Genetic markers in malignant progression of Barrett’s esophagus
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Assessment of chromosomal gains as compared to DNA content changes is more useful to detect dysplasia in Barrett’s esophagus brush cytology specimens

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Submitted
ABSTRACT

**Background & Aims:** Abnormal DNA ploidy status has been suggested as a prognostic factor for Barrett’s esophagus progression into esophageal adenocarcinoma (EAC). The aim was to compare image cytometry DNA analysis (ICDA) and fluorescent in situ hybridization (FISH) in the assessment of DNA ploidy status in BE brush cytology specimens, and to determine the value of these abnormalities as an adjunct to conventional cytology in detection of dysplasia and EAC.

**Methods:** Brush cytology specimens of 90 BE patients were examined using ICDA (the DNA index) and FISH with peri-centromeric probes for chromosome 7 and 17. The results of ICDA and FISH were compared with each other, and with dysplasia grade or EAC as determined by histology and cytology.

**Results:** FISH and ICDA detected abnormalities in 41% (37/90) and 22% (19/90) of the BE cases, respectively. These two methods were concordant in 76% of the cases. Gains of chromosome 7 and/or 17 as detected by FISH were present in 13% of non-dysplasia cases which further increased with dysplasia stage and EAC, while aneuploidy as determined by ICDA was detected predominantly in high grade dysplasia (HGD) and EAC. Using FISH results combined with cytology, we were able to identify IND or LGD (indefinite - or low grade dysplasia) with a sensitivity and specificity of 75% and 76%, respectively. FISH alone detected HGD or EAC with a high sensitivity and specificity of 85% and 84%, which was superior to that of cytology alone.

**Conclusions:** FISH seems to be more sensitive than ICDA to detect chromosomal abnormalities in BE brush cytology specimens. FISH detects chromosomal gains in early stages of BE and represents a valuable adjunct to conventional cytology to detect dysplasia or EAC.
INTRODUCTION

Barrett’s esophagus (BE) is the metaplastic replacement of the normal squamous epithelium of the lower esophagus by the columnar epithelium developing as a consequence of longstanding gastroesophageal reflux disease (GERD).\textsuperscript{1} BE is a premalignant condition that predisposes to the development of esophageal adenocarcinoma (EAC), a tumor with increasing frequency in most Western countries.\textsuperscript{2} EAC develops through a multistep process which is characterized by increasing grade of dysplasia (intraepithelial neoplasia).\textsuperscript{3} Therefore, routine endoscopy with biopsies for histopathological evaluations of BE patients is considered mandatory. It appears, however, that the present endoscopic and histopathologic evaluations of BE patients are insufficient for effective identification of high-risk patients at early stage.\textsuperscript{4,5} For the clinical management of BE patients it is, therefore, of great importance to identify specific markers associated with development of EAC and its precursor lesions in BE.\textsuperscript{1}

Parallel to the metaplasia-dysplasia-adenocarcinoma sequence, numerous studies demonstrated the accumulation of genetic abnormalities from normal cells to invasive malignant cells.\textsuperscript{6,7} Some of these genetic abnormalities i.e., DNA ploidy changes have been suggested to be of potential help in the surveillance of BE patients.\textsuperscript{8,9} There is accumulating evidence that DNA aneuploidy, defined as any other formulation of chromosomes then 2N, is a early key event in carcinogenesis and may be a cause rather then a consequence of malignancy.\textsuperscript{10-12} Indeed, the Seattle group using flow cytometry, has shown in a prospective study that patients with DNA aneuploidy have an increased risk of developing HGD or EAC.\textsuperscript{13} The same group demonstrated that among patients with non-dysplasia, indefinite for dysplasia, LGD, the risk of cancer was strongly related to the presence of DNA aneuploidy or increased tetraploid populations.\textsuperscript{14} Although several studies confirmed that DNA aneuploidy is a prognostic factor for malignant transformation of BE\textsuperscript{15}, other groups reported discordance between histology and DNA ploidy\textsuperscript{16}, which at partially may be due to technical issues.

