N-myc downstream-regulated gene 2 (NDRG2) promoter methylation and expression in pituitary adenoma

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Abstract

Background: Pituitary adenoma (PA) is a benign primary tumor that arises from the pituitary gland and is associated with ophthalmological, neurological and endocrinological abnormalities. However, causes that increase tumor progressing recurrence and invasiveness are still undetermined. Several studies have shown N-myc downstream regulated gene 2 (NDRG2) as a tumor suppressor gene, but the role of NDRG2 gene in pituitary adenoma pathogenesis has not been elucidated. The aim of our research has been to examine NDRG2 mRNA expression in PA and to determine the associations between the NDRG2 gene epigenetic changes and the development of recurrence or invasiveness of PA and patient clinical data.

Methods: The MS-PCR was used for NDRG2 promoter methylation analysis and gene mRNA expression levels were evaluated by qRT-PCR in 68 non-functioning and 73 functioning adenomas. Invasiveness was evaluated using magnetic resonance imaging with Hardy’s modified criteria. Statistical analysis was performed to find correlations between NDRG2 gene mRNA expression, promoter methylation and patient clinical characteristics and PA activity.

Results: The NDRG2 mRNA expression was significantly lower in the case of acromegaly (GH and IGF-1 hypersecretion) than in other diagnoses of PAs (p < 0.05). Also, the NDRG2 expression was significantly higher in prolactinoma (PRL hypersecretion) than in in other diagnoses of PAs (p < 0.05). The promoter of NDRG2 was methylated in 22.69% (12/58 functioning and 15/61 non-functioning) of patients with PA. However, the NDRG2 gene mRNA expression was not significantly related to its methylation status. Clinical factors, such as: age, gender, relapse and diagnoses of Cushing syndrome were of no significance for NDRG2 promoter methylation and mRNA expression levels, as well as secreting or non-secreting PAs and the invasiveness of PAs.

Conclusion: The different NDRG2 promoter methylation and expression levels in PA samples showed tumor heterogeneity and indicates a potential role of this gene in pituitary adenoma pathogenesis, but the corresponding details require intensive research.

Keywords: Pituitary adenoma, N-myc downstream-regulated gene 2, NDRG2, DNA methylation, mRNA expression, Invasiveness

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Background
Pituitary adenoma (PA) is a common intracranial neoplasm with reported estimated prevalence rates to be 14.4% to 22.5% in pooled autopsy and radiological series, respectively [1]. PAs are generally benign but can behave clinically in different ways. Some of pituitary tumors are hormonally inactive, others secrete hormones in excess, and some of PAs can cause morbidity because of dysregulation of hormone production and/or symptoms of mass effect [2, 3]. The pituitary gland is localized in a dual bag attached to the inferior aspect of the diaphragm of the sella and surrounded by venous spaces that correspond laterally to the cavernous sinuses [4]. Cavernous sinus invasion has an influence on the management and prognosis of PA [5], because dual wall invasion usually implies partial surgical removal of the tumor [6]. Early prediction of which pituitary tumor will recur and/or exhibit an invasive phenotype remains difficult despite the introduction of several tissue-based molecular markers [7].

Associations between tumors (including glioblastoma, gastric cancer, colorectal cancer, breast and liver cancers, meningioma, bladder and thyroid cancers) and N-myc downstream-regulated gene 2 (NDRG2) have been reported in numerous studies [8–16]. NDRG2 is a member of the NDRG family, which consists of NDRG1, NDRG2, NDRG3 and NDRG4 [17] and is located at chromosome 14q11.2, a region that has been reported to harbor a tumor suppressor gene [18]. NDRG2 is highly expressed in the brain and skeletal muscle, while it is marginally expressed or almost undetectable in the several human cancer cell lines [17, 19]. NDRG proteins are reported to be involved in cell proliferation, differentiation, migration, invasion and stress response [19]. It was shown that NDRG2 reduce tumor cell proliferation in glioblastomas [20]. Also, NDRG2 upregulation was associated with Alzheimer’s disease or cerebral ischemia [21, 22]. Several studies have shown NDRG2 promoter CpG island methylation and down-regulation in liver [13], gastric [10], colorectal cancers (CRC) [23, 24], glioblastomas [8, 9] and anaplastic meningioma [25]. However, NDRG2 promoter methylation and mRNA expression levels in PAs has not been investigated.

