffer (Roche Diagnostics GmbH) were added. The subsequent steps of cDNA synthesis had been described earlier (36).

RNA expression pattern analysis was performed using a LightCycler[®] 2.0 (Roche Diagnostics GmbH). Primer sequences and TaqMan[®] hydrolysis probe positions for the *EZH2* and *SMYD3* were determined using the Roche Universal ProbeLibrary (UPL) Assay Design Center (<http://qpcr.probefinder.com>; last accessed on May 10, 2017). Ensembl (<http://www.ensembl.org/>), and protein-coding sequences for *EZH2* (ENST00000320356.7, ENST00000460911.5, ENST00000476773.5, ENST00000478654.5, ENST00000350995.6, ENST00000483967.5) were used to design the primers and hydrolysis probes (Roche TaqMan Probe UPL #35; cat. no. 04687680001). The forward (5'-tgttgatactctccaaggaa-3') and reverse (5'-gaggagcctctcttttca-3') primers flank the 90 nt amplicon (Fig. 1A). The set of forward (5'-cctgcctttgaccttttga-3') and reverse (5'-agatactgggatagccaacac-3') primers for *SMYD3* covered the 106 nt amplicons with the TaqMan UPL #4 in between (Roche Diagnostics GmbH; cat. no. 04685016001). The assay was designed for both transcript variants (NCBI NM_001167740 and NM_022743.2) (Fig. 1B). The hypoxanthine-guanine phosphoribosyltransferase (*HPRT*) gene assay (Roche cat. no. 05532957001) served as an internal control.

The quantitative polymerase chain reactions had been standardized earlier and conducted as described before (36) in a total volume of $20 \mu\text{l}$ with a 1X LightCycler[®] FastStart TaqMan[®] Probe Master mix. Each reaction was performed in duplicate on independently synthesized cDNA, and the mean values were used for statistical analyses. Reaction efficiencies were obtained from the genes' standard curves (36). Raw data for threshold values were analyzed by comparing them to

Table I. Clinical data of the study and the control groups.

Characteristics	Papillary thyroid cancer group (n=62)	Control group (n=30)	P-value
Mean ± SD age, years	51±16	46±16	0.17
Sex			0.15
Female	43	25	
Male	19	5	
Histological variant			-
Conventional	54	-	
Follicular	4	-	
Oncocytic	3	-	
Tall cell	1	-	
Staging at diagnosis			-
I	35	-	
II	12	-	
III	13	-	
IV	12	-	
Metastases to the lymph nodes (%)	14 (22.6)	-	-
Multifocality (%)	27 (43.5)	-	-
Distant metastases (%)	1 (1.6)	-	-

appropriately selected standard curves and the reference gene assay with the use of LC 5.0.0.38 software, and presented as concentration ratios (Cr).

Statistical analysis. Statistical analysis was performed with MedCalc Statistical Software version 19.1.5 (MedCalc Software bv; <https://www.medcalc.org>; 2020). The D'Agostino-Pearson test analyzed normality. $P < 0.05$ was considered to indicate a statistically significant difference. The Mann-Whitney test was used to compare non-normally distributed parameters between the study and control groups, as well as between analyzed subgroups. When data followed a normal distribution, Student's t-test was used for comparison between groups. The χ^2 test was applied to compare descriptive parameters. The Kruskal-Wallis test with Conover post-hoc test was used to compare gene expression between thyroid cancer samples staged 1, 2, 3, and 4. The Spearman's correlation coefficient test was used to find relationships between analyzed parameters.

Results

Patient characteristics. The study group consisted of 62 patients with papillary thyroid cancers. There were 30 tissue samples in the healthy control group. Clinical data are presented in Table I. The study, and the control groups did not differ according to patients' age or sex.

EZH2 expression. EZH2 expression was found in all thyroid cancer samples and 25 out of 30 samples of benign lesions. We found EZH2 overexpression in thyroid cancers ($P = 0.0002$) (Fig. 2). EZH2 expression positively correlated with tumor stage ($P < 0.0001$; $r = 0.504$; Fig. 3), and multiple comparison analysis revealed the highest expression in samples staged pT4

($P = 0.0001$) (Fig. 4). We did not observe EZH2 overexpression in patients with lymph node involvement (Fig. 5), and there was no association between EZH2 expression and multifocality ($P = 0.13$ and $P = 0.49$, respectively). Also, patients' age did not correlate with EZH2 expression levels ($P = 0.66$) (Fig. 6).

