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Original Contribution

Quantitative analysis of p16 methylation in Barrett's carcinogenesis

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ABSTRACT

p16 hypermethylation in Barrett's carcinogenesis has been evaluated in studies which did not take into account sample heterogeneity and yielded qualitative (methylated/unmethylated) instead of accurate quantitative (percentage of CpG methylation) data. We aimed to measure the degree of p16 methylation in pure samples representing all the steps of Barrett's tumorogenesis and to evaluate the influence of sample heterogeneity in methylation analysis. Methods: 77 paraffin-embedded human esophageal samples were analyzed. Histological grading was established by two pathologists in: negative for dysplasia, indefinite for dysplasia, low-grade dysplasia, high-grade dysplasia and adenocarcinoma. Areas of interest were selected by laser-capture microdissection. p16 methylation was quantified by pyrosequencing. An adjacent section of the whole sample was also analyzed to compare methylation data. Results: After microdissection, we obtained 15 samples of squamous epithelium, 36 non-dysplastic Barrett's esophagus, 3 indefinite for dysplasia, 24 low-grade dysplasia, 4 high-grade dysplasia and 12 adenocarcinoma. Squamous epithelium showed the lowest methylation rates: 6% (IQR 5–11) vs. 11%(7–39.50) in negative/indefinite for dysplasia, $p < 0.01$; 10.60%(6–24) in low-grade dysplasia, $p < 0.05$; and 44.50%(9–66.75) in high-grade dysplasia/adenocarcinoma, $p < 0.01$. This latter group also exhibited higher methylation rates than Barrett's epithelium with and without low-grade dysplasia ($p < 0.05$). p16 methylation rates of microdissected and non-microdissected samples did not correlate unless the considered histological alteration comprised > 71% of the sample. Conclusions: p16 methylation is an early event in Barrett's carcinogenesis which increases with the severity of histological alteration. p16 methylation rates are profoundly influenced by sample heterogeneity, so selection of samples is crucial in order to detect differences.

1. Introduction

Barrett's esophagus (BE) is widely accepted as the main risk factor for developing esophageal adenocarcinoma (EAC) [1]. The overall mortality of this tumor remains high because most patients with EAC have incurable advanced disease at symptomatic presentation, with 5-year survival rates < 20% [2]. This points out to a need for effective screening strategies to detect EAC at early and more curable stages. Currently, the “gold standard” method used in the screening of EAC is the grade of dysplasia presented under endoscopical surveillance of patients with BE, dividing lesions in negative for dysplasia (ND), low grade dysplasia (LGD), indefinite for dysplasia (ID), and high grade dysplasia (HGD). The last one is the most severe lesion, considered to be immediately before the EAC, and involves therapeutic interventions, not only surveillance.

However, this screening strategy has two main disadvantages. First, this prognosis grading system on haematoxylin-eosin samples has problems of reproducibility among different pathologists. Therefore, according to the College of American Pathologists (CAP), the opinion of two professionals is required for the diagnosis of HGD [3]. On the other hand, the cost-effective impact of endoscopy surveillance is controversial because of the low percentage of BE patients (0.5% patients-year) who will actually progress to EAC [4]. Taking these issues into account, it is necessary to find better biomarkers which allow a more

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effective stratification of the risk of neoplastic progression, especially in patients with ND and LGD, thus improving the efficacy of surveillance programs in the early detection of EAC in high-risk patients and reducing the number of endoscopies in low-risk patients.

Molecular alterations usually precede morphological changes. Among them, hypermethylation of CpG sites surrounding the transcription start site (TSS) of tumor suppressor genes are a common feature in many tumors [5-7]. In Barrett's carcinogenesis, methylation of the promoter region of *CDKN2A* (*p16*) gene has been observed as is a frequent and early event in the progression to EAC [8,9]. However, these studies yielded qualitative instead of quantitative data which did not allow to accurately evaluate the degree of *p16* methylation. Quantitative measurement of methylation is important because different levels of methylation of a given gene may affect its expression. On the other hand, sample heterogeneity may influence the results of methylation analysis. Therefore, in the present study we aimed to assess the methylation status of *p16* in a quantitative manner in pure samples obtained by Laser Capture Microdissection (LCM) representing all the steps of the neoplastic transformation of Barrett's epithelium (normal esophagus, BE without dysplasia, low grade dysplasia, high grade dysplasia and EAC).

