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Research Article
Expression of MSH2 in Head and Neck Lymphomas (A Study Utilizing Immunohistochemistry and Real-time Polymerase Chain Reaction)

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Abstract

Background and Objective: Lymphomas are malignant neoplastic proliferations that cause major health problems worldwide. However, the precise pathogenesis and underlying molecular mechanisms of both Hodgkin's lymphoma (HL) and non-Hodgkin's lymphoma (NHL) are not fully elucidated. Accordingly, the aim of the present study was to detect the expression of MSH2 at the gene and protein levels in HL and NHL of the head and neck. **Materials and Methods:** Expression of MSH2 on gene and protein levels were detected using quantitative real-time polymerase chain reaction (qRT-PCR) and immunohistochemistry respectively. A total of 26 archival specimens were selected including normal lymph nodes (6 cases) which were used as a control group, lingual tonsils with lymphoid hyperplasia (6 cases) and 14 cases of lymphomas of submandibular and sublingual lymph nodes. Lymphomas were classified into of HL (6 cases of mixed cellularity type) and NHL (8 cases of diffuse B cell lymphoma). **Results:** Lower expression of MSH2 in NHL than HL was also observed. Moreover, a positive correlation was detected between the expression of MSH2 protein and its mRNA among the examined groups. **Conclusion:** Lower expression of MSH2 in NHL than that of HL suggested a more aggressive behavior and poor prognosis in this lesion. The reduced expression of MSH2 could be considered as a molecular biomarker for loss of MMR activities and it might serve as a prognostic biomarker for head and neck lymphomas.

Key words: MSH2, Lymphoma, immunohistochemistry, polymerase chain reaction, prognostic biomarker

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Competing Interest: The author has declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Lymphomas represent one of the major health problems throughout the world, as they account for over 3.5% of cancers occurring worldwide. They are heterogeneous group of malignancies of immune system, which are formed due to neoplastic clonal proliferation of lymphoid cells and present as a solid tumor. The head and neck region has been one of the most common sites for extra nodal lymphomas, about 1.5-8.8% occurred in the oral and para oral regions. Lymphomas are divided into Hodgkin's lymphoma (HL) and non-Hodgkin's lymphoma (NHL). The NHL is subdivided into B-cell (accounting for 80-85% of all cases), T-cell and natural killer (NK)-cell types. The HL has two major subtypes classical (accounting for 95% of all cases) and nodular lymphocyte predominant types[1,2].

In Egypt, lymphomas have represented an important oncologic problem, especially NHL which was considered as one of the five most common cancers, accounting for 4.64% of all cancers. Moreover, a progressive increase in the general number of incident cancer cases has been expected in the period from 2013 to 2050[3]. Therefore, it is extremely urgent to unravel the underlying molecular mechanisms of both HL and NHL for better understanding of these common malignancies in community.

The DNA mismatch repair (MMR) genes are responsible for maintaining the fidelity of DNA replication by recognizing mis-incorporation errors and facilitating their excision. Several MMR proteins have been identified such as Mut-S homologs (MSH) and the Mut-L homologs (MLH)[4,5]. Defects in any of these can result in an increased rate of mutation, which were identified in many cancers. A crucial part of this system is MSH2 gene, which is located on chromosome 2p22, its protein recognizes DNA mismatches by forming two functional heterodimers: MSH2-MSH6 and MSH2-MSH3[6].

Furthermore, MSH2 is principally involved in a phenomenon known as microsatellite instability (MSI). This phenomenon is caused by accumulation of DNA replication errors, particularly in areas of the genome with short repetitive nucleotide sequences[7]. In the past few years, a growing body of evidence has accumulated about the importance of MSI as a distinct, measurable form of genomic damage and its useful prognostic and predictive value in different tumors[8]. Being defined as a finding in lymphomas, MSI and MMR proteins might be involved in the pathogenesis of these lesions. In the same context, studies have proposed that mutations in MSH2, could be one of the critical genes that underlie a novel pathway of tumorigenesis for some cancers, especially lymphomas[9,10].

Despite the advances in our knowledge of the role of MSH2 in different cancers and human lymphomagenesis, to date, no sufficient data supported its role in lymphomas of the head and neck. Accordingly, the aim of the present study was to detect the expression of MSH2 at the gene and protein levels in HL and NHL of the head and neck. This could contribute to better understanding of its role in development, tumorigenesis, prognosis and treatment of these malignancies.

