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Frequency of gene *hMLH1* promoter methylation in the stomach antral and body area tissue of chronic atrophic pangastritis patients

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Aim. To compare *hMLH1* methylation frequency in stomach antral and body area tissue in chronic atrophic pangastritis patients, and to evaluate possible correlation severity of chronic atrophic gastritis markers.

Methods. The study population consisted of 24 participants (with histologically confirmed chronic atrophic pangastritis), who underwent upper endoscopy. Biopsy specimens were taken from the gastric antral and body area. The methylation status of the gene *hMlH1* was investigated.

Results. Methylation of the *CpG* island of gene *hMLH1* was found in the antral stomach area group 9/24, compared with the body area 2/24. There was a significant difference in gene methylation frequencies in the observed stomach parts (Fisher's exact test, $p = 0.04$). There was a significant association between gene *hMLH1* methylation and the occurrence of severe atrophic gastritis, intestinal metaplasia and the presence of hyperplastic mucosal changes (Fisher's exact test, $p < 0.05$).

Conclusions. *hMLH1* gene methylation frequency is higher in the stomach antral tissue compared with the stomach body tissue, and increases with higher level of atrophic gastritis and intestinal metaplasia.

Key words: atrophic gastritis, intestinal metaplasia, *hMLH1*, methylation

INTRODUCTION

Atrophic gastritis is a histopathologic entity characterized by chronic inflammation of the gastric mucosa with loss of gastric glandular cells and replacement by intestinal-type epithelium, pyloric-type glands, and fibrous tissue. *H. pylori* infection is the most important factor for stomach mucosal atrophy (1). Chronic atrophic gastritis (CAG) is an essential precursor lesion in the development of the intestinal type of gastric cancer (2). It is known that patients with chronic atrophic gastritis may have up to a 16-fold increased risk of developing gastric cancer, compared to general population (3, 4).

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Gene regulation by *CpG* methylation is involved in a large spectrum of biological processes, from development to aging, including inflammatory and infectious diseases, and cancer (5). *hMLH1* protein coding gene is a member of the family of proteins required for DNA mismatch repair (6). Many studies confirmed the *hMLH1* gene function loss by promoter methylation in gastric cancer (7–12). There is data that *hMLH1* methylation is associated with stomach intestinal carcinoma type (7) and this epigenetic event occurs in early gastric cancer (8). Studies also revealed *hMLH1* gene hypermethylation in atrophic gastritis and intestinal metaplasia in damaged stomach tissue (13, 14). But there is a lack of data about gene *hMLH1 CpG* island methylation and its frequency in different stomach areas affected by atrophic gastritis. This is an important issue, considering the data showing the association of *hMLH1* gene methylation with a distal part of stomach cancer (7, 15).

METHODS

Study population and tissue samples

The study population consisted of 24 participants (with histologically confirmed chronic atrophic gastritis of stomach antral and body area) who underwent upper endoscopy at the Hospital of Lithuanian University of Health Sciences Kaunas Clinics during the period of 2011–2012. Biopsy specimens were taken from the gastric antral and body area. The specimens were cut into two pieces. Then one part was fixed in 10% buffered formalin and embedded in paraffin for histological evaluation. The other part was frozen in liquid nitrogen and stored at –80 °C. Tissue sections were cut and stained with hematoxylin and eosin. The biopsy cases were analyzed in an attempt to assess major histopathological features of gastritis according to updated Sydney systems (Dixon MF. The Updated Sydney System. International Workshop), and to identify the *H. pylori* status. Representative cases are presented in Figs. 1, 2, 3.

A written informed consent was obtained from all study participants. The study was approved by the Kaunas Regional Research Bioethical Committee (Protocol No.: BE-2-16).