The aim of this study was to determine aberrant promoter methylation and mRNA expression of NDRG2 in PAs and to evaluate the associations between the methylation profile of gene, mRNA expression, patients’ clinical characteristics and tumor invasiveness and recurrence.

Methods
Description of subject
One hundred forty one pituitary adenoma tissues and clinical patient data were collected at the Department of Neurosurgery of Hospital of Lithuanian University of Health Sciences between 2010 and 2016. Tumor tissues were frozen in liquid nitrogen immediately after their surgical resection. The age at the time of the operation, gender, relapse, size and diagnoses of Cushing syndrome, acromegaly or prolactinoma were collected for each patient. The endocrinological features were: 73 functioning and 68 nonfunctioning adenomas. According to the clinical findings functioning adenomas were: 7 growth hormone (GH) - secreting adenomas, 2 insulin-like growth factor 1 (IGF-1) - secreting adenomas, 1 cortisol (COR) - secreting adenoma, 44 prolactin (PRL) - secreting adenomas, 1 adenocorticotropic hormone (ACTH) - secreting adenoma and 18 adenomas secreting more than one hormone. According to tumor size all PAs were macroadenomas (greater than 10 mm).

Invasion of pituitary adenomas were analyzed using MR imaging findings and classified according to Hardy classification, modified by Wilson [5]. The Knosp classification system was used to quantify the invasion of the cavernous sinus [6]. Invasiveness was established in 71 patients with pituitary adenoma. From them, 51 invasive and 20 non-invasive PAs were found.

Nucleic acid extraction
Tissue specimens were pulverized and stored at -80 °C until DNA and RNA was obtained. Genomic DNA was extracted from 119 PA specimens by SDS/proteinase K treatment, followed by phenol–chloroform extraction and ethanol precipitation. The remaining 22 samples were missing because of containing too small an amount of tumor tissue. All the samples were stored at -20 °C until DNA was modified with sodium bisulfite.

Total RNA was extracted from 141 PAs using Trizol reagent, according to the manufacturer’s protocol (Ambion, Life Technologies) and stored at -80 °C until cDNA synthesis. However, 10 mRNA samples were lost because the concentrations for cDNA synthesis were too small. The genomic DNA and RNA concentrations and purity was determined using Nanodrop spectrophotometer (Eppendorf). For pure DNA, A260/280 is ~1.8 and for pure RNA A260/280 is ~2.

Bisulfite Modification and MS-PCR
Extracted genomic DNA of 119 PA samples was modified with EZ DNA methylation kit™ (Zymo Research), according to the manufacturer’s instructions. The sodium bisulfite treated DNA was eluted in 40 μL of nuclease-free water.

After bisulfite modification, the methylation-specific polymerase chain reactions (MS-PCR) were performed in 15 μL of 7.5 μL Maxima™ Hot Start PCR Master Mix (ThermoFisher Scientific) with Hot Start Taq DNA polymerase, 10 pmol of each primer (Metabion International AG) and nuclease-free water. Primers for methylated
were performed in triplicate. A main PCR product and primer-dimers. All measurements were performed after denaturation step at 95 °C for 5 min. The amplifications were carried out in a thermal cycler (Eppendorf) for 38 cycles, each of which consisted of denaturation at 92 °C for 15 s, annealing at 60 °C for 30 s, and extension at 72 °C for 15 s, followed by a final 5 min extension at 72 °C. For each set of methylation-specific PCR reactions methylated (Bisulfite-Converted Universal Methylated Human DNA Standard (Zymo Research, USA)), unmethylated (human blood lymphocyte DNA, treated with bisulfite) and negative (nuclease-free water) controls were included in all reactions. The MS-PCR products were analyzed by electrophoresis on a 2% agarose gel stained with ethidium bromide and visualized under UV illumination. cDNA synthesis and qRT-PCR First-strand cDNA was produced from total RNA by using RevertAid H Minus M-MuLV Reverse Transcriptase (ThermoFisher Scientific) and random hexamer primers (ThermoFisher Scientific), according to the manufacturer’s protocol. Negative controls were prepared as above, but without Reverse Transcriptase. For the NDRG2 gene mRNA expression, Quantitative real-time PCR (qRT-PCR) was performed using the SYBR Green chemistry in a Real-Time PCR System “Applied Biosystems 7500 Fast” (Applied Biosystems, USA). The 12 µl reaction mixture contained of 6 µl Maxima SYBR Green/ROX qPCR Master Mix (2×) (ThermoFisher Scientific), 15 ng of the cDNA, nuclease-free water and gene-specific primers: NDRG2 forward 5’-AGAGGTATTAGGATTTTTGG TAGC-3’ (forward) and 5’-GCTAAGGCGAAAAAT CTCGC-3’ (reverse) for unmethylated allele: 5’- AGAGGTATTAGGATTTTTGGTGATGA-3’ (forward) and 5’-CCACTAAGGACAAAAACTCTACC-3’ (reverse), according to the published data [26]. The PCR amplification was carried out between PA normalized threshold cycle (CT) values and healthy human brain (RHB) normalized Ct values: $\Delta\Delta\text{Ct} = (\text{Ct}_{\text{NDRG2}} - \text{Ct}_{\beta\text{-actin}})_{\text{PA sample}} x - (\text{Ct}_{\text{RHB}} - \text{Ct}_{\beta\text{-actin}})_{\text{RHB}}$ [27]. The final result was given as $\log_2(2^{-\Delta\Delta\text{Ct}})$ calculation.