SMYD3 expression. SMYD3 expression was found in all thyroid cancer samples and 29 out of 30 healthy tissues, and the expression levels were similar in both groups ($P = 0.90$) (Fig. 7). Also, there were no differences in SMYD3 expression between tumors staged pT1, pT2, pT3 or pT4 ($P = 0.37$) (Fig. 8). Patients with metastases to the lymph nodes did not have higher SMYD3 expression than those without ($P = 0.83$) (Fig. 9). We did not observe any correlation between SMYD3 expression and multifocality ($P = 0.45$).

Discussion

We found histone methyltransferase EZH2 overexpression in papillary thyroid cancer (PTC), while SMYD3 expression was not elevated. EZH2 gene expression was found in all papillary thyroid cancer samples, but also in most, as many as five sixths of healthy thyroid tissue samples. These were significantly higher expression rates, in both the study and control groups, than those obtained by Xue *et al* (35). However, they examined the expression of the EZH2 gene both by real-time PCR, as in our study, and immunohistochemistry (IHC), and they presented the expression percentages for IHC (35). However, in papillary thyroid carcinomas, statistically significant overexpression of the EZH2 gene was found in our study. Therefore, the EZH2 gene may be associated with the development of papillary thyroid cancer. Xue *et al* (35) obtained similar results. This is the case in papillary thyroid cancer, as well as in other thyroid cancers, as shown in Table II (24,34,35).

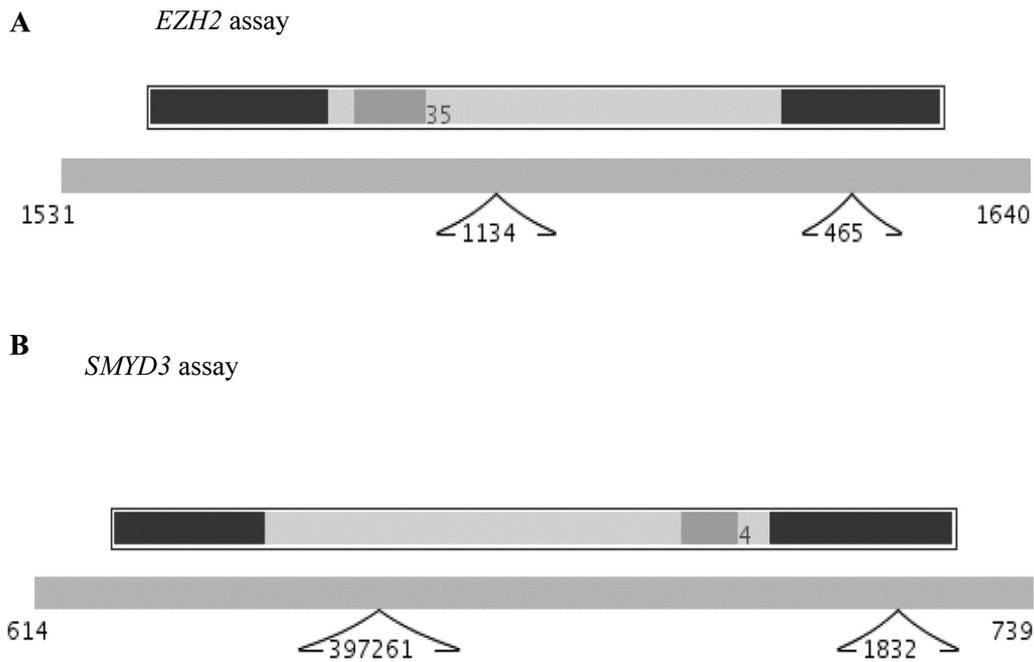


Figure 1. Amplicons of analyzed genes (A) *EZH2* and (B) *SMYD3* with the primers, TaqMan® Probes and introns positions. Trade secrets protect hypoxanthine-guanine phosphoribosyltransferase (reference) probe assays. Black boxes represent the primers position, the dark-gray box with the number represent probe positions and their Roche Universal ProbeLibrary numbers. The numbers in the brackets represent introns and their lengths. Numbers at the beginning and end represent amplicon positioning relative to the basic mRNA sequence.

