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# Expression of DNA repair proteins MSH2, MLH1 and MGMT in human benign and malignant thyroid lesions: An immunohistochemical study

**Authors' Contribution:**
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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## Summary

**Background:** DNA repair is a major defense mechanism, which contributes to the maintenance of genetic sequence, and minimizes cell death, mutation rates, replication errors, DNA damage persistence and genomic instability. Alterations in the expression levels of proteins participating in DNA repair mechanisms have been associated with several aspects of cancer biology. The present study aimed to evaluate the clinical significance of DNA repair proteins MSH2, MLH1 and MGMT in benign and malignant thyroid lesions.

**Material/Methods:** MSH2, MLH1 and MGMT protein expression was assessed immunohistochemically on paraffin-embedded thyroid tissues from 90 patients with benign and malignant lesions.

**Results:** The expression levels of MLH1 was significantly upregulated in cases with malignant compared to those with benign thyroid lesions (p=0.038). The expression levels of MGMT was significantly downregulated in malignant compared to benign thyroid lesions (p=0.001). Similar associations for both MLH1 and MGMT between cases with papillary carcinoma and hyperplastic nodules were also noted (p=0.014 and p=0.026, respectively). In the subgroup of malignant thyroid lesions, MSH2 downregulation was significantly associated with larger tumor size (p=0.031), while MLH1 upregulation was significantly associated with the presence of lymphatic and vascular invasion (p=0.006 and p=0.002, respectively).

**Conclusions:** Alterations in the mismatch repair proteins MSH2 and MLH1 and the direct repair protein MGMT may result from tumor development and/or progression. Further studies are recommended to draw definite conclusions on the clinical significance of DNA repair proteins in thyroid neoplasia.

**key words:** **DNA repair proteins • immunohistochemistry • thyroid malignancy • clinicopathological parameters • diagnosis**

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## Background

Benign and malignant thyroid lesions constitute the most common malignancy of endocrine glands, with rates increasing during the last 2 decades [1,2]. The rapidly rising incidence of thyroid cancer has mainly been attributed to improved small papillary tumor detection [3]. However, increased diagnostic scrutiny is not the sole reason, as large and more advanced cancers, which are clinically apparent and associated with a less favourable prognosis, are increasing nearly as fast as small tumors [4]. Papillary thyroid carcinoma is the most common thyroid malignancy, accounting for more than 80% of all thyroid cancers. Together, papillary and follicular thyroid carcinomas are termed differentiated thyroid carcinomas, and represent approximately 90% of all thyroid cancers [5]. The rare forms of thyroid cancer mainly consist of medullary carcinoma arising from parafollicular C-cells, anaplastic carcinoma, and, less frequently, thyroid lymphoma, Hurthle cell and squamous cell carcinoma, and intrathyroid sarcoma [6]. Although thyroid cancer generally has a favorable outcome, a significant proportion of patients ultimately die from the disease due to local recurrences and/or distant metastases [7]. Thyroid-stimulating hormone levels, thyroid ultrasound and fine-needle aspiration biopsy are key clinical tests to guide patient management [8]. However, in many cases the pathologist is confronted by thyroid lesions in which the distinction between benign and malignant can be quite subtle, and the decision favouring one or another has clinical consequences and implies different treatment modalities [9]. In this respect, the identification of molecular markers, which contribute to the discrimination of benign from malignant thyroid tumors, represents a diagnostic challenge. Recently, there has been considerable progress in identifying biomarkers in thyroid tumors, improving the accuracy of fine-needle aspiration biopsy and contributing to the estimation of tumor aggressiveness or behavior [10–12].

DNA repair is an important defense mechanism against DNA damage caused by normal metabolic activities and environmental factors [13]. It includes several distinct pathways: direct repair (DR), base and nucleotide excision repair (BER and NER), mismatch repair (MMR), double strand break repair (DSBR), and interstrand crosslink repair systems [14–16]. Inherited or acquired deficiencies in DNA repair proteins participating in the above mechanisms have generally been considered to contribute to the onset of carcinogenesis [14–16]. In the last few years, alterations in expression levels and polymorphisms of DNA repair genes have been associated with increased risk for developing thyroid cancer [17]. Among them, MMR proteins such as MSH2 (Mut-S-Homologin-2) and MLH1 (Mut-L-Homologon-1) have recently been implicated in the development, progression and metastatis of several types of head and neck neoplasia, including thyroid cancer [18–21]. MSH2 is located on chromosome 2p22-p21 and acts as a heterodimer with either MSH6 or MSH3 (MutSα and MutSβ complex, respectively) in order to bind to and recognize base-base mismatches and 1-10 nucleotides insertion/deletion loops [22]. MLH1 forms heterodimers with PMS2 and MLH3 (MutLα complex) to discriminate the old from the new DNA strand and to signal downstream repair factors, such as helicases and exonucleases [23]. Notably, germ line mutations in MMR genes, in which MSH2 and MLH1 alterations account for the vast majority of cases, have been implicated in hereditary non-polyposis colorectal cancer [24].

Beyond MMR proteins, the methylation status of methyl guanine DNA methyltranferase (MGMT), a direct repair enzyme located on chromosome 10q26, has also been investigated in thyroid neoplasia [25,26]. MGMT protein acts through a self-destruction mechanism, removing abnormal adducts from the $O^6$ position of guanine, providing protection from mutagenic agents and conferring resistance to alkylating chemotherapeutic drugs [27]. Loss of MGMT expression has been associated with aggressive tumor behaviour and progression in several types of neoplasia, including esophageal, hepatocellular, lung, gastric and breast carcinomas [28–32]. However, the available data evaluating the immunohistochemical expression of MSH2, MLH1 and MGMT in benign and malignant thyroid lesions thus far remains sparce. The present study aimed to evaluate the immunohistochemical expression of MSH2, MLH1 and MGMT in patients with benign and malignant thyroid lesions. The association of MSH2, MLH1 and MGMT protein expression with important clinicopathological characteristics, such as tumor size and lymph node metastases, capsular, lymphatic and vascular invasion, was also examined.