DNA aneuploidy is usually measured by flow cytometry analysis (FCDA).\textsuperscript{13-15} The sensitivity of this method, however, is limited in diagnosing DNA content abnormalities, because it analyses both affected epithelial cells as well as stromal cells in the sample. Moreover, the need for time-consuming, special tissue preparation also limits the usefulness of DNA FCDA in clinical practice.\textsuperscript{17,18} Image cytometric DNA analysis (ICDA) seems to be a more convenient method for DNA ploidy analysis. This technique was previously applied to measure DNA content in disaggregated cytospin preparations or in microscopically identified epithelial cells.\textsuperscript{11} Some reports indicate that ICDA is more sensitive then FCDA to detect DNA ploidy changes.\textsuperscript{19,20} Both FCDA and ICDA are based on stoichiometrically binding of a dye to the DNA that can be measured quantitatively. An alternative
means of estimating ploidy status can be performed by DNA fluorescence in situ hybridization (FISH), which can accurately detect copy numbers of specific chromosomes using (peri-)centromeric DNA probes. DNA FISH can be applied successfully on interphase nuclei in tissues and cellular specimens. Such interphase DNA FISH analysis can easily identify chromosomal gains and losses in populations of (epithelial) cells. Previously, we and others successfully applied DNA-FISH on brush cytology specimens to detect a variety of cytogenetic abnormalities in BE.\textsuperscript{21-23} The advantage of brush cytology, for sampling of cells in BE patients, includes simplicity, lower cost and the potential to sample a larger area of the BE epithelium when compared to taking random biopsies. Furthermore, cytology samples are more reliable for the enumeration of FISH signals and assessment of ploidy status by ICDA, since there is no artifact caused by nuclear truncation, as is the case when using tissue sections.\textsuperscript{24}

The purpose of the present study was first, to compare the DNA ploidy status as assessed by ICDA to chromosomal gains by DNA-FISH analysis on BE brush cytology specimens. The secondary aim was to determine the value of the observed abnormalities as an adjunct to conventional cytology in detection of dysplasia and EAC in BE.

**MATERIALS and METHODS**

**Patients**

A cohort of 90 Barrett’s esophagus patients, who underwent endoscopy at the Academic Medical Center in Amsterdam between 2002 and 2007 were included. Only patients that had proven (incomplete) intestinal type of metaplasia in biopsies, taken during and prior to surveillance were included. All patients were on long term proton pump inhibition of 40 to 80 mg daily to prevent reflux esophagitis. During endoscopy, the brush cytology specimens were taken prior to biopsies. The brushes of the normal squamous epithelium were taken from each patient at least 3 cm above the BE segment, and were used for control purposes. Biopsies for routine histological examination were taken immediately after brushing and at least per 2 cm in 4 quadrants and of all suspected visible lesions using the protocol of Reid \textit{et al.}\textsuperscript{25} The Ethics Committee of the Academic Medical Center approved the study. All patients signed informed consent for the use of their biopsy and brush cytology material.

**Brush Cytology preparation**

Cytological brush material was sampled using the Wilson-Cook (Winston-Salem, NC) brush type LCB-220-3-1.5-S. Directly before brushing, the mucosal surface was sprayed with acetylcysteine (50mg/ml) for dissolving the mucus layer. Separate brush samples from Barrett’s and normal squamous mucosa were taken from each
BE patient. After the procedure the brushes were inserted in a vial with 20ml of 5% acetylcysteine in 0.9% of NaCl and mixed gently to obtain a homogeneous cell suspension. Subsequently, a Cytospin (Shandon Cytospin 4 Cytocentrifuge, Thermo, Waltham, MA) was used to first generate a single layer of the Barrett cells on the top of the glass slide. A second cytospin spot of the squamous epithelial cells of the same patient was made on the bottom of the same slide. These squamous spots served as internal control for FISH and the ploidy analysis procedures. The cytospin procedure was performed as described previously. 22 After the procedure the cytospin slides were dried at RT, and then stored at - 80°C until further processing.

Cytological evaluation
The brush cytology samples were thawed and stained with Giemsa. Subsequently, two investigators (F. ten Kate and J.G. de Groot) independently scored the brush cytology samples for dysplasia. Any discrepancies in diagnosis were discussed until consensus was reached. BE was defined when goblet cells or cylindrical cells were identified. For the cytological diagnosis of dysplasia or adenocarcinoma, we used widely accepted standards for nuclear atypia such as increased nuclear size, elongated rather then oval nuclear shape and loss of polarity. Cases were categorized into four groups: 1) no dysplasia, 2) indefinite for dysplasia, including inflammatory cases with reactive changes, 3) LGD and 4) HGD or EAC.