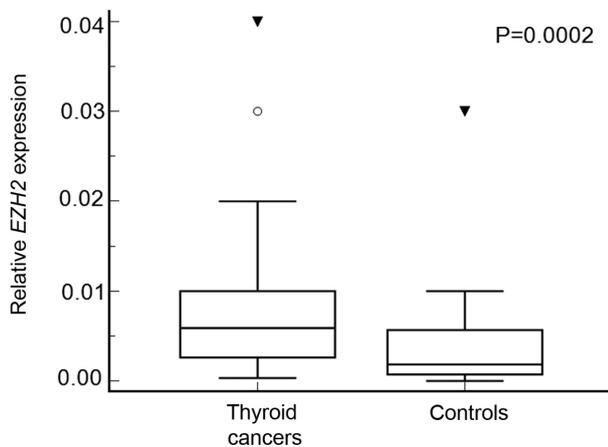


Figure 2. Comparison of relative *EZH2* gene expression in thyroid cancers and healthy controls.

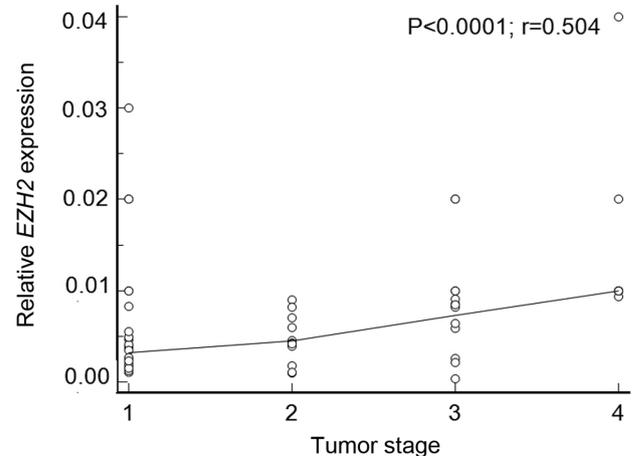


Figure 3. Correlation between *EZH2* expression and tumor staging.

The expression of the *EZH2* gene positively correlated with the tumor stage, in the case of tumorous staged pT4, it was the highest. Xue *et al* (35) did not observe statistically significant differences between tumors ≤ 1 cm and tumors >1 cm, or between those that extended beyond the thyroid tissue and those that did not. However, we did not observe *EZH2* overexpression in patients with lymph node involvement, as had been obtained by Xue *et al* (35). Also, we did not find a relationship between *EZH2* expression and multifocality. In both studies, age did not significantly correlate with *EZH2* gene expression. Correlation with aggressiveness in thyroid cancers was described by Sponziello *et al* (24) based on their study on medullary thyroid carcinomas (MTC). Sponziello *et al* (24) examined the expression of epigenetic regulators in medullary

thyroid carcinomas (MTCs) and correlated this with clinical outcome and *RET* or *RAS* mutational status. In the case of a more aggressive disease, they noted a significant increase in *EZH2* and *SMYD3* gene expression (more than 3 and 2-fold, respectively). They determined the aggressiveness of the disease, according to the current guidelines (37), based on the occurrence of lymph nodes and distant metastases, persistence after primary treatment and disease-related death. Noticeably, they did not observe a significant correlation between the overexpression of *EZH2* and *SMYD3* and the mutational status of *RET* or *RAS* genes. Therefore, the researchers suggested that *EZH2* and *SMYD3* mRNA expression may be useful prognostic biomarkers, and further studies are needed to investigate their possibility of use in therapy of MTC patients (24). Also, Masudo *et al* (34) claim that *EZH2* overexpression may be a

Table II. Studies on *EZH2* gene in thyroid cancers.