Statistical analysis
The SPSS Statistics 19 (SPSS Inc., Chicago, IL) software package was used for statistical analysis. Chi-square test was used to evaluate associations among NDRG2 gene promoter methylation, mRNA expression levels and clinical characteristics (age, gender, relapse, Cushing syndrome, acromegaly, prolactinoma, invasiveness, secreting and non-secreting pituitary adenomas and hormone groups). The correlation between NDRG2 gene expression and methylation and the other clinical factors were evaluated by use of the Mann-Whitney test. Kruskal–Wallis test was used to reveal the difference across medians of NDRG2 mRNA expression in all hormone groups. The significance level was defined as $p$ value less than 0.05.

Results
NDRG2 gene methylation frequency in PAs and associations with patient clinical data
Methylation specific PCR analysis was performed to determine the methylation status of NDRG2 gene in 119 PA samples (Fig. 1c). The gene was methylated in 22.69% (27/119) of cases. Representative chart is shown in Fig. 1a. These results indicate that NDRG2 gene has low methylation status in PAs.

To characterize the correlation between methylation of NDRG2 promoter and clinical features of PAs, several clinicopathological characteristics including age, gender, relapse, invasiveness, diagnoses of acromegaly, prolactinoma and Cushing syndrome were compared between methylated and unmethylated NDRG2 promoter groups. However, Chi-square test showed that methylation of NDRG2 promoter was not associated with any of this clinical data ($p > 0.05$) (Table 1).

We then analyzed the relationship between NDRG2 promoter methylation and PA hormonal activity (Fig. 1b). The histogram of hormones distribution in methylated and unmethylated gene groups revealed that hypersecretion of PRL, IGF-1, GH, ACTH and more than one hormone mostly occur in PAs with unmethylated promoter of NDRG2 gene. Also, in most cases nonfunctioning PAs have unmethylated NDRG2 gene. However, the data showed no statistically significant differences between these groups ($p > 0.05$) (Table 1).
NDRG2 gene expression in PAs and associations with NDRG2 promoter methylation and patient clinical data
The expression of NDRG2 mRNA in 131 PAs was determined by qRT-PCR method using the SYBR Green chemistry. The values were normalized with internal β-actin control. To investigate the correlation between NDRG2 promoter methylation and mRNA expression, statistical analysis was performed in 109 PA samples that have the values of methylation and expression. However, Mann-Whitney test showed no significant differences of NDRG2 mRNA expression between the group of NDRG2 methylation samples (23 tumors) and the group of unmethylated NDRG2 gene (86 tumors, \( p = 0.323 \)).

We then analyzed the correlation between NDRG2 gene mRNA expression and patient clinical characteristics. Using the Mann–Whitney test, we found that the expression of NDRG2 had no correlation with age, gender, presence or absence of repeated surgery, secreting or non-secreting PAs and Cushing syndrome (\( p = 0.076, p = 0.545, p = 0.783, p = 0.927 \) and \( p = 0.980 \), respectively). Nevertheless, NDRG2 expression was increased with diagnoses of prolactinoma and decreased with diagnoses of acromegaly, compared to patients with other symptoms (\( p < 0.05 \)) (Fig. 2a, b).