Methylation-specific PCR

DNA was extracted from 25–30 mg of the frozen tissue using the ZS Genomic DNA™ Tissue Mini Prep Kit (Zymo Research, USA) according to the manufacturer's instructions. The methylation status of the *hMLH1* gene promoter was determined by bisulfite treatment of DNA. The bisulfite treatment was performed using the EZ DNA Methylation Gold Kit™ (Zymo Research, USA) according to the manufacturer's instructions / protocol. Human genomic DNA from peripheral blood lymphocytes treated with bisulfite served as a negative control. Human genomic DNA treated *in vitro* with Sss I methyltransferase (New England Biolabs, UK) was

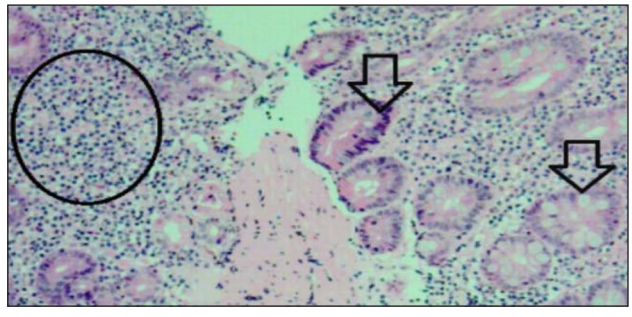

Fig. 2. Gastric atrophy with extensive intestinal metaplasia (arrows) and mononuclear cell infiltration (circumscribed)

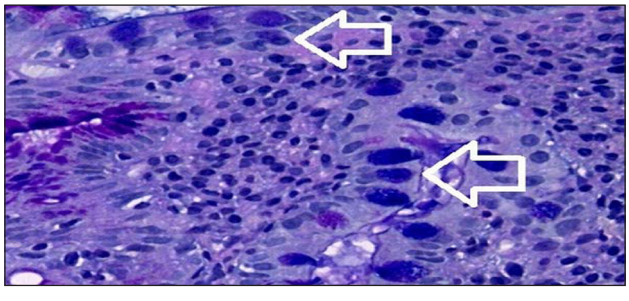

Fig. 1. Complete intestinal metaplasia (arrows) highlighted with combination of alcian blue and PAS stains

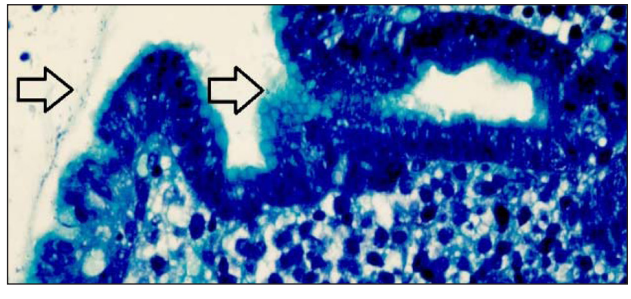

Fig. 3. *Helicobacter pylori* organisms (arrows) in atrophic gastritis. Giemsa stain

used as a positive control. The methylation status of the promoters was detected by methylation-specific PCR (MSP). The methylation-specific PCR was done with the primers: methylated forward (MF) 5'-CGG ATA GCG ATT TTT AAC GC-3', methylated reverse (MR) sequence 5'-CCT AAA ACG ACT ACT ACC CG-3' which amplify 64 bp (16), and unmethylated DNA sequence primers UF 5'-AAT GAA TTA ATA GGA AGA GTG GAT AGT-3', UR 5'-TCT CTT CAT CCC TCC CTA AAA CA-3'– product size 97 bp (16). PCR reactions were done in a total volume of 20 µL, using 10 µL Maxima® Hot Start PCR Master Mix with Hot Start Taq DNA polymerase (Thermo Fisher Scientific, USA) and 10 µM of each primer (Metabion International AG, Germany). MSP was performed for 38 cycles with start of 94 °C for 30 s, annealing for 45 s at 56 °C, and extension at 72 °C for 1 min. PCR products were separated by 3% gel electrophoresis. In both cases, appearance of methylated and unmethylated signals in a gel and methylation of the gene were considered. Representative cases are presented in Fig. 4.