MATERIALS AND METHODS

The research project was conducted from April 2016 to February 2017.

Case selection: A total of 26 paraffin blocks were used in this study including six cases of normal lymph nodes (NLs) obtained from elective neck dissection of oral squamous cell carcinoma with a clinical staging N=0 (used as control group), six cases of lingual tonsils with lymphoid hyperplasia (LH) and fourteen cases of lymphomas of the submandibular and sublingual lymph nodes. All cases were retrieved from the archival paraffin blocks from files of Pathology Department, National cancer institute, Cairo University. Four micron paraffin sections were cut from each block, stained with Haematoxylin and Eosin (H&E) and re-examined for the confirmation of diagnosis. The normal lymph nodes were re-diagnosed as normal and free from any histological features of malignancy. On the other hand, the Lymphoma cases were classified into HL (6 cases of mixed cellularity type) and NHL (8 cases of diffuse B cell lymphoma) according to the REAL classification (Revised European American Lymphoma Classification).[11]

Paraffin sections of each case were mounted on positively charged glass slides (Optiplus, Biogenex, Milmont Drive, CA, USA) for immunostaining with anti-MSH2 antibody. In addition, five sections of 5 µm thickness were cut from each case and placed in plastic Eppendorf tubes. These tissue sections were used to extract total RNA for quantitative determination of MSH2 gene expression.

Immunohistochemical staining protocol: The 26 paraffin embedded tissue sections on positively charged slides were immunostained with anti-MSH2 antibody with super sensitive biotin-streptavidin staining technique. Tissue sections were de-paraffinized, rehydrated and treated with endogenous peroxidase in 0.3% H_2O_2 for 30 min to block the endogenous peroxidase activity. For antigen retrieval, the slides were boiled in 10 mM citrate buffer, pH 6.0 for 10-20 min followed by cooling at room temperature for 20 min. The positive test

slides were incubated with the primary antibody mouse monoclonal anti-MSH2 antibody (Cat #MS-1498-R7, Thermo Scientific, Labvision, Kalamazoo, MI, USA) for 30 min at room temperature in a humidified chamber. On the other hand, the negative control slides were not exposed to the primary antibody. After washing with phosphate buffer solution (PBS), the slides were treated with the biotin-labeled link antibody for 30 min, then the streptavidin conjugated horseradish peroxidase was used. The diaminobenzidine chromogen was applied to visualize the antigen antibody reaction. All these reagents belong to the universal Labeled Streptavidin-Biotin 2 System, Horseradish Peroxidase (code no. K0673 Dako Cytomation, Glostrup, Denmark). All the slides were immersed in Mayer's hematoxylin for counterstaining. Finally, the sections were covered by cover slips using aqueous mounting medium.

Immunohistochemical evaluation: The ordinary light microscope was used to detect and localize the immunostaining of anti-MSH2 antibody. Cells with nuclear staining were considered positive. Then, all the sections were examined by an image analyzer computer system using the software Leica Qwin 500 (Leica Microsystems Imaging Solutions Ltd, Cambridge, UK). Five random fields in each specimen were captured using a magnification (X400) then MSH2 positive cells were counted in the five fields. The average number of anti-MSH2 positive cells for each specimen was calculated[12].

Quantitative real-time PCR (qRT-PCR) analysis for MSH2 expression: Total RNA from the examined cases were extracted with Trizol reagent. The RNA integrity and concentration were determined by nanodrop measurement at 260 nm. The extracted RNA was reverse transcribed to cDNA with High cDNA Reverse Transcriptase Kit. cDNA was amplified for the expression of MSH2 and β-actin with SYBR Green Universal Master Mix (2X) (Applied Biosystems, Warrington, WA1 4SR, UK) according to the manufacturer's protocol. The sequence of primers used for Real-time PCR analysis were: MSH2-Fprimer 5'-GCCATGTGAGTCAGCAGAAG-3' and the MSH2-R primer 5'-CCCAAATCCATCGTAGGTAGAAG-3 and β actin-F primer 5'-GTGGACATCCGCAAAGAC-3' and the βActin-R primer (5'AAAGGGTGTAACGCAACTA-3'). The relative quantification of MSH2 gene was determined using the comparative CT method. The ΔCt was calculated as the difference between the average Ct values of the β-actin from the average Ct value of MSH2 gene. The ΔΔCt was determined by subtracting the ΔCt of the control from the ΔCt of the HL and NHL cases. The thermal cycling profile consisted of 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 30 sec, annealing at 60°C for 1min. Samples were run in triplicate. Relative expression of the target gene was calculated by the equation $2^{-\Delta\Delta Ct}$, which was the amount of MSH2 product, normalized to the endogenous control (β-actin) and relative to the control sample[13].