To further investigate whether NDRG2 gene mRNA expression was associated with hormones, we compared medians of NDRG2 mRNA expression in PRL, IGF-1, GH, ACTH, COR and multi hormone groups and nonfunctioning PAs (Fig. 3a). Kruskal–Wallis test revealed that the medians of NDRG2 mRNA expression in all hormone groups statistically differ (\( p < 0.05 \)), and the box plots showed the tendency that GH-secreting, ACTH-secreting and more than one hormone secreting PAs are at lower gene expression level than other hormone groups. To specify these findings, we then analyzed whether the hormone groups and nonfunctioning PAs reflect on expression levels of NDRG2 gene. For this matter, the NDRG2 mRNA expression values were divided into three gene mRNA expression groups: “low” - values 1.0-fold lower than the NDRG2 gene mRNA expression median, “medium” - values ranging in between “low” and “high”, and “high” - values 1.0-fold higher than the gene median) As shown in Fig. 3b, distribution of hormones in NDRG2 expression levels were different. In most cases PRL hypersecretion was detected with medium NDRG2 gene expression level (\( p < 0.05 \)). IGF-1, COR, GH, and ACTH hormones showed no statistically significant differences (\( p = 0.730, p = 0.172, p = 0.075, p = 0.379, p = 0.379 \), respectively) as well as multiple (more than one hormone) and non-secreting PAs (\( p = 0.096, p = 0.584, p = 0.096 \), respectively). Nevertheless, the histogram of hormone groups distribution showed a tendency, that the hypersecretion of IGF-1 hormone mostly occur with medium and low NDRG2 gene expression levels, COR - with high NDRG2 mRNA expression level, GH, ACTH and multiple - at low gene expression level (Fig. 3b).

Finally, we analyzed the relationship between NDRG2 gene promoter methylation, mRNA expression, and invasiveness of 71 pituitary adenomas (51 invasive and 20 noninvasive). However, the analysis (Chi-square and Mann-Whitney test) showed no significant differences between these groups (\( p = 0.452, p = 0.472, \) respectively). Additionally, secreting and non-secreting PAs also had no correlations with invasiveness (\( p = 0.571 \)). We also wanted
to reveal the tendency of invasiveness in PRL, IGF-1, GH, COR, ACTH and more than one hormone secreting PAs. In addition to this, the dot plot analysis was performed (Fig. 4). The comparison showed that the means of NDRG2 gene mRNA expression of hormones in invasive and non-invasive tumor groups were various. The lowest NDRG2 expression was detected in PAs secreting more than one hormone, and the highest in PRL-secreting and non-secreting pituitary adenomas (Fig. 4).

**Discussion**

Pituitary adenoma is a common benign monoclonal neoplasm [28]. Early prediction of which pituitary tumors will recur and/or exhibit an invasive phenotype remains difficult [7]. NDRG2 gene may be a promising target for cancer, because NDRG2 down-regulation is associated with cancer development and progression, including such features as malignant clinical manifestations and increased pathological grade. Moreover, this gene is a relevant biomarker for predicting aggressive behavior, tumor recurrence and overall patient survival [29]. Therefore, should be further studies to show that NDRG2 up-regulation may be a promising therapeutic strategy for the treatment of cancer and that might be associated with PA development, as well.

We began our study by defining NDRG2 promoter methylation status in PA samples, including all the clinically functioning and hormonally inactive types. We have determined 22.69% (27/119) NDRG2 methylation frequency. These results indicate that NDRG2 gene has low methylation status in PAs. A number of studies using various techniques have shown that epigenetic silencing of the NDRG2 promoter has been found in the majority of primary tumors, and different cancer cell lines and other tumor tissues such as glioma (46.3 - 62%), primary gastric (54%) and colorectal carcinoma (64.28%) cancers [8–11]. NDRG2 promoter methylation was observed to be associated with the invasiveness in gastric and colorectal cancer and with the aggressiveness of glioma tumor [8–10]. However, our analysis has shown no significant association between NDRG2 gene methylation and pituitary adenoma invasiveness. Meanwhile, we have demonstrated that hypersecretion of PRL, IGF-1, GH, ACTH hormones appear with a methylated NDRG2 gene more often than with an unmethylated gene. Also, in most cases nonfunctioning PAs have an unmethylated NDRG2 gene. However, the mechanisms related to this are still unknown.