First author, year	Number of patients, type of thyroid cancer	Number of patients in the control group	Analytical technique	Results	Refs.
Masudo, 2018	67 cases of PDTC and 48 cases of ATC	30 adjacent healthy and differentiated thyroid carcinoma tissue	IHC	<i>EZH2</i> was expressed in PDTC and ATC, but not in the normal thyroid gland or DTC; <i>EZH</i> -positivity increased in the order of DTC, PDTC, and ATC ($P < 0.01$); higher <i>EZH2</i> expression correlated with more reduced survival in PDTC ($P = 0.004$) and ATC ($P = 0.166$)	(34)
Sponziello, 2014	54 MTCs; 13 familial MTCs and 41 sporadic forms; 33 hosted an <i>RET</i> mutation and 13 an <i>RAS</i> somatic mutation	-	qPCR	A significant increase in <i>EZH2</i> and <i>SMYD3</i> gene expression in more aggressive diseases (i.e. occurrence of metastases; persistent disease; disease-related death); the increase in <i>EZH2</i> and <i>SMYD3</i> did not correlate with the mutational status of <i>RET</i> or <i>RAS</i> genes	(24)
Xue, 2019	65 PTCs	30 adjacent healthy thyroid tissues	qPCR and IHC	Higher <i>EZH2</i> expression in PTC tissues than in healthy thyroid tissues; <i>EZH2</i> expression is associated with lymph node metastasis and is recurrent; inhibition of <i>EZH2</i> in PTC cell lines downregulates cellular proliferation and migration. PTC is a disease with a high incidence in females and E2-ER α upregulates <i>EZH2</i> expression	(35)

DTC, differentiated thyroid cancer; PDTC, poorly differentiated thyroid cancer; ATC, anaplastic thyroid carcinoma; MTCs, medullary thyroid cancer; PTC, papillary thyroid cancer; IHC, immunohistochemistry; qPCR, quantitative PCR.

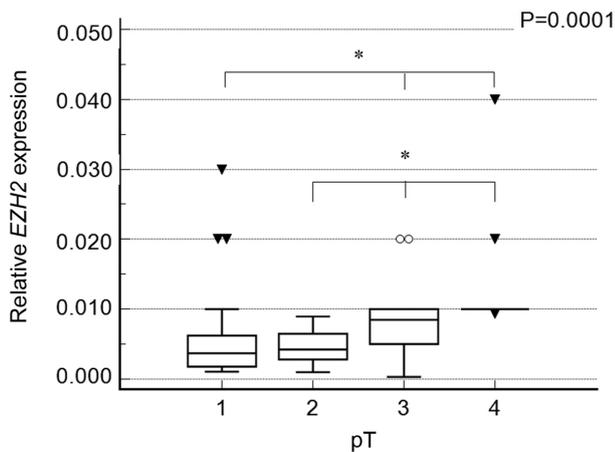


Figure 4. Comparison of relative *EZH2* gene expression in tumors staged pT1, pT2, pT3 or pT4. Values from the lower to the upper quartile are represented by the central box, while the middle line shows the median. The thin vertical lines extending up or down from the boxes to the horizontal lines extend to a multiple of 1.5X the distance of the upper and lower quartile, respectively. Outliers are any values beyond the whiskers. * $P \leq 0.05$.

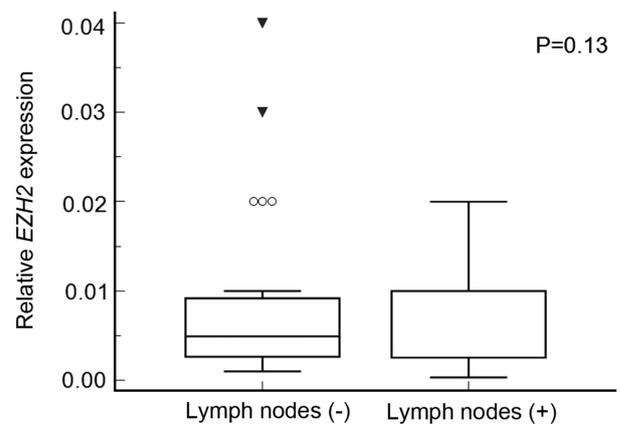


Figure 5. Comparison between relative *EZH2* expression in patients with lymph nodes involvement and without.

useful prognostic marker for more aggressive thyroid cancers. This is justified by their statistically significant increase in

EZH-positivity in order from differentiated (DTC), then poorly differentiated (PDTC) to anaplastic forms of thyroid cancers (ATC). Also, higher *EZH2* expression correlated with more reduced survival in the case of less differentiated cancers (34). Similarly, the prognostic significance of the *EZH2* gene has already been observed in cancers of other organs, including