2. Materials and methods

2.1. Study population

In this study, archived esophageal biopsy specimens from patients with BE and/or EAC collected between 2000 and 2010 were obtained from the Service of Pathology of Miguel Servet University Hospital (Zaragoza, Spain). In all cases, haematoxylin-eosin-stained slides were re-evaluated by two pathologists to ensure accurate histological diagnosis according to Riddell criteria, classifying samples in normal squamous epithelium (NE), ND, ID, LGD, HGD and EAC. Indefinite for dysplasia category was considered as non-dysplastic BE because, despite the nuclear atypia found on this group, it does not fill all dysplasia criteria for LGD. The last two categories of the Riddell criteria were jointed because of its similar evolution behavior, as we know the short interval between the detection of HGD and the development of EAC [10]. Biopsies containing different degrees of BE were classified according to the highest degree of lesion present in the sample. NE cases were obtained from normal squamous mucosa areas present in the biopsy samples.

Each specimen was labeled with a study code, which did not include patient identifiers. Clinical and demographic information were obtained for each patient by reviewing medical records including gender, age, tobacco and alcohol use in case the information was available. In EAC patients we also reviewed whether patients had received chemo (EOX, cisplatin-vinorelbine or paclitaxel-CDDP-5FU) and radiotherapy prior to the collection of the samples.

2.2. Membrane slide preparation and microdissection

For each sample, the histopathological lesions of interest were first identified on routinely stained sections. Following this step, 8-μm thick sections were cut from tissue blocks in a microtome (Leica RM2255 rotary microtome, Barcelona, Spain) and mounted on Glass PEN (polyethylene naphthalate) membrane slides (Leica). After mounting the tissue sections, the membrane slides were incubated at 60 °C for 2 h in a dry oven to further improve tissue adhesion to the membrane. Samples were deparaffined and haematoxylin-eosin stained. After that, LCM was performed in a Leica Laser Microdissection AS in order to obtain pure material, selecting only the zones of the tissue containing the lesion of interest. In this system, microdissectates were captured in the cap of Eppendorf tubes containing 50 μl of lysis buffer (Qiagen, Hilden, Germany). A minimum of 2500 cells were obtained for each sample.

LCM is a very useful but laborious technique, therefore handling and time requirements of the procedure makes it difficult to apply to routine practice of pathologists. In order to evaluate whether the results obtained by LCM were maintained when the whole tissue sample present in the biopsy was analyzed, we obtained again 8 μm-slices from tissue blocks just adjacent to the previous ones used for LCM. DNA was isolated and quantified for *p16* methylation in the same manner. In addition, the pathologists evaluated the area occupied by the histological lesion considered in relation to the total tissue area in the biopsy.

2.3. DNA extraction, bisulphite treatment and pyrosequencing methylation analysis

DNA from microdissected cells was extracted with QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's instructions. DNA extracted was subjected to sodium bisulfite treatment, using the Epitect Bisulfite kit (Qiagen) according to the manufacturer's protocol for low DNA concentrations. By this procedure, all unmethylated cytosines were converted to uracil, and then subsequently to thymidine during PCR, so that allowed us to identify methylated and non-methylated cytosines. Hot-start PCR was carried out with HotStar Taq Master Mix Kit (Qiagen) using 5 μl of bisulphate-treated DNA. After the PCR amplification of the target loci, the degree of methylation of seven CpG sites was quantified by pyrosequencing using the PyroMark Q24 *p16* assay (Biotage, Uppsala, Sweden), which detects the level of methylation in the region +148 to +182 in exon 1 of the *p16* gene (Ensembl gene: ENSG0000014889). We included a non-CpG cytosine in the sequence analyzed by pyrosequencing and a set of methylated and unmethylated DNAs (Zymo Research, Freiburg, Germany and Qiagen), as controls for completion of bisulfite treatment and PCR reaction. Confirmation of PCR product quality and absence of contamination was established on a 3% agarose gel containing Sybr Safe staining (Invitrogen, Carlsbad, USA).