Fluorescent in situ hybridization (FISH)
We used directly labeled fluorescent chromosomal centromeric probes (CEP) for chromosome 7 and 17 (SpectrumGreen each) obtained from Vysis (Downers Grove, IL). DNA-FISH was performed on BE brush cytology according to the manufacturer’s instructions provided by Vysis as described previously. 22

Criteria for determining FISH ploidy abnormalities
Enumeration of the number of hybridization signals per nucleus after applying centromeric DNA FISH probes on interphase nuclei reveals the copy number of the corresponding chromosome (Figure 1). Although FISH by itself cannot measure the entire ploidy status of the cells, abnormal ploidy status can be indicated by a change in the copy numbers of the individual chromosomes within the interphase nuclei, which is mostly indicated by chromosomal gains. In this study, 100 to 200 interphase nuclei of BE cells, after FISH with two peri-centromeric probes (CEP 7 and 17), were scored per slide by an experienced scorer (A.M Rygiel) using Olympus BX61 fluorescent microscope (Germany). The cases were evaluated without prior knowledge of histology and cytology findings. Damaged cells and cells with indistinct and blurry signals were excluded from the analysis. Chromosomal gains by FISH were defined when the sample contained ≥ 3 % of the nuclei with three or more signals for the peri-centromeric probes (CEP 7 or 17). The cutoff value of
≥ 3 % was obtained from counts in the normal squamous epithelia taken from 20 BE patients without dysplasia and calculated as the mean percentage of squamous nuclei with signal gain plus 3xSD (standard deviation) as described previously.22

**Feulgen staining**

Each brush cytology slide, apart of the spot with BE cells, contained also a separate cytospin spot generated from normal squamous epithelium, which served as the internal diploid control. The slides were stained with Feulgen dye using ImagePath Blue Feulgen Stain kit (ImagePath System, Inc., Edwardsburg, Michigan), according to manufacture’s protocol. The staining involves treatment with concentrated hydrochloric acid that strips the cells of non-nuclear substances, and hydrolyses chromatin. The Feulgen dye then stoichiometrically binds to the nucleic acids, imparting a blue color. The intensity of the blue color is directly proportional to the amount of DNA content.

**Image cytometry**

After Feulgen staining, DNA ploidy analysis was performed using the Ariol system with software version 3.0 (Applied Imagining, New Castle, UK). The analysis was performed in a blinded manner according to histology and cytology findings. For each brush cytology slide, two separate areas of BE and squamous cytospin spots were scanned (40 x magnification), digitalized and stored in the same file (Figure 1). Subsequently, the nuclei of interest from the squamous and the BE area (cylindrical or/and atypical cells) were marked for analysis. For the BE cells, we used widely accepted standards for nuclear atypia such as increased nuclei size, elongated rather than oval nuclear shape, loss of polarity, and marked these cells for DNA quantification. BE or adenocarcinoma nuclei were quantified for DNA content and compared with the DNA content of normal squamous cells from the same slides. The digitalized images of nuclei were converted into a series of pixels, which were quantified on the basis of the integrated optical density (IOD), reflecting the DNA content. Overlapping nuclei, nuclear debris and other artifacts that escaped auto-detection and removal by the system were deleted by the operator. At least 100 BE cells and 100 control squamous cells (range 100-650 and 100-375, respectively) within the same brush cytology slide were quantified. The DNA content histogram was automatically plotted in a separate window when using the ploidy software (Figure 1). The mean integrated optical density (IOD) of control squamous cells was assigned as a DNA index (DI) of 1, which served as an internal diploid (2N) standard and reference for DI calculation of the BE cells. DI values of 0.9 -1.1 were considered to be within the diploid range. Aneuploidy was defined when more then 10% of BE cells had DI of main peak greater then 1.2.26,27 The cutoff of less than 10% of the coefficient of variation (CV) for diploid and aneuploid peak was applied.
Statistical analysis
Differences in frequencies of abnormalities were tested using a chi-square test (2-sided) and statistical significance was set at a $P$ value of $<0.05$. The statistical analyses were conducted using SPSS software (version 12.0; SPSS, Inc, Chicago, IL). Sensitivity and specificity of the different methodologies (FISH, ICDA or cytology) were determined for patients classified as abnormal (dysplasia or EAC) and normal (non-dysplasia or IND/LGD, depending on the analysis) by histology, respectively, which was considered the gold standard.