Moreover, previous studies have shown that NDRG2 mRNA expression is low in numerous types of tumor tissues and cancer cell lines, and is a novel tumor suppressor candidate gene [8, 12–16]. It was observed that NDRG2 expression loss is significantly correlated with aggressive tumor behaviors such as late tumor-node-metastasis stage, differentiation grade, portal vein thrombi, infiltrative growth pattern, nodal/distant metastasis, as well as shorter patient survival rates in liver cancer [13]. Also, NDRG2 overexpression can inhibit tumor growth and invasion in vitro in bladder and breast cancer [12, 15]. Meanwhile, our results have showed no correlation between NDRG2 gene mRNA expression and clinical characteristics, PA invasiveness and NDRG2 mRNA expression.

### Table 1

| NDRG2 gene methylation | Number of patients M (%) | U (%) | p-value |
|------------------------|--------------------------|-------|---------|
| Cases                  | 119                      |       |         |
| Age (years)            |                          |       |         |
| ≤60                    | 48                       | 13 (27.08) | 35 (72.92) | 0.368 |
| >60                    | 70                       | 14 (20.00) | 56 (80.00) |       |
| Gender                 |                          |       |         |
| Female                 | 68                       | 15 (22.06) | 53 (77.94) | 0.850 |
| Male                   | 51                       | 12 (23.53) | 39 (76.47) |       |
| PA function            |                          |       |         |
| Secreting              | 61                       | 15 (24.59) | 46 (75.41) | 0.612 |
| Non-secreting          | 58                       | 12 (20.69) | 46 (79.31) |       |
| Relapse                |                          |       |         |
| Appear                 | 10                       | 2 (20.00) | 8 (80.00) | 0.832 |
| None                   | 109                      | 25 (22.94) | 84 (77.06) |       |
| Prolactinoma           |                          |       |         |
| Appear                 | 37                       | 9 (24.32) | 28 (75.68) | 0.952 |
| None                   | 24                       | 6 (25.00) | 18 (75.00) |       |
| Acromegaly             |                          |       |         |
| Appear                 | 12                       | 4 (33.33) | 8 (66.67) | 0.433 |
| None                   | 49                       | 11 (22.45) | 38 (77.55) |       |
| Cushing syndrome       |                          |       |         |
| Appear                 | 1                        | 0 (0.00) | 1 (100.00) | 0.565 |
| None                   | 60                       | 15 (25.00) | 45 (75.00) |       |
| Hormones               |                          |       |         |
| PRL                    | 37                       | 9 (24.32) | 28 (75.68) | 0.801 |
| IGF-1                  | 2                        | 0 (0.00) | 2 (100.00) | 0.401 |
| GH                     | 6                        | 2 (33.33) | 4 (66.67) | 0.639 |
| ACTH                   | 1                        | 0 (0.00) | 1 (100.00) | 0.556 |
| Multiple               | 13                       | 4 (30.77) | 9 (69.23) | 0.616 |
| Invasiveness           |                          |       |         |
| Invasive               | 41                       | 10 (24.39) | 31 (75.61) | 0.452 |
| Non-invasive           | 19                       | 3 (15.79) | 16 (84.21) |       |
| NDRG2 mRNA expression  |                          |       |         |
| Low                    | 22                       | 3 (13.64) | 19 (86.36) |       |
| Medium                 | 50                       | 13 (26.00) | 37 (74.00) | 0.458 |
| High                   | 37                       | 7 (18.92) | 30 (81.08) |       |

| M methylated, U unmethylated, PRL prolactin, IGF-1 insulin-like grow factor 1, GH growth hormone, ACTH adrenocorticotropic hormone, multiple PAs secreting more than one hormone |
expression and pituitary adenoma invasiveness. The mechanism of NDRG2 expression in pituitary adenoma proliferation and invasion has not yet been reported, making it necessary to further elucidate the role of NDRG2 gene in pathogenesis of PA.