## Material and Methods

### Patients

Ninety formalin-fixed, paraffin-embedded thyroid tissues from an equal number of patients who had undergone thyroid surgery for benign or malignant disease were included in this study. None of the patients received any kind of anti-cancer treatment prior to surgery. None of the patients had a history of head and neck irradiation or a history of other cancer types. Each case was classified according to the WHO histological classification of thyroid tumors [33]. The clinical material consisted of 36 benign (30 hyperplastic nodules and 6 Hashimoto thyroiditis) and 54 malignant (40 papillary, 5 medullary, 7 follicular and 2 anaplastic thyroid carcinomas) cases. The characteristics of the population under study classified as benign and malignant thyroid lesions are summarized in Table 1.

### Immunohistochemistry

Immunostainings for MSH2, MLH1 and MGMT were performed on formalin-fixed, paraffin-embedded thyroid tissue sections using mouse monoclonal anti-MSH2 (CM 219 BK, Biocare Medical, Walnut Creek, CA, USA), anti-MLH1 (CM 220 CK, Biocare Medical) and anti-MGMT (sc-56432, Santa Cruz Biotechnology, Santa Cruz, CA, USA), and $IgG_1$ antibodies, respectively. Briefly, 4μm thick tissue sections were dewaxed in xylene and were brought to water through graded alcohols. Antigen retrieval was performed by microwaving slides in 10mM citrate buffer (pH 6.1) for 15 min at high power, according to the manufacturer's instructions. To remove the endogenous peroxidase activity, sections were then treated with freshly prepared 0.3% hydrogen peroxide in methanol in the dark for 30 min at room temperature. Non-specific antibody binding was blocked using Sniper, a specific blocking reagent for mouse primary antibodies (Sniper, Biocare Medical) for 5 min. The sections were incubated for 1 h, at room temperature, with

**Table 1.** Study population characteristics.

| Clinicopathological parameters | Benign | Malignant |
|---|---|---|
| **N=90** | 36 (38%) | 54 (62%) |
| **Age (mean ±SD; ys)** 50.18±15.06 | 49.23±14.74 | 50.32±15.14 |
| **Gender** | | |
| Male | 17 (13%) | 16 (12%) |
| Female | 56 (41%) | 46 (34%) |
| **Histopathology** | Hyperplastic nodule 30 (33%) | Papillary 40 (44%) |
| | Hashimoto thyroiditis 6 (7%) | Medullary 5 (6%) |
| | | Follicular 7 (8%) |
| | | Anaplastic 2 (2%) |
| **Tumor size (T)** | N/A* | |
| T1 | | 38 (70%)** |
| T2 | | 11 (20%)** |
| T3 | | 2 (4%)** |
| T4 | | 6 (6%)** |
| **Lymph node metastases (N)** | N/A | |
| N0 | | 49 (91%)** |
| N1 | | 5 (9%)** |
| **Distant metastases (M)** | N/A | |
| M0 | | 54 (100%) |
| **Capsular invasion** | N/A | |
| No | | 43 (80%)** |
| Yes | | 11 (20%)** |
| **Lymphatic invasion** | N/A | |
| No | | 44 (81%)** |
| Yes | | 10 (19%)** |
| **Vessel invasion** | N/A | |
| No | | 47 (87%)** |
| Yes | | 7 (13)** |
| **Ki-67 protein statement** | | p-value=0.0001 |
| Negative | 31 (34%) | 28 (31%) |
| Positive | 5 (6%) | 26 (29%) |

* N/A: not applicable; ** Percentages in parentheses correspond to the number of malignant thyroid cases.

the primary antibodies against MSH2, MLH1 and MGMT diluted 1:100 in Van Gogh (Biocare Medical), Renoir Red (Biocare Medical) and phosphate buffered saline (PBS), respectively, according to the manufacturers' instructions. After washing 3 times with PBS, sections were then incubated at room temperature with biotinylated linking reagent (Biocare Medical) for 10 min, followed by incubation with peroxidase-conjugated streptavidin label (Biocare Medical) for 10 min. The resultant immune peroxidase activity was developed using a DAB substrate kit (Vector Laboratories, USA) for 10 min. Sections were counterstained with Harris' hematoxylin and mounted in Entellan (Merck, Darmstadt, Germany). Appropriate negative controls were performed by omitting the primary antibody and/or substituting it with an irrelevant anti-serum. As a positive control, colon cancer tissue sections with known increased MLH1, MSH2 and MGMT immunoreactivity were used [34]. Follicular cells' proliferative capacity was assessed immunohistochemically,