2.4. Statistical analysis

Data analysis was performed using SPSS 22.0 for Windows (SPSS Ibérica, Madrid, Spain). Comparison of *p16* methylation between groups was performed using non-parametric tests (Kruskal-Wallis and Mann-Whitney *U* test). Correlation between *p16* methylation data in samples obtained with or without LCM was analyzed by theSpearman test. A p value < 0.05 was considered to be significant.

2.5. Patient and public involvement

The study was approved by the Ethical Committee of Clinical Research of Aragón (CEICA). As we used archived paraffin embedded tissue and clinical data anonymously, including samples from deceased patients; no consent was considered to be obtained.

3. Results

3.1. Study population

We studied 55 patients, 45 of them having only one biopsy and the rest, between two and five. Finally, 77 biopsies containing BE and/or EAC were obtained. Microdissection of the 77 specimens yielded 94 samples available for the study. Two samples were obtained from patients displaying different degrees of dysplasia in the same specimen. The distribution of microdissected samples according to the different degrees of histological lesion was the following: 15 NE, 39 ND/ID, 24 LGD, and 16 columnar intestinal epithelium with HGD or EAC. In the latter group the distribution was 4 HGD and 12 EAC (2 stage I, 4 stage IB, 1 stage IIA, 1 stage IIB, 3 stage III and 1 stage IVB).

There was a higher male prevalence (70.90%) and the median age was 61.36 years (57.96–64.77).

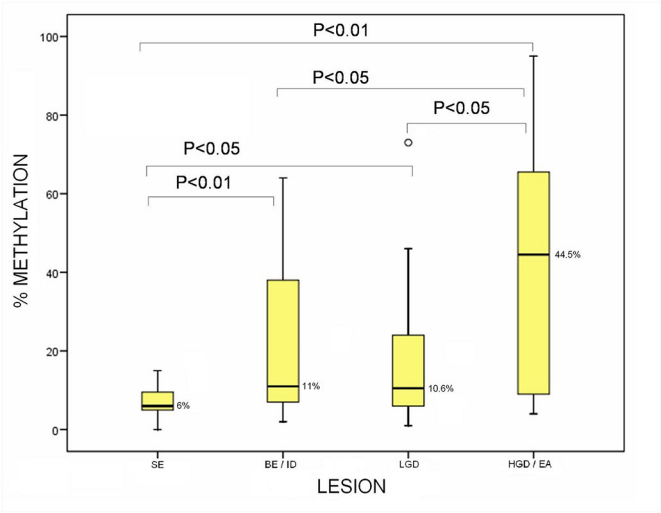

Fig. 1. Box plots showing methylation results of the average of methylation from seven CpG sites in the region +148 to +182 in exon 1 of the *p16* gene grouped by lesion. SE: squamous epithelium; BE/ID: Barrett's esophagus without dysplasia/indefinite for dysplasia; LGD: low grade dysplasia; HGD/EG: high grade dysplasia/esophageal adenocarcinoma.

Data for alcohol and tobacco consummation was also reviewed, and no significant effect was observed on *p16* methylation in the samples evaluated. However, data for alcohol and tobacco consummation were obtained only in 13 patients (46.1% no drinking, 30.8% moderate drinking and 23.1% high drinking) and in 22 patients (40.9% non-smokers, 36.4% ex-smokers and 22.7% current smokers) respectively, which could influence the results obtained.