Statistical analysis: The anti-MSH2 positive cells in the studied groups were represented as median and range (Min-Max).The expression values of MSH2 mRNA in normal lymph nodes, lymphoid hyperplasia, HL and NHL were also presented as were represented as median and range (Min-Max). The Kruskal Wallis test was used to investigate the difference between the median values among the studied groups. Man Whitney test was used to investigates the difference between each two groups. Spearman's rank correlation test was used to test the correlation between MSH2 protein positive cells and MSH2 mRNA expression among the studied cases. P-value was considered significant when $p \leq 0.05$.

RESULTS

Immunohistochemical detection of MSH2: All normal lymph nodes specimens (100%) were immunopositive. The positive cells were concentrated within the germinal centers (Fig. 1). Similarly, all cases of lingual tonsils with lymphoid hyperplasia (100%) showed positive immunostaining. The positivity was detected in cells within germinal centers as well as within diffuse lymphocytes (Fig. 2). Regarding cases of lymphomas, 83% of the cases of HL were anti-MSH2 positive. The immunopositivity was mainly localized to malignant histiocytes, however, some malignant lymphocytes were positive (Fig. 3). In NHL, 62.5% cases were immunopositive. Few malignant lymphocytes were anti-MSH2 positive (Fig. 4). The count of anti-MSH2 positive cells was the highest in the normal lymph nodes (control group), followed by lymphoid hyperplasia, HL to NHL which revealed the least count of positive cells.

Expression of MSH2 mRNA by qRT-PCR: MSH2 mRNA was detected in all studied cases. Concentration of MSH2 mRNA showed its highest levels in normal lymph nodes (control group) followed by lymphoid hyperplasia, HL and the lowest levels were observed in examined NHL cases.

Statistical analysis: A statistically significant decrease in the count of MSH2 positive cells was detected from normal

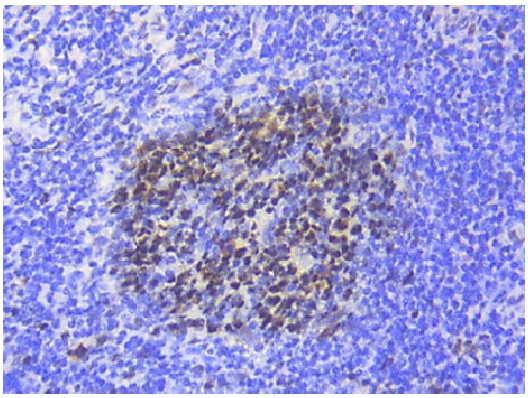

Fig. 1: A photomicrograph of normal lymph node showing positive MSH2 immunostaining of lymphocytes within germinal center (Anti-MSH2 antibody X400)

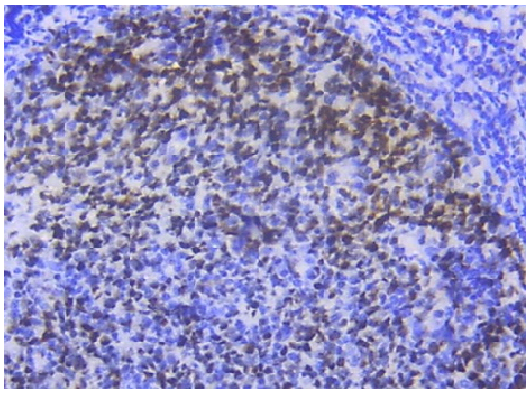

Fig. 2: A photomicrograph showing positive MSH2 immunostaining of lymphocytes in hyperplastic inflamed lingual tonsil (Anti-MSH2 antibody X400)