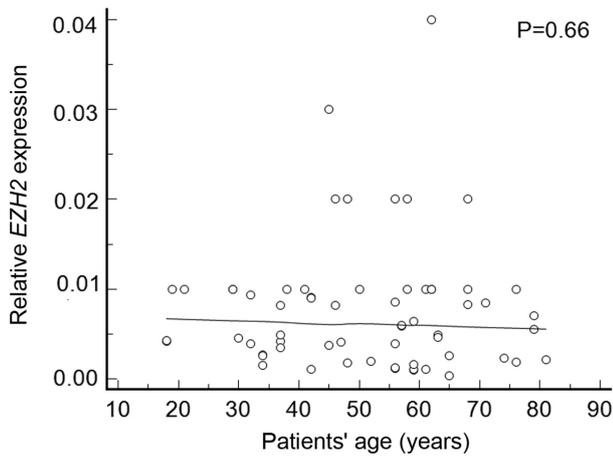


Figure 6. Correlation between relative *EZH2* gene expression and patients' age.

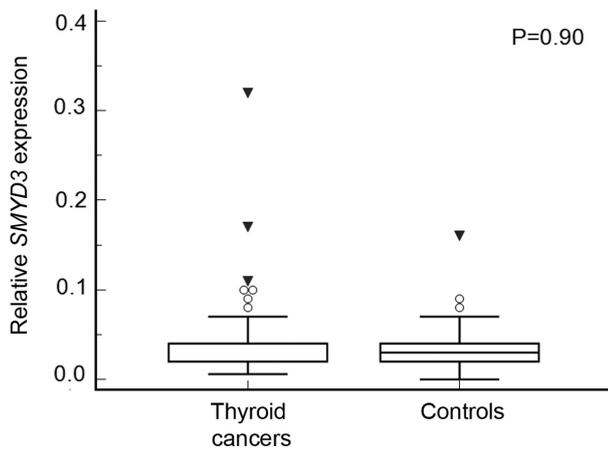


Figure 7. Comparison of *SMYD3* gene expression between thyroid cancers and controls.

the prostate, lung or lymphomas (28). Currently, increasing numbers of studies are being developed that expand the range of thyroid cancers tested, as well as molecular mechanisms associated with the impact of the *EZH2* gene on carcinogenesis (38). *EZH2* is important in medullary thyroid cancer by affecting ERK and AKT signaling pathways. It also controls genes of the Wnt/ β -catenin (24). It has been observed that increased expression of *EZH2* in PTC cell lines upregulates cellular proliferation and migration by affecting the E2-ER α signaling pathway (35). Researchers have observed the role of long noncoding RNA PVT1 in the development of thyroid cancer through its involvement in the modulation of cell proliferation by recruiting *EZH2* and regulating the thyroid-stimulating hormone receptor (TSHR) (39). In their search for differences between thyroid follicular cancer and thyroid follicular adenoma scientists have used network-based genetic profiling, which includes the *EZH2* gene (40).

Overexpression of *SMYD3* was not characteristic of papillary thyroid cancer in our study. Expression of this gene was observed in every test sample and in almost every control sample. Moreover, expression levels in study and control samples were similar. There was also no correlation between *SMYD3* gene expression and the markers of greater disease

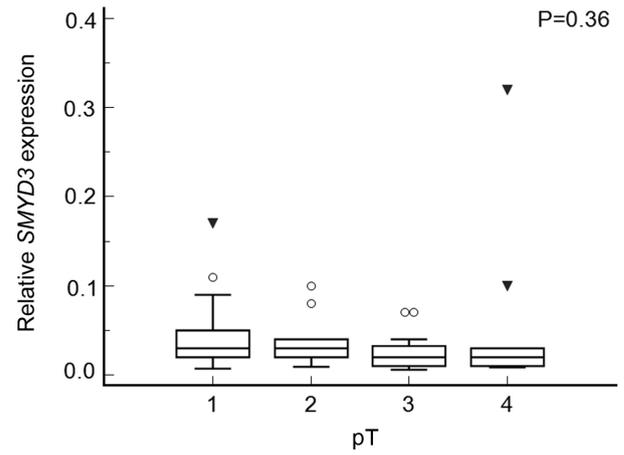


Figure 8. Comparison of relative *SMYD3* gene expression in tumors staged pT1, pT2, pT3 or pT4. Values from the lower to the upper quartile are represented by the central box, while the middle line shows the median. The thin vertical lines extending up or down from the boxes to the horizontal lines extend to a multiple of 1.5X the distance of the upper and lower quartile, respectively. Outliers are any values beyond the whiskers.