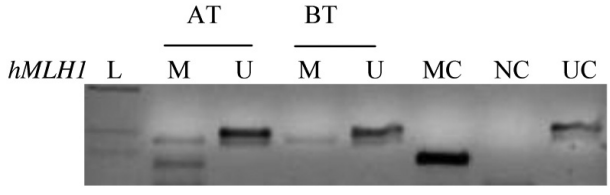

Fig. 4. Representative methylation specific PCR for hMLH1 gene. L – DNA ladder marker, M – methylated allele, U – unmethylated allele, MC – methylated control, NC – negative control, UC – unmethylated control, AT – tissue sample from antral gastric area, BT – tissue sample from gastric body area

Statistical analysis

Statistical analysis was carried out with the IBM SPSS Statistics 19 Software (IBM SPSS Inc., Chicago, IL). Quantitative data was presented as mean and standard deviation (SD). To show the reliability of the estimate, the confidence interval (CI) of 95% was selected. For testing the statistical hypothesis about the independence of two variables, the Fisher exact test was used. The significance level of 0.05 was selected.

RESULTS

In total, the study involved 24 participants: 14 women and 10 men. The median age was 66.7 ± 11.7 (mean SD), range 35–78. For majority of patients (62.5%) tissue samples were positive for *H. pylori* infection. We did not find any correlation between gene *hMLH1* methylation and patients' sex and age. 75% of patients in the antral and body stomach area had various levels of intestinal metaplasia. Gene *hMLH1* CpG island promoter methylation frequencies and distribution in respect of stomach area groups are presented in Fig. 5.

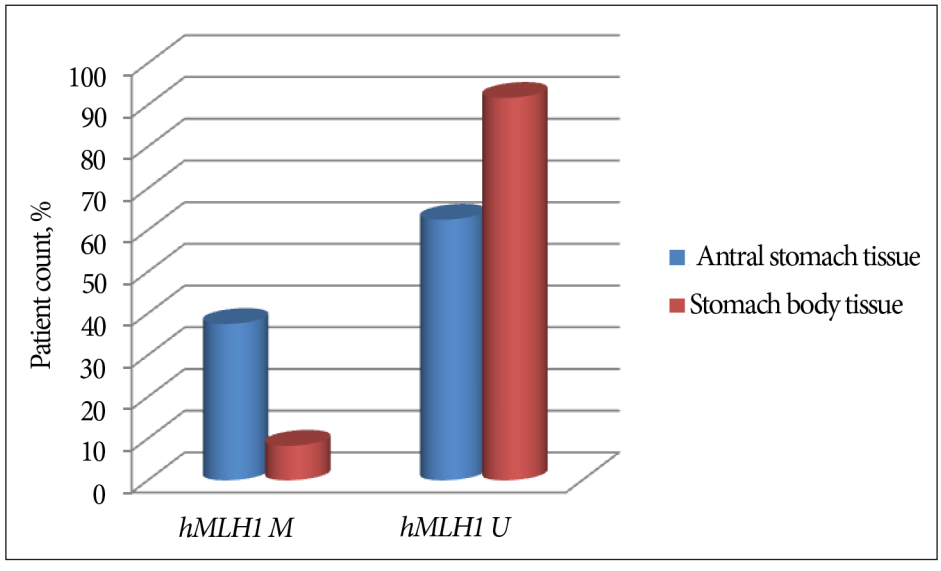

Fig. 5. Gene hMLH1 promoter methylation frequencies and distribution in stomach area groups. *M* – methylated allele, *U* – unmethylated allele

There is a significant difference between gene *hMLH1* methylation frequencies in the antral and stomach body tissue (Fisher's exact test, p = 0.04). Further evaluation of gene *hMLH1* methylation frequencies in the antral area determined significant correlations between histopathological features. *hMLH1* gene methylation frequency increases with the level of atrophic gastritis and intestinal metaplasia. There was a different frequency of the methylated gene *hMLH1* in patients with severe atrophy (Fisher exact test, p = 0.003) compared with mild and moderate atrophy in the tissue. The same significant tendency was observed in the level of intestinal metaplasia in atrophic gastritis tissue. Severe intestinal metaplasia is related to gene *hMLH1* methylation (Fisher exact test, p = 0.003). We also found the association between gene *hMLH1* promoter methylation and the presence of hyperplastic mucosal changes in the gastric tissue (Fisher exact test, p = 0.042).