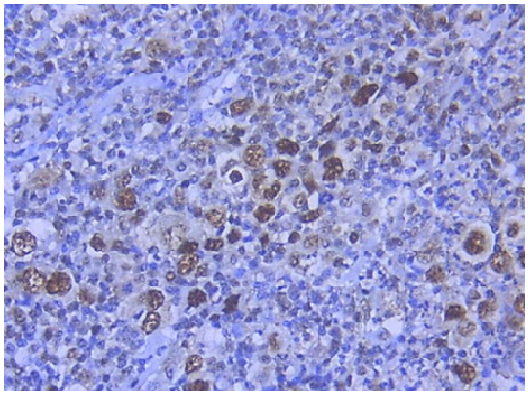

Fig. 3: A photomicrograph of HL showing MSH2 immunoexpression in malignant histiocytes. (Anti-MSH2 antibody X400)

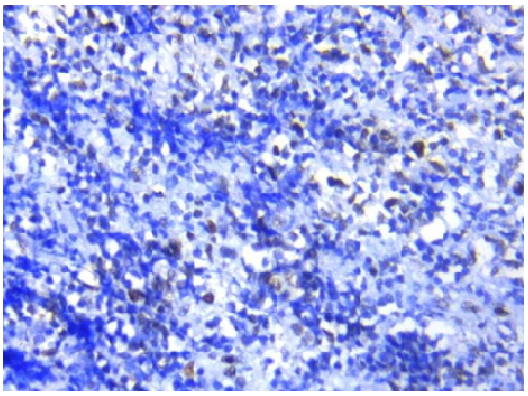

Fig. 4: A photomicrograph of NHL showing MSH2 immunoexpression in few malignant lymphocytes (Anti-MSH2 antibody X400)

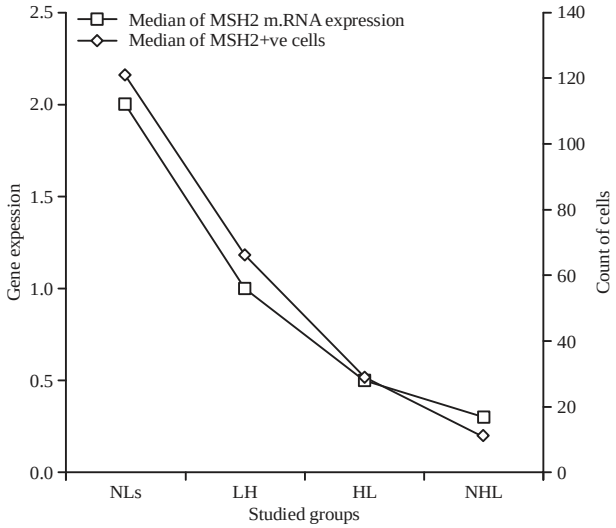

Fig. 5: Graph showing positive linear correlation between count of MSH2 protein positive cells and its gene expression among the studies groups of cases

finally NHL ($p \leq 0.0046$). Similar results were obtained by MSH2 m.RNA expression, where statistically significant decrease was detected among the studied cases ($p \leq 0.007$). Statistical significant difference was detected in both count of MSH2 positive cells and m.RNA expression between group of normal lymph nodes and Hodgkin's as well as non-Hodgkin's lymphoma ($p \leq 0.05$). Statistical significant difference in both count of MSH2 positive cells and m.RNA expression was found between group of lymphoid hyperplasia and Hodgkin's as well as non-Hodgkin's lymphoma ($p \leq 0.05$). Moreover, a strong positive linear correlation was detected between the expression of MSH2 protein and its mRNA among the studied cases (R = 1), (Table 1) (Fig. 5).

Table 1: Statistical difference in protein expression of MSH2 and its gene expression among the studied cases

Studied cases	Anti-MSH2 positive cells Median (Minimum-Maximum)	MSH2 m.RNA Median (Minimum-Maximum)
Control group	121	2.00
Lymphoid hyperplasia	66	1.00
HL	29	0.50
NHL	11	0.30
p-value	<0.0047*	<0.007