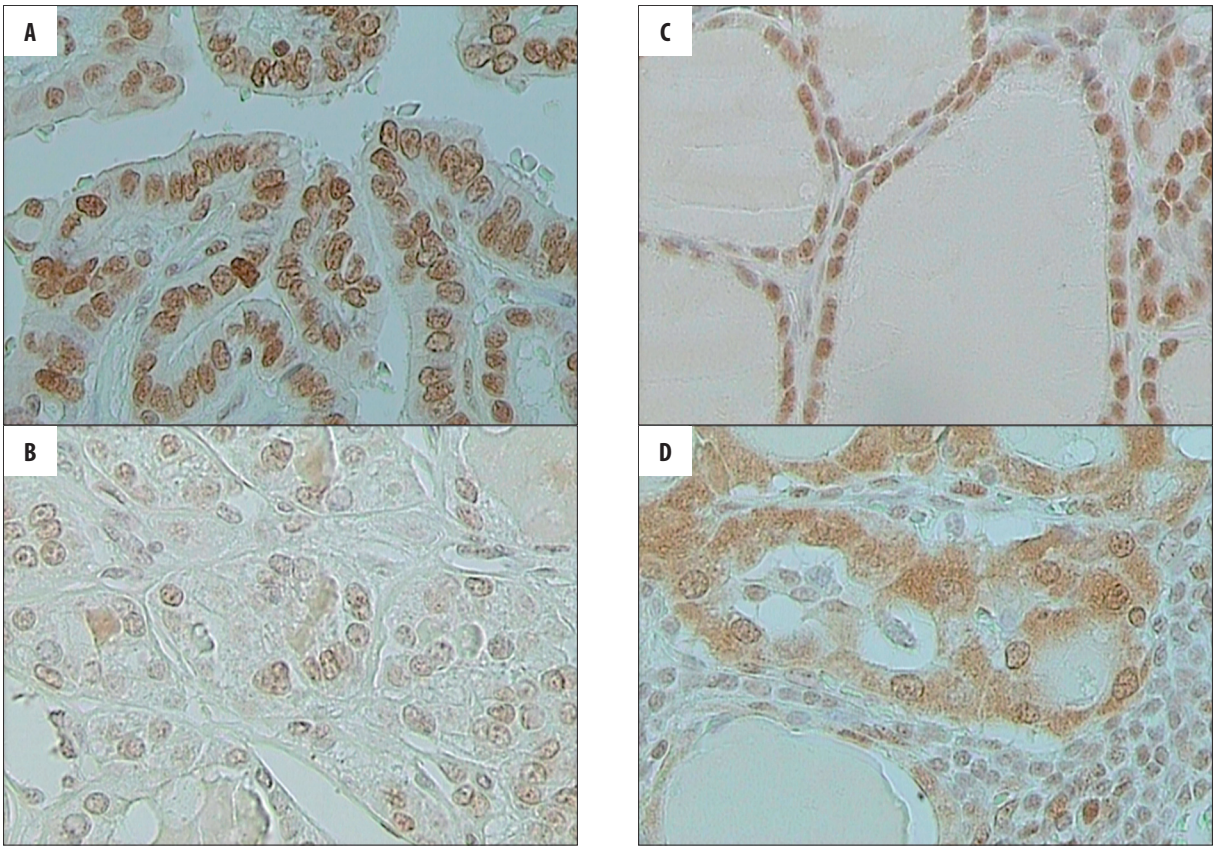


**Figure 1.** Representative immunostainings of MSH2 protein expression in malignant thyroid cases with (**A**) Papillary carcinoma and (**B**) Follicular carcinoma and in benign thyroid cases with (**C**) Hyperplastic nodules and (**D**) Hashimoto thyroiditis.

using a mouse anti-human Ki-67 antigen; $IgG_{1k}$ antibody (clone MIB-1, Dakopatts, Glostrup, Denmark), as previously described [35,36].

### Evaluation of immunohistochemistry

The percentages of positively stained follicular cells were obtained by counting at least 1000 cells in each case by 2 independent observers (S.T. and P.A) blinded to the clinical data, with complete observer agreement ($\kappa$=0.959, SE: 0.024). Specimens were considered "positive" for MSH2, MLH1 and MGMT when more than 5% of the follicular cells were stained. A semi-quantitative scoring system was applied based on previously published reports of other immunohistochemical markers on thyroid tissue lesions [36–39]. The immunoreactivity of the follicular cells for MSH2, MLH1 and MGMT was scored according to the percentage of MSH2, MLH1 and MGMT positive follicular cells as 0: negative staining – 0–4% of follicular cells positive; 1: 5–24% of follicular cells positive; 2: 25–49% of follicular cells positive; 3: 50–100% of follicular cells positive, and its intensity as 0: negative staining, 1: mild staining; 2: intermediate staining; 3: intense staining. Finally, the immunoreactivity of MSH2, MLH1 and MGMT was classified as negative/weak if the total score was 0–2, and moderate/strong if the total score was ≥3. In this way we ensured that each group had a sufficient and more homogeneous number of cases in order to be comparable with the other groups [35,36]. Both nuclear and cytoplasmic immunostaining was taken into consideration for the immunohistochemical scoring. Ki-67 immunoreactivity was classified according to the percentage of positively stained follicular cells exceeded the mean percentage value into 2 categories (below and above mean value), as previously reported [35,36].

### Statistical analysis

Chi-square tests were used to assess the difference of MSH2, MLH1 and MGMT immunoreactivity between malignant and benign thyroid lesions, as well as between papillary carcinoma cases and hyperplastic nodules, which comprise the most numerous histopathological entities of malignant and benign cases, respectively. Chi-square tests were also used to assess the associations between MSH2, MLH1 and MGMT immunoreactivity and clinicopathological characteristics in the subgroup of patients with malignant thyroid lesions. A 2-tailed $p<0.05$ was considered statistically significant. Statistical analyses were performed using SPSS for Windows (version 11.0; SPSS Inc., Chicago, IL, USA).