3.2. Methylation status of p16 in BE and EAC

Samples containing normal squamous epithelium showed homogenously very low levels [median 6% (IQR: 5–11)] which were significantly lower than methylation levels of samples with intestinal metaplasia ND/ID [11 (7–39); $p < 0.01$], LGD [10.60 (6–24); $p < 0.05$], and HGD/EAC [44.50 (9–66.75); $p < 0.01$]. There were no differences in the degree of methylation between ND/ID BE and LGD BE samples, which showed mild methylation rates significantly different both of them to HGD/EAC ($p < 0.05$). It is remarkable the great heterogeneity of *p16* methylation rates observed in adenocarcinomas, as it is illustrated by the box and whisker plots of *p16* methylation rates distribution in Fig. 1. This heterogeneity might be due to the effect of previous therapies in this group and thus, we sought to evaluate the influence that previous chemotherapy or radiation therapy had on methylation levels in HGD/EAC patients. A group of six EAC samples from patients who had not received any previous therapy (diagnosed with stages II and IB) were compared with those who had underwent chemo and/or radiotherapy (diagnosed with EAC in stages II, III and IV). The results showed that EAC samples from patients who had received previous treatment exhibited significantly lower levels of methylation than untreated patients [mean 24.14% ± 9.27 SEM *vs* 62.66% ± 11.90; $p < 0.05$]. The comparison between the methylation levels in HGD/EAC patients without previous therapies and the rest of the groups reached similar results than those showed before. Methylation was significantly lower in normal squamous epithelium ($p < 0.01$), ND/ID ($p < 0.01$) and LGD ($p < 0.01$) than in HGD/EAC samples without previous treatment.

Focusing on the different CpG sites analyzed, the results are similar to those observed considering the average of all of them (Fig. 2). However, comparison between normal epithelium and LGD showed significant differences only in CpG site 4. Moreover, the percentage of methylation at each individual CpG did not vary in a characteristic pattern between the different degrees of lesion.

3.3. Influence of sample heterogeneity in methylation analysis

When we analyzed *p16* methylation in the samples obtained without LCM we observed very similar levels of methylation between the different groups: [13 (7–26)] for ND/ID; [8 (1.25–29.75)] for LGD and [12.50 (6–30.75)] in samples with HGD/EAC, and the results showed no statistical differences ($p = 0.6$) between the different groups evaluated (Fig. 3A). It should be noticed that we could only evaluate 53 of the 94 samples included in the first analysis since in some cases there was not enough sample left after the first analysis and also because some of the biopsies contained more than one degree of lesion and were therefore classified according to the highest degree of lesion present in the sample. For that reason, any normal squamous epithelium sample was included in the whole tissue sample methylation analysis. No differences were observed when each CpG site was analyzed separately, too. As shown in Fig. 3B, the correlation between *p16* methylation rates in samples obtained by LCM *versus* those obtained in the whole slice was low and non-significant. However, when we considered sample's representativeness (calculated as the percentage of the total sample area occupied by the histological grade of interest), we found a positive correlation between microdissected and non-microdissected samples only when the degree of lesion considered comprised > 71% of the sample ($p = 0.035$) (Fig. 3C).

4. Discussion

p16 inactivation and specifically *p16* hypermethylation has been reported in different tumors as well in preneoplastic lesions [4,11,12]. In this study we evaluated *p16* methylation through the metaplasia-dysplasia-adenocarcinoma sequence in BE, and the results showed increasing rates of *p16* methylation as histological lesion appears and progresses.

In agreement with previous studies [8,13], we observed significant increasing methylation values between NE, BE; and HGD/EAC, which supports the idea that *p16* plays a key role in the genesis of EAC. Samples with HGD/EAC displayed the higher degree of methylation and also great methylation heterogeneity, which might be a consequence of the previous antineoplastic therapy. In our study we observed lower methylation rates in patients undergoing previous therapy, which agrees with the decrease in DNA methylation of several genes (*DTNB*, *USP2*, *SMAD3* and *TMEM49*) in peripheral blood observed in patients with breast cancer who received chemotherapy prior to the extraction of the study sample [14]. The reduced *p16* methylation observed in those patients might be due to the DNA breaking effects of antitumor therapies, which activate DNA reparation pathways that add cytosines instead of methyl-cytosines, thus decreasing global DNA methylation. Chemo and radiotherapy are well known for inducing epigenetic changes in a wide variety of tumors *in vitro*, but the precise effects of both therapies on DNA methylation in patients with solid tumors have not been studied to date and should be taken into account in further methylation studies.