**p-value is significant when it is ≤0.05

DISCUSSION

In the present study, the statistical significant decrease of MSH2 levels in both HL and NHL in comparison to normal and hyperplastic lymphoid tissues could reflect the possible role of MSH2 in pathogenesis of head and neck lymphomas as well as expression of a malignant phenotype of lymphoid cells. This was in accordance with previous studies, which have shown that MSH2 knockout mice were prone to develop aggressive lymphomas[9]. Profiling of the DNA repair pathways in malignancy might allow the development of robust molecular biomarkers which might improve prediction of prognosis and suitable therapeutic selection of such lesions[8]. Being one of the critical genes of the MMR system that has been most abundantly expressed, MSH2 was the biomarker of choice in the present study. Furthermore, Pereira *et al.*[6] pointed out that inactivation of MSH2 might show profound pathologic consequences. However, formal evidence linking expression of MSH2 gene and protein, with pathogenesis and prognosis of head and neck lymphomas is still lacking.

For the best of existing knowledge, this was the first study that had examined MSH2 expression in normal and hyperplastic lymph node tissues as well as lymphomas of the head and neck. Expression analysis using various approaches such as immunohistochemistry (IHC) and RT-PCR had yielded insights into the possible role of MSH2 in pathogenesis of both head and neck HL and NHL. Previous studies of Hampel *et al.*[14] and Barnetson *et al.*[15] revealed that the effectiveness of IHC screening of the MMR proteins seemed to be similar to that of the more complex strategy of MSI genotyping. Thus, IHC was used in the present work, in addition to its advantages as a rapid, much easier to perform, cost-effective and accurate method[7].

The down-regulation of MSH2 at the gene and protein levels in NHL and HL which was demonstrated in this study could be attributed to pathogenic mutations in MMR proteins. Thus, reduced expression of MSH2 could be considered as a biomarker for loss of MMR activities in malignant cells[14,15]. This finding was consistent with several studies on different human cancers as colorectal cancer[14-16], cervical cancer[17], non-small-cell lung cancer[18], in addition to head and neck squamous cell carcinoma[6].

Gu *et al.*[19] pointed out that deficiency of MSH2 had caused genomic instability and development of diffuse large B-cell lymphoma in a murine model. In the same context, Pereira *et al.*[6], reported that low expression of MSH2 might contribute to a higher genomic instability resulting in a worse prognosis for patients. Contrarily, Robledo *et al.*[20] proposed that defects of the MMR system do not contribute to the development of lymphomas as MSI was an inconsistent feature in such malignancies.

Moreover, the lower expression of MSH2 in NHL in comparison to HL in this study suggested a more aggressive behavior and poorer prognosis of NHL which might be attributed to increased molecular disturbances and higher genomic instability. In the same context, El-Naggar *et al.*[1] reported that 85% of HL cases have been curable by radiation and chemotherapy, while the prognosis of NHL seemed to be highly variable.

Furthermore, a positive correlation between the gene and protein levels of MSH2 was observed in the present study which was obtained by IHC and RT-PCR. This finding might show that mutations in MSH2 genes could have resulted in total loss of the protein expression, rather than loss of function only[7].

The present study shed the light on the role of MSH2 in pathogenesis of human head and neck lymphomas, in addition to the more aggressive behavior and poorer prognosis of NHL than HL. Therefore, the reduced expression of MSH2 could be considered as a biomarker for loss of MMR activities in malignant cells and might aid in prediction of prognosis of such lesions. Moreover, it might be used as a potential diagnostic marker to assess risk of malignant transformation in lymphoid tissues. In addition, it is recommended to do more investigations on restoring this protein in lymphomas as the present work provided insights into developing a possible novel therapeutic aid for patients with HL and NHL of the head and neck.

CONCLUSION

Lower expression of MSH2 in NHL than that of HL suggested a more aggressive behavior and poor prognosis in this lesion. The reduced expression of MSH2 could be

considered as a molecular biomarker for loss of MMR activities and it might serve as a prognostic biomarker for head and neck lymphomas.

SIGNIFICANCE STATEMENT

This study investigated the expression of MSH2 on protein and gene levels in normal, hyperplastic and malignant lymphoid tissue, which could be beneficial for better understanding of the role of MSH2 in tumorigenesis of lymphomas and malignant transformation of lymphocytes. This study will help the researchers to identify the differences between behaviors and prognoses of Hodgkin's and non Hodgkin's lymphoma and will introduce a possible way for distinguishing between both of them. These points still not fully explored by many researchers.

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