## Results

MSH2 positivity (IHC score >0) was noted in 82 (91%) out of 90 cases with thyroid lesions. More than half (50/90, 56%) of the examined cases presented moderate/strong immunoreactivity for MSH2 protein (IHC score ≥3). The pattern of MSH2 distribution was predominantly nuclear, and occasionally cytoplasmic in cases with malignant thyroid lesions (Figure 1A, B). The pattern of MSH2 distribution was predominantly nuclear in cases with hyperplastic

**Table 2.** Associations of MSH2, MLH1 and MGMT expression with patients' age and gender, type of histopathology (Benign *vs* Malignant thyroid lesions and Hyperplasic nodules *vs* Papillary carcinomas) and Ki-67 protein statement in 90 thyroid lesions cases.

| Clinicopathological characteristics | MSH2 expression | | | MLH1 expression | | | MGMT expression | | |
|---|---|---|---|---|---|---|---|---|---|
| | Negative/ weak (%) | Moderate/ strong (%) | p-value | Negative/ weak (%) | Moderate/ strong (%) | p-value | Negative/ weak (%) | Moderate/ strong (%) | p-value |
| **N=90** | 40 (44) | 50 (56) | | 67 (74) | 23 (26) | | 24 (27) | 66 (73) | |
| **Age** (mean±SD;ys) | 51.6±14.4 | 48.3±15.6 | 0.340 | 51.1±14.3 | 46.1±17.0 | 0.382 | 49.1±14.9 | 50.0±15.3 | 0.701 |
| **Gender** | | | **0.019** | | | 0.434 | | | 0.176 |
| Female | 26 (29) | 43 (48) | | 50 (56) | 19 (21) | | 16 (18) | 53 (59) | |
| Male | 14 (16) | 7 (8) | | 17 (19) | 4 (4) | | 8 (9) | 13 (14) | |
| **Histopathology** | | | 0.665 | | | **0.038** | | | **0.001** |
| Benign | 15 (17) | 21 (23) | | 31 (34) | 5 (6) | | 3 (3) | 33 (37) | |
| Malignant | 25 (28) | 29 (32) | | 36 (40) | 18 (20) | | 21 (23) | 33 (37) | |
| **Histopathology (N=70)** | | | 0.228 | | | **0.014** | | | **0.026** |
| Hyperplastic nodules | 14 (20) | 16 (3) | | 26 (37) | 4 (6) | | 3 (4) | 27 (39) | |
| Papillary | 13 (19) | 27 (39) | | 24 (34) | 16 (23) | | 13 (19) | 27 (39) | |
| **Ki-67 protein statement** | | | 0.427 | | | **0.038** | | | 0.893 |
| Negative | 28 (31) | 31 (34) | | 48 (53) | 11 (12) | | 16 (18) | 43 (48) | |
| Positive | 12 (13) | 19 (21) | | 19 (21) | 12 (13) | | 8 (9) | 23 (26) | |

**Table 3.** MSH2, MLH1 and MGMT immunoreactivity in the distinct subgroup of benign and malignant thyroid lesions.

| Thyroid lesions | N | MSH2 expression | | MLH1 expression | | MGMT expression | |
|---|---|---|---|---|---|---|---|
| | | Negative/ Weak (%) | Moderate/ Strong (%) | Negative/ Weak (%) | Moderate/ Strong (%) | Negative/ Weak (%) | Moderate/ Strong (%) |
| Papillary carcinoma | 40 | 13 (33) | 27 (67) | 24 (60) | 16 (40) | 13 (33) | 27 (67) |
| Medullary carcinoma | 5 | 4 (80) | 1 (20) | 4 (80) | 1 (20) | 2 (40) | 3 (60) |
| Follicular carcinoma | 7 | 7 (100) | 0 (0) | 6 (86) | 1 (14) | 5 (71) | 2 (29) |
| Anaplastic carcinoma | 2 | 1 (50) | 1 (50) | 2 (100) | 0 (0) | 1 (50) | 1 (50) |
| Hyperplastic nodule | 30 | 14 (47) | 16 (53) | 26 (87) | 4 (13) | 3 (10) | 27 (90) |
| Hashimoto's thyroiditis | 6 | 1 (17) | 5 (83) | 5 (83) | 1 (17) | 0 (0) | 6 (100) |

nodules, whereas Hashimoto thyroiditis cases showed both nuclear and cytoplasmic staining to an equivalent extent (Figure 1C, D). In cross-tables, female patients presented significantly higher incidence of moderate/strong MSH2 immunoreactivity compared to male patients (Table 2, p=0.019). MSH2 immunoreactivity was not significantly different between benign and malignant thyroid lesions (Table 2, p=0.665). Moderate/strong MSH2 immunoreactivity was more frequently observed in cases with papillary carcinoma (27/40, 68%) compared to those with hyperplastic nodules (16/30, 53%), without reaching statistical significance (Table 2, p=0.228). The vast majority of follicular (6/7, 85.71%) and medullary (4/5, 80%) carcinoma cases showed negative/weak MSH2 immunostaining (Table 3). Five (83%) out of 6 cases with Hashimoto thyroiditis and 1 (50%) out of 2 cases with anaplastic carcinoma showed moderate/strong MSH2 immunoreactivity (Table 3). In the subgroup of malignant thyroid lesions, moderate/strong MSH2 immunoreactivity was significantly associated with small tumor size and borderline with enhanced follicular cells' proliferative capacity (Table 4, p=0.031 and p=0.067, respectively).