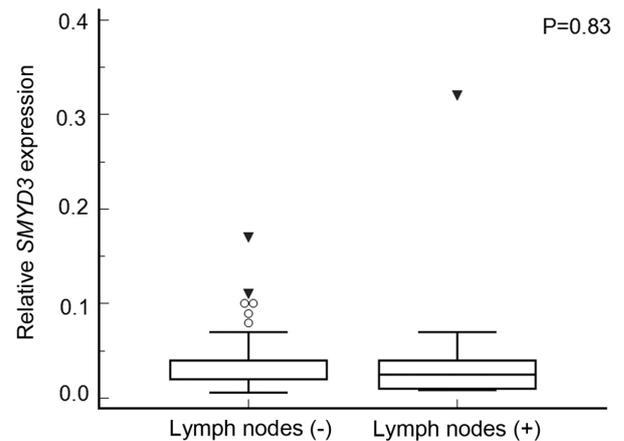


Figure 9. Comparison between relative *SMYD3* expression in patients with lymph nodes involvement and without.

aggression. No studies on the expression of the *SMYD3* gene in papillary thyroid cancer have previously been performed. However, our study was justified due to the overexpression of both the *EZH2* and *SMYD3* genes observed by Sponziello *et al.*, as well as the correlation of both genes with greater aggressiveness of medullary thyroid cancers (MTCs) (24). A similar correlation between the expression level of these genes and tumor aggression has been observed in cancers of other organs, e.g. liver or prostate (3,16). Chemical probes are being developed to target *SMYD3* selectively (41).

In both the study and the control groups, the majority of patients were women. It has been known for many years that PTC is a disease with a high prevalence in women, which is also confirmed by the current research. This tendency is emphasized by Xue *et al.* (35) who recently published their research on the *EZH2* gene in PTC on similarly numerous research (n=65) and control (n=30) groups. Moreover, the latest trends show that among women, the highest increase in incidence was observed in 2014: 22.2 new cases were diagnosed per 100,000 people (42).

Also, researchers have noted that the disproportion between women and men is particularly intensified during the reproductive period (35,43). It has been found that estrogen can increase PTC growth, progression and metastasis and that E2 treatment can significantly increase EZH2 levels (35,44,45). The effectiveness of the specific EZH2 inhibitor GSK126 also confirms the above observations (35). Most, as many as 25 thyroid cancers were in stage I. This means that often the tumor was not larger than 2 cm and did not grow outside the thyroid gland (37). Samples in stages II-IV were similarly numerous. In thyroid cancers, metastases to the lymph nodes were observed in 23% of patients. Although PTC is often localized, the lymphatic tract is the most common for metastasis, and the site of metastasis is usually local lymph nodes (46). Literature data show the influence of both genes on the fate of individual cells, so it would be reasonable to compare cells from the same thyroid that are neoplastic to those unchanged. On the other hand, thyroid cancers might be multifocal, and molecular alterations proceed cancer. So it could also potentially affect achieved results.

Our results indicate that overexpression of the *EZH2* gene may be associated with the development of papillary thyroid cancer. Therefore, the *EZH2* gene may also be a potential therapeutic target in papillary thyroid cancer.

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Availability of data and materials

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

Authors' contributions

NSG designed the study, was involved in data collection, analyzed data, and wrote and revised the manuscript. SS and PZ conducted the manuscript preparation and data analysis/interpretation. MA carried out the experimental studies and data analysis. AC was involved in data collection and data analysis. PG conceived the study and was involved in data analysis. MR made substantial contribution to acquisition of samples and clinical data, and revised the paper. NSG and MA confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The Ethical Committee of Poznan University of Medical Sciences approved the present study (approval no. 228/14). Written informed consent was obtained from all patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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