The significantly different levels of *p16* methylation between normal esophagus, in which methylation is very rarely seen, and first steps of the carcinogenesis process suggests that this is an early event in the development of the BE [15]. In patients with HGD and/or EAC, abnormal hypermethylation occurs both in the dysplastic and malignant tissues, thus indicating a preservation of the event during the evolution to cancer. In the present study we could observe high variability in *p16* methylation rates in samples of BE, and even though the meaning of these differences has not been addressed in the present study, it would be interesting to further evaluate in a prospective longitudinal study a group of BE patients who developed EAC with available biopsies from all the steps of the neoplastic sequence, in order to evaluate whether there is a threshold in *p16* methylation which can

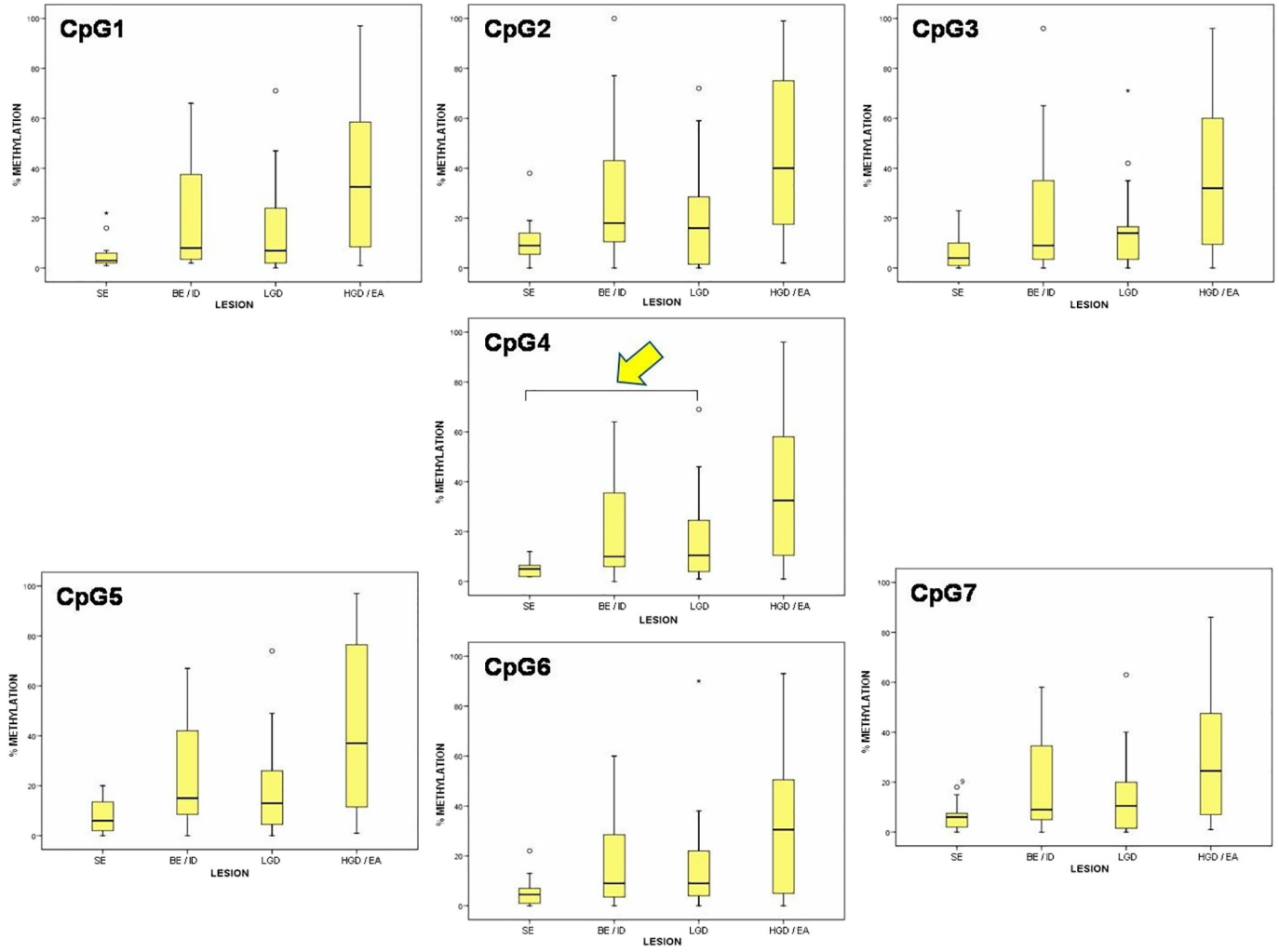

Fig. 2. Box plots of methylation results by lesion in the seven different CpG sites evaluated in region +148 to +182 of p16 exon 1. The results in all CpG sites correlate to those observed considering the average of all of them except for CpG 4, in which statistical differences were found only between normal squamous epithelium and low-grade dysplasia. Significant level (arrow) for $p < 0.05$.

discriminate those patients with more risk to develop an adenocarcinoma.