DISCUSSION

According to the previous studies, gene *hMLH1* methylation in the stomach tissue affected by intestinal metaplasia ranges from 6 to 7% (14, 17). Our data are comparable with results from other studies in terms of corpus atrophic gastritis complicated intestinal metaplasia (8%). We found a higher frequency of the examined gene promoter methylation in the antral area gastritis group reaching 37%. This difference reached a statistically significant level. The reason for such discrepancy could be the fact that the previous studies did not examine methylation frequency separately for the gastric antral and body area, and those studies also do not mention from what part of the stomach the tissue was obtained. In this case we found a new significant tendency, which needs to be tested in further studies involving a higher number of patients. We also detected that the gene *hMLH1* methylation frequency is related to intestinal metaplasia, higher level of atrophic gastritis and presence of hyperplastic changes in the mucosal area. We did not find any published data in literature which would address these associations.

This research is important because we managed to form a homogeneous study group (patients with pangastritis, and most of the patients had intestinal metaplasia in both antral and body stomach area), and therefore we could evaluate the epigenetic changes in different parts of stomach. Our data logically correspond with the studies confirming that *hMLH1* methylation occurs more often in the distal part of the gastric cancer tissue (1, 18). Our findings could be the basis for further research in order to evaluate a possible *hMLH1* gene methylation role for determination of gastric cancer development risk in patients with atrophic gastritis.

CONCLUSIONS

hMLH1 gene methylation frequency is higher in the stomach antral tissue compared with the stomach body tissue, and increases with higher level of atrophic gastritis and intestinal metaplasia.

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LĖTINIU ATROFINIU PANGASTRITU SERGANČIŲ PACIENTŲ *hMLH1* GENO PROMOTORIAUS METILINIMO DAŽNIO SKIRTUMAI SKRANDŽIO ANTRALINĖS DALIES IR KŪNO AUDINIUOSE

Santrauka

Tikslas. Palyginti *hMLH1* geno metilinimo dažnį sergančiųjų lėtiniu atrofiniu pangastritu skrandžio antralinės ir kūno dalių audinyje, įvertinti galimas sąsajas su morfologiniais atrofinio gastrito sunkumo kriterijais.

Metodai. Studijoje dalyvavo 24 pacientai su morfologiškai patvirtinta atrofinio pangastrito diagnoze. Jiems atlikto skrandžio endoskopinio tyrimo metu buvo paimtos biopsijos iš skrandžio urvo ir kūno. *hMLH1* promotoriaus metilinimui nustatyti atliktas metilinimui jautrios grandininės polimerazės reakcijos tyrimas.

Rezultatai. *hMLH1* geno promotoriaus metilinimas skrandžio antralinėje dalyje nustatytas 9 iš 24, skrandžio kūno audinyje – 2 iš 24 pacientams, skirtumas tarp grupių yra statistiškai reikšmingas (Fisherio testas p = 0,04). Tyrimas atskleidė reikšmingas sąsajas tarp tirtojo geno metilinimo ir sunkaus atrofinio gastrito laipsnio, pažengusios žarninės metaplazijos bei nustatomų hiperplastinių gleivinės pokyčių.

Išvados. *hMLH1* geno metilinimas yra dažnesnis reiškinys skrandžio antralinėje dalyje, palyginti su kūnu. Geno metilinimas susijęs su sunkiu atrofinio gastrito laipsniu, pažengusia žarnine metaplazija bei hiperplastiniais gleivinės pokyčiais.

Raktažodžiai: atrofinis gastritas, žarninė metaplazija, *hMLH1*, metilinimas