MLH1 positivity (IHC score >0) was noted in 26 (29%) out of 90 cases with thyroid lesions. Twenty-three (26%) out of 90 thyroid lesions showed moderate/strong MLH1 immunoreactivity (IHC score ⩾3). The pattern of MLH1 distribution was both nuclear and cytoplasmic in malignant thyroid lesions, except for papillary carcinoma cases, which showed perinuclear and cytoplasmic staining (Figure 2A, B). The

**Table 4.** Associations of MSH2, MLH1 and MGMT expression with clinicopathological characteristics in 54 patients with malignant thyroid lesions.

| Clinicopathological Characteristics | MSH2 expression | | | MLH1 expression | | | MGMT expression | | |
|---|---|---|---|---|---|---|---|---|---|
| | Negative/ Weak (%) | Moderate/ Strong (%) | p-value | Negative/ Weak (%) | Moderate/ Strong (%) | p-value | Negative/ Weak (%) | Moderate/ Strong (%) | p-value |
| **N=54** | 25 (46) | 29 (54) | | 36 (67) | 18 (33) | | 21 (39) | 33 (61) | |
| **Age** (mean ±SD; ys) | 51.9±13.9 | 47.0±15.4 | 0.270 | 50.2±13.9 | 47.4±16.7 | 0.883 | 48.6±14.4 | 49.7±15.2 | 0.570 |
| Tumor size (T) | | | **0.031** | | | 0.399 | | | **0.079** |
| T1 | 14 (26) | 24 (44) | | 24 (44) | 14 (26) | | 12 (22) | 26 (48) | |
| T2-4 | 11 (20) | 5 (9) | | 12 (22) | 4 (7) | | 9 (17) | 7 (13) | |
| **Lymph node metastasis (N)** | | | 0.518 | | | 0.184 | | | 0.309 |
| N0 | 22 (41) | 27 (50) | | 34 (63) | 15 (28) | | 18 (33) | 31 (57) | |
| N1 | 3 (6) | 2 (4) | | 2 (4) | 3 (6) | | 3 (6) | 2 (4) | |
| **Capsular invasion** | | | 0.949 | | | **0.094** | | | 0.616 |
| No | 20 (37) | 23 (43) | | 31 (57) | 12 (22) | | 16 (30) | 27 (50) | |
| Yes | 5 (9) | 6 (11) | | 5 (9) | 6 (11) | | 5 (9) | 6 (11) | |
| **Lymphatic invasion** | | | 0.794 | | | **0.006** | | | 0.522 |
| No | 20 (37) | 24 (44) | | 33 (61) | 11 (20) | | 18 (33) | 26 (48) | |
| Yes | 5 (9) | 5 (9) | | 3 (6) | 7 (13) | | 3 (6) | 7 (13) | |
| **Vascular invasion** | | | 0.313 | | | **0.002** | | | 0.548 |
| No | 23 (43) | 24 (44) | | 35 (65) | 12 (22) | | 19 (35) | 28 (52) | |
| Yes | 2 (4) | 5 (9) | | 1 (2) | 6 (11) | | 2 (4) | 5 (9) | |
| **Ki-67 protein statement** | | | **0.067** | | | 0.177 | | | 0.238 |
| Negative | 16 (30) | 12 (22) | | 21 (39) | 7 (13) | | 13 (24) | 15 (28) | |
| Positive | 9 (17) | 17 (31) | | 15 (28) | 11 (20) | | 8 (15) | 18 (33) | |

pattern of MLH1 distribution was nuclear in cases with hyperplastic nodules, whereas those with Hashimoto thyroiditis showed predominantly cytoplasmic and occasionally nuclear patterns of staining (Figure 2C, D). MLH1 immunoreactivity was not significantly associated with patients' sex (Table 2, p>0.05). Moderate/strong MLH1 immunoreactivity was significantly more frequently observed in cases with malignant compared to those with benign thyroid lesions (Table 2, p=0.038). A similar discrimination between cases with papillary carcinoma and hyperplastic nodules was also noted (Table 2, p=0.014). Sixteen (40.00%) out of 40 cases with papillary carcinoma presented moderate/strong MLH1 immunoreactivity, whereas only 4 (13%) out of 30 cases with hyperplastic nodules showed moderate/strong MLH1 immunoreactivity. Thyroid lesions with enhanced follicular cell proliferative capacity, reflected by Ki-67 labeling index, showed significantly increased incidence of moderate/strong MLH1 immunoreactivity (Table 2, p=0.038). The vast majority of follicular (6/7, 86%) and medullary (4/5, 80%) carcinomas showed negative/weak MLH1 immunostaining (Table 3). Five (83%) out of 6 cases with Hashimoto thyroiditis and all (2/2, 100%) anaplastic carcinomas showed negative/weak MLH1 immunoreactivity (Table 3). In the subgroup of malignant thyroid lesions, moderate/strong MLH1 immunoreactivity was significantly associated with the presence of lymphatic and vascular invasion (Table 4, p=0.006, p=0.002, respectively), whereas a trend of correlation with capsular invasion was also noted (Table 4, p=0.094). Malignant thyroid cases with enhanced follicular cells showed increased incidence of moderate/strong MLH1 immunoreactivity, without reaching statistical significance (Table 4, p=0.177).