Among the risk factors evaluated, tobacco consumption had previously shown to increase the risk for developing BE and EAC [16-18]. Cigarette smoking has been linked to modifications in DNA methylation of tumor suppressor genes as *p16* between smokers and non-smokers, differences which persisted after smoking cessation [19-22]. In our study we also observed a trend to an increase in methylation levels of *p16* in patients with BE and EAC who are current smokers or ex-smokers, but these differences did not reach statistical significance possibly due to the small number of patients in which tobacco consumption was registered.

In this study we evaluated the methylation of seven CpG sites in exon 1. While it has been extensively investigated that CpG methylation in promoter regions is linked to gene silencing, in recent years it has been found that once methylated, some DNA regions including the first exon play a key role in gene inactivation. The CDKN2A gene has a large CpG island region that spans the promoter and exon 1, and methylation of exon 1 constitutes a common mechanism in silencing *p16* [7,23-26]. The study of DNA methylation has several advantages over other molecular alterations, for example the possibility to use formalin-fixed paraffin-embedded samples for the analysis. In addition, DNA is more stable than RNA or protein and the location of CpG islands in gene promoter regions is similar between individuals, therefore the detection of changes in the methylation pattern is easier than the identification of mutations. Unlike most of the studies carried out to date about the role of methylation in BE carcinogenesis [8,9,11,13,27], which use qualitative assays and thus do not quantify the degree of methylation, in the present study the presence of methylation was assessed by pyrosequencing, therefore allowing the accurate quantification of the percentage of methylated or unmethylated cytosines at each CpG site analyzed in the samples.

Only a small portion of Barrett's epithelium is usually taken as a biopsy in routine clinical practice, and the area containing the highest degree of lesion might not be present in the biopsy sample because of incomplete sampling. Therefore, in order to increase the diagnostic sensitivity, the American Gastroenterological Association (AGA) recommends obtaining biopsy samples in four quadrants, starting from the proximal border of the stomach and ascending every 2 cm [28]. The heterogeneity of each biopsy sample can affect the results of methylation analysis since every biopsy specimen usually contains a significant proportion of cells, such as immune and stromal cells, which are not of interest for the analysis and may have distinct methylation levels than the target cells, thus interfering with the results. One of the advantages of our study with respect to others is the use of LCM, a time-consuming technique which makes it difficult to be applicated in routine clinical practice but serves as a valuable tool on research studies allowing to identify and select the population of cells with the lesion of interest