In addition, we have analyzed the associations of NDRG2 gene mRNA expression with clinical features of PAs. Our study has revealed that in the case of acromegaly, NDRG2 gene mRNA expression is significantly lower than in other diagnoses of PAs. It is known that acromegaly is an insiduous disorder characterized by excess secretion of growth hormone and elevated circulating levels of insulin-like growth factor-I [30], and in our examination, in most cases the hypersecretion of GH and IGF-1 hormones were determined with low NDRG2 gene expression level as well. Moreover, NDRG2 gene mRNA expression is significantly higher with diagnoses of prolactinoma than in other diagnoses of PAs, and in most cases the hypersecretion of PRL hormone that causes prolactinoma have been detected with medium NDRG2 expression level. These results are

Fig. 2 NDRG2 gene expression associations with diagnoses of prolactinoma and acromegaly. a Box plots of relative NDRG2 expression associated with diagnoses of prolactinoma. b Box plots of relative NDRG2 expression associated with diagnoses of acromegaly. None – no diagnoses of acromegaly. The line inside each box represents the median, the lower and upper edges of the boxes represent the 25th and 75th percentiles, respectively, and upper and lower lines outside the boxes represent minimum and maximum values (error bars). The Mann-Whitney test showed, that the NDRG2 expression level was increased with diagnoses of prolactinoma and decreased with diagnoses of acromegaly (p < 0.05)
consistent with previous reports that the expression of NDRG2 is regulated by many hormones, including adrenal steroids [31], dexamethasone [32], insulin [33], androgen [34] and aldosterone [35]. It was also shown that hormone estrogen can enhance the expression of NDRG2 and may influence Na+/K + -ATPase activation as well as ion transport in salivary glands, brain, heart, skeletal muscle, and kidney where Na+/K + -ATPases were enriched [36]. Also, in human and in animal models estrogen stimulates PRL secretion in vitro and induces PRL adenomas in vivo [37]. However, more studies for signal pathway are needed to show the mechanism underlying and the significant results we showed in this study.

**Conclusion**

This is the first study that has demonstrated the NDRG2 gene promoter methylation and mRNA expression in patients with diagnoses of pituitary adenoma and analyzed the relationships between NDRG2 epigenetic changes and the association with PA clinical features including patient age, gender, relapse, hormone groups, invasiveness, diagnoses of prolactinoma, acromegaly and Cushing syndrome. Our data have revealed that in the case of acromegaly, NDRG2 gene mRNA expression is significantly lower than in other diagnosis of PAs and PA that secretes hormones GH and IGF-1 hormones have low NDRG2 gene mRNA expression is significantly lower than NDRG2 gene expression in invasive and non-invasive hormones of PAs. The horizontal bars represent the mean values with 95% confidence interval, the spots represent the amount of samples. PRL – prolactin, IGF-1 – insulin-like grow factor 1, multiple – PAs secreting more than one hormone, NS – non-secreting PAs; I – invasive, N – non-invasive PA.

**Abbreviations**

ACTH: Adrenocorticotropic hormone; COR: Cortisol; CRC: Colorectal cancer; Ct: Threshold cycle; GH: Growth hormone; IGF-1: Insulin-like growth factor 1; MR: Magnetic resonance; MS-PCR: Methylation-specific polymerase chain reaction; NDRG2: N-myc downstream-regulated gene 2; PA: Pituitary adenoma; PRL: Prolactin; qRT-PCR: Quantitative real-time polymerase chain reaction; RHB: Human brain reference RNA.

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**Availability of data and materials**

Please contact author for data requests.

**Authors’ contributions**

PV, RL and BG generated the idea. RL and BG gathered postoperative pituitary adenoma tissue and patient data. PV and IV extracted tissue DNA and RNA, and performed bisulfite modification of DNA, MS-PCR experiments, and qRT-PCR. PV and IV did statistical analysis of data. PV, IV, RL and BG wrote a manuscript. All authors read and approved the final manuscript.

**Competing interests**

The authors declare that they have no competing interests.

**Consent for publication**

Not applicable.

**Ethics approval and consent to participate**

Permission (Number P2-9/2003) to undertake the study was obtained from the Kaunas Regional Biomedical Research Ethics Committee. Written patient consent under the approval of Ethics Committee of Lithuanian University of Health Sciences was obtained for every patient.

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