MGMT positivity (IHC score >0) was noted in 70 (78%) out of 90 cases with thyroid lesions. Sixty-six (73%) out of 90 thyroid lesions showed moderate/strong MGMT immunoreactivity (IHC score ⩾3). The pattern of MGMT distribution was both nuclear and cytoplasmic in malignant thyroid lesions (Figure 3A, B). The pattern of MGMT distribution was predominately nuclear in cases with hyperplastic nodules, whereas in Hashimoto thyroiditis perinuclear and cytoplasmic staining was noted (Figure 3C, D). MGMT immunoreactivity was not significantly associated with patients' sex or follicular cell proliferative capacity (Table 2, p>0.05). Negative/weak MGMT immunoreactivity was significantly more frequently observed in malignant compared to benign thyroid lesions (Table 2, p=0.001). A similar discrimination between cases with papillary carcinoma and hyperplastic

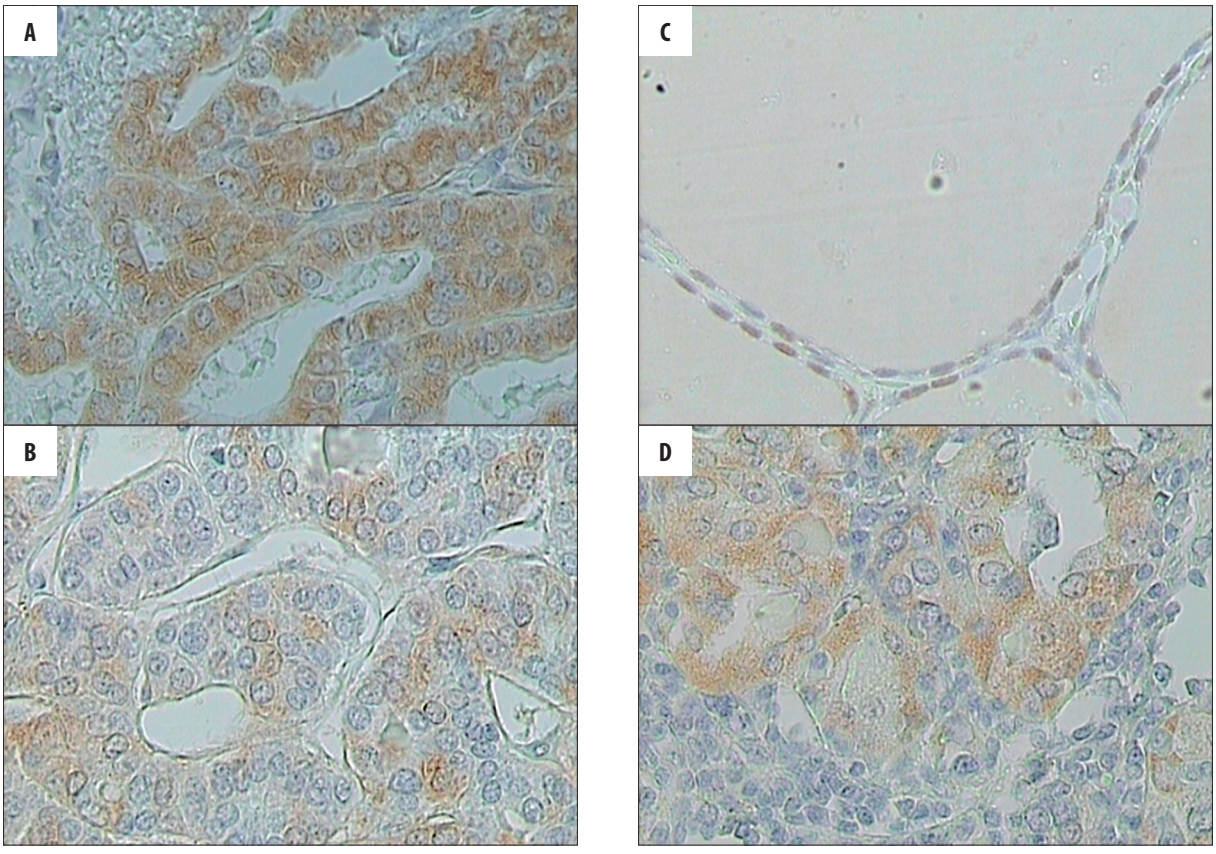


**Figure 2.** Representative immunostainings of MLH1 protein expression in malignant thyroid cases with (**A**) Papillary carcinoma and (**B**) Follicular carcinoma and in benign thyroid cases with (**C**) Hyperplastic nodules and (**D**) Hashimoto thyroiditis.

nodules was also noted (Table 2, p=0.026). Thirteen (32%) out of 40 cases with papillary carcinoma presented negative/weak MGMT immunoreactivity, whereas only 3 (10%) out of 30 cases with hyperplastic nodules showed analogous MGMT immunoreactivity (Table 3). Three (60%) out of 5 medullary, 2 (29%) out of 7 follicular, and 1 (50%) out of 2 anaplastic carcinomas presented moderate/strong MGMT immunoreactivity (Table 3). All (6/6, 100%) Hashimoto thyroiditis cases showed moderate/strong MGMT immunoreactivity (Table 3). In the subgroup of malignant thyroid lesions, MGMT immunoreactivity was not associated with clinicopathological parameters, except for a trend of correlation with tumor size, as larger tumors presented increased frequency of negative/weak MGMT immunoreactivity (Table 4, p= 0.079). Moderate/strong MGMT immunoreactivity was more frequently observed in malignant cases with enhanced follicular cell proliferative capacity, but not at a significant level (Table 4, p=0.238).

## Discussion

It is well established that inherited or acquired deficiencies in DNA repair proteins may lead to deleterious mutation rates, genomic instability and cell death associated with development, differentiation and progression of cancer [15,27]. Notably, alterations in expression levels and polymorphisms of DNA repair genes have been considered responsible for induction of thyroid carcinogenesis [17]. In this regard, the present study evaluated the immunohistochemical expression of MSH2 and MLH1 proteins, which participate in MMR mechanism, as well as that of MGMT, a direct DNA repair protein, in human benign and malignant thyroid lesions.