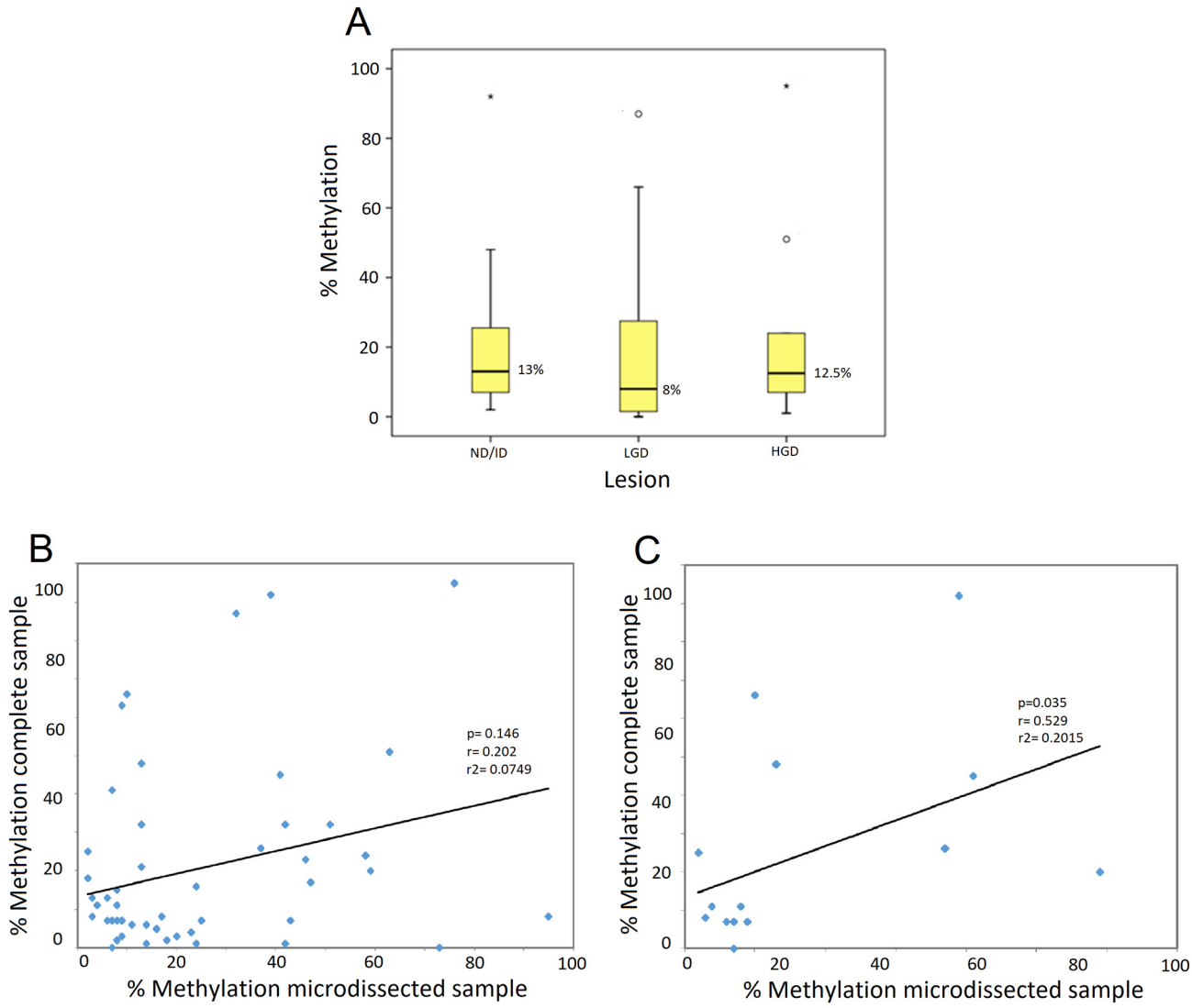

Fig. 3. Methylation analysis in samples obtained without microdissection. Box plots showing methylation results in samples obtained without microdissection (A). The numbers over the whisker plots correspond to extreme values. Correlation in methylation values between microdissected samples and whole samples (B) and between microdissected samples and whole samples in which the degree of lesion considered comprised > 71% of the sample (C).

within each biopsy sample, increasing the accuracy of the methylation analysis.

In this study we also evaluated the impact of LCM in methylation analysis. As previously reported in gastric cancer biopsies, the heterogeneity of the sample can mask changes in methylation in a subset of cells with the lesion of interest [29]. In agreement with that previous study, which indicated the need of at least a 70% of cells of interest in the sample to obtain accurate methylation results, we observed that if biopsy samples contained a percentage of cells of interest below 71% of total, the sensitivity in detecting differences between the different degrees of lesion remained drastically affected. Therefore, the results observed indicate that an accurate quantification of the percentage of BE in the sample seems to be a key factor to avoid the interferences that the surrounding tissue can exert on the methylation levels.

Another advantage of our study is the use of pyrosequencing. In contrast to standard methylation methods, which provide qualitative data and can lead to inaccurate conclusions, bisulfite pyrosequencing provides reproducible and accurate quantification of methylation levels at individual CpG sites.

In conclusion, our data suggest that methylation of *p16* is increased through the neoplastic progression of BE to HGD and EAC thus addressing its influence in the carcinogenesis process. In addition, this study also confirmed the impact of sample heterogeneity in methylation analysis.

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Data statement

Technical appendix, statistical code, and all available data can be obtained by contacting corresponding author.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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