Among MMR proteins, the expression levels of MLH1 were upregulated in cases with malignant compared to those with benign thyroid lesions. This discrimination is mainly ascribed to the increased frequency of moderate/strong MLH1 expression in cases with papillary carcinoma compared to those with hyperplastic nodules. On the other hand, MSH2 expression was not significantly different between malignant and benign thyroid lesions, while in cases of papillary carcinoma an increased frequency of moderate/strong MSH2 immunoreactivity compared to those with hyperplastic nodules was noted, but at a non-significant statistical level. Ruschenburg et al documented that the expression levels of 3 MMR proteins, MLH1, MSH2 and PMS1, were generally elevated in malignant compared to benign thyroid lesions [20]. The present study included hyperplastic nodules and follicular adenomas as benign, and follicular carcinomas as malignant thyroid lesions, and did not detect point mutations in MSH2 (exon 12, 13) and MLH1 (exon 15, 16) genes [20]. On the other hand, we found negative/weak MSH2 and MLH1 expression in the vast majority of follicular carcinoma cases (7/7 and 6/7, respectively), which needs to be confirmed by a larger cohort study in order for precise conclusions to be drawn. The vast majority of medullary carcinoma cases also showed increased incidence of negative/weak expression for both MSH2 and MLH1 proteins, which further suggests that the role of MMR proteins

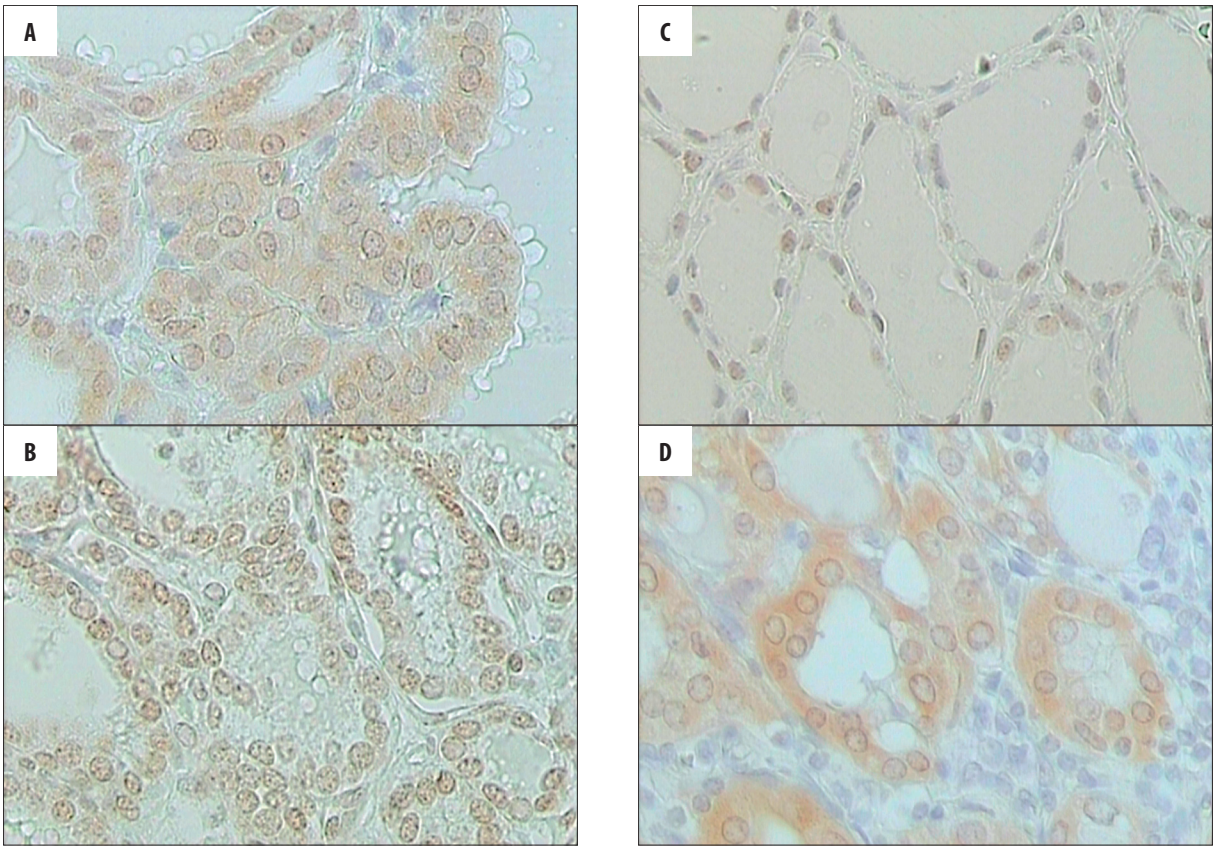


**Figure 3.** Representative immunostainings of MGMT protein expression in malignant thyroid cases with (**A**) Papillary carcinoma and (**B**) Follicular carcinoma and in benign thyroid cases with (**C**) Hyperplastic nodules and (**D**) Hashimoto thyroiditis.

in thyroid neoplastic transformation may vary among the different thyroid carcinoma variants.

The present study also showed that MLH1 upregulation was positively associated with the presence of capsular, lymphatic and vascular invasion, while MSH2 downregulation was positively associated with larger tumor size. These differences in the expression levels of MSH2 and MLH1 may result from tumor development and/or progression of thyroid cancer. In this context, epigenetic alterations on MLH1 have been reported, as methylation of the MLH1 gene was associated with lymph node metastasis and T1799A BRAF mutation in patients with papillary thyroid carcinoma [21]. Moreover, microsatellite instability (MSI), a form of genomic instability associated with MMR deficiency, has recently been implicated in the pathogenesis of thyroid cancer. In fact, Mitmaker et al showed increased incidence of MSI in papillary (9/14) and follicular (10/16) carcinoma [40]. In addition, a significant difference in MSI frequency between follicular adenomas and follicular carcinomas was noted [40]. On the other hand, several studies reported higher MSI frequency in benign compared to malignant thyroid lesions [41–43]. However, a more recent study documented MSI in 70% of benign and 65% of malignant thyroid lesions, and stated that iodine deficiency may influence MSI by altering the molecular pathway and leading especially to follicular and anaplastic carcinoma [44]. Immunohistochemical analysis of MMR proteins has been considered as a better first-line screening tool than MSI testing for detecting mutation rates of particular genes [45].

The present study further documented that moderate/strong MSH2 and MLH1 immunoreactivity was more frequently observed in cases with enhanced follicular cell proliferative capacity. This finding implies that MMR proteins may be involved in the cell proliferation state of thyroid neoplasia, as highly proliferating follicular cells are expected to have increased necessity for DNA repair systems. Such an association may enhance the diagnostic utility of DNA repair protein detection in the thyroid neoplasia decision-making process, as the proliferative capacity of tumor cells is a characteristic feature in whole growing tumors, while enhanced cellular proliferative activity and cell death have already been associated with thyroid malignancy [46–48].

Differences in the cellular localization of MMR proteins were also noted. Papillary carcinoma cases presented nuclear (or perinuclear) and cytoplasmic distribution for MSH2 and MLH1 proteins, in contrast to hyperplastic nodules, which showed only nuclear staining. This finding may suggest that destabilization of the MMR protein complexes could be occurring progressively from hyperplasia to malignancy, as MMR proteins may be no longer bound to the nuclear DNA. Such a distribution pattern may be ascribed to certain types of MMR gene mutation and may reflect protein deregulation, as MMR proteins could be incompletely synthesized in the cytoplasmic ribosomes due to gene mutations that prevent transport to the nucleus [45,49–52]. Hashimoto thyroiditis cases also showed both nuclear and cytoplasmic MSH2 and MLH1 protein distribution. It has been shown that there is an overlap in the morphological

features, immunohistochemical staining pattern, and most importantly, molecular profile between papillary thyroid carcinoma and Hashimoto thyroiditis [53]. Although considered as a benign condition, Hashimoto thyroiditis almost always harbours a genetic rearrangement strongly associated with and highly specific for papillary thyroid carcinoma [53]. The RET/PTC-RAS-BRAF cascade was been shown to be involved in the development of papillary thyroid carcinoma and oxyphil cell metaplasia in Hashimoto thyroiditis, raising the possibility of a molecular link between the 2 disorders [54].

We further showed that the expression levels of MGMT were downregulated in cases with malignant compared to those with benign thyroid lesions. Papillary carcinoma cases also showed increased incidence of MGMT downregulation compared to hyperplastic nodules. Moreover, MGMT downregulation was associated with large tumor size in thyroid cancer cases. Research involving methylation status of MGMT showed an incidence of 15% methylation in papillary thyroid tumors [26]. Moreover, Schagdarsurengin et al reported that MGMT hypermethylation preferentially occurred in undifferentiated thyroid carcinomas compared to differentiated ones [25]. Accordingly, loss of MGMT expression in several cancer tissues, including hepatocellular, gastric, breast, esophageal and biliary tract carcinoma, has been correlated with clinicopathological parameters and poor prognosis [28,29,55]. Reduced MGMT expression was associated with advanced stage, lymph node positivity and poor prognosis in oral squamous cell carcinoma patients [31]. In precancerous oral lesions, significant loss of MGMT expression was noted from hyperplasia to dysplasia, supporting the assumption that MGMT deregulation may be an early event in oral tumorogenesis [31]. Low MGMT expression was also correlated with hepatic invasion and poor prognosis in biliary tract carcinomas [29]. Hepatocellular carcinoma patients with reduced MGMT presented advanced disease stage and poor prognosis [30]. Low MGMT expression was also associated with serosal invasion, advanced disease stage, lymph node positivity, undifferentiated histopathological type and poor prognosis in gastric carcinoma patients [28]. Collectively, the reduced expression levels of MGMT protein in malignant thyroid lesions, in accordance with evidence from other types of cancer, reinforces the assumption that downregulation of MGMT expression may result from tumor development and/or progression of thyroid neoplasia. The co-localization of MGMT in the nucleus and cytoplasm of follicular cells in malignant thyroid lesions, in contrast to hyperplastic nodules, which showed predominately nuclear distribution, may be ascribed to MGMT gene mutation, as has been suggested for other types of cancer [56]. A similar pattern of distribution was observed in cases with Hashimoto's thyroiditis, which may be ascribed to the fact that it almost always harbours a genetic rearrangement that is strongly associated with and is highly specific for papillary thyroid carcinoma, raising the possibility of a molecular link between the 2 disorders [53,54].

## Conclusions

Alterations in the expression levels of MLH1 and MGMT proteins detected by immunohistochemistry were associated with thyroid malignancy. MSH2, MLH1 and MGMT also showed correlations with clinicopathological parameters crucial for patient management. In this context, the present study suggests that alterations in the expression levels of MMR and MGMT proteins may result from tumor development and/or progression of thyroid neoplasia. Larger cohort studies are recommended in order to draw more definite conclusions on the clinical significance of the DNA repair proteins, with the aim to improve diagnostic scrutiny in thyroid neoplasia. Subset analysis of histological subtypes of papillary and follicular carcinoma, with the latter being the most difficult for pathologists to diagnose, is also recommended. Further research should also been conducted to elucidation the precise molecular mechanisms through which DNA repair proteins participate in thyroid cancer development and progression.

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