RESULTS

Histopatology and Cytology
In this prospective study, 90 BE patients were included. Of these 90 BE patients 86 were men and 6 women, median age was 60 (range 30-84), median length of the BE segment was 3 cm (range 1-13cm). The BE population included 38 patients with ND, 19 patients with IND or LGD, 18 patients with HGD and 15 patients with EAC as assessed by histopathology. Brush cytology specimens of 85 of the 90 BE cases were available for cytology diagnosis. There were 45 cases of ND, 20 cases of indefinite for dysplasia or LGD, 20 cases of HGD or EAC as classified by cytology diagnosis. Table 1 compares cytology and histology results.

<table>
<thead>
<tr>
<th>Histology</th>
<th>Cytology No.</th>
<th>Total nr</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ND</td>
<td>LGD/IND</td>
</tr>
<tr>
<td>ND</td>
<td>34 (91%)</td>
<td>3</td>
</tr>
<tr>
<td>IND/LGD</td>
<td>7</td>
<td>8 (50%)</td>
</tr>
<tr>
<td>HGD/EAC</td>
<td>4</td>
<td>9</td>
</tr>
</tbody>
</table>

Table 1: Correlation of cytology and histology diagnosis of BE. ND- no dysplasia, IND – indefinite for dysplasia, LGD- low grade dysplasia, HGD- high grade dysplasia, EAC- esophageal adenocarcinoma

Concordance between FISH and ICDA
Gains of chromosome 7 and 17 were determined by FISH analysis and overall DNA ploidy status was assessed by ICDA analysis in brush cytology specimens of the 90 BE patients. In the FISH analysis, gains of chromosome 7 and 17 were detected on average in 30 % of cells (median 16, range 3-87). The number of signal gain for each of the chromosomes varied from 3 to 6 signals per cell. In the ICDA analysis, the median DNA index (DI) value for control cells and normal BE cells (diploid peak) was 1.0 (range 0.9-1.1) with median coefficient of variation (CV) of 4.5 (range 2.97-7.24) and for abnormal cells (aneuploidy peak) the median DI value was 1.8 (range 1.4-2.0) with a median CV of 6.3 (range 3.47- 9.5). On average aneuploidy detected in 40% of cells (median 30, range 11- 79%).
Figure 1: DNA content analysis by ICDA and detection of chromosomal gains by FISH.
(A) ICDA analysis. Brush cytology specimen containing a cytospin spot with BE cells (top) and a spot with squamous cells (bottom) were stained with Feulgen’s stain. The final classification of DNA ploidy is depicted in a DNA histogram generated from the DNA content data using the Ariol system (Applied Imaging, New Castle, UK). The histogram is representative for one BE case with DNA content aneuploidy. In the histogram, the results for the control squamous cells are shown in black (control peak), results for BE cells in green (diploid peak) and in red (aneuploid peak). The y axis shows the number of cell nuclei, and the x axis shows the nuclear DNA content defined as DNA index (DI).
(B) FISH analysis. BE brush cytology specimens after FISH with peri-cetromeric probes for chromosome 7 (CEP 7, top) and 17 (CEP 17, bottom). The graph shows frequency of BE nuclei with CEP7/17 gains and is representative for the same case as shown in panel A. The y axis shows percentages of BE nuclei, and the x axis shows the number of signals for CEP 7 and CEP 17. For color figure, see page 144.
Table 2 illustrates the overall comparison of FISH and ICDA results. FISH and ICDA detected abnormalities in 41% (37/90) and 22% (19/90) of the BE cases, respectively (p<0.0001, chi-square test). Overall these two methods were concordant in 76% (68/90) of the cases. There were 22 discordant cases, which are listed in Table 3. Of the 22 discordant cases there was 1 case with ND and 1 case with EAC classified as aneuploid by ICDA but was found to be normal by FISH. The remaining 20 discordant cases were classified as diploid by ICDA but chromosomal gains were detected by FISH. In most of these cases (18 out of 20), however, only gain of a single chromosome (chromosome 7 or 17) was detected. Nine of the 22 discordant cases displayed chromosomal gains in a relatively small fraction of cells (3-5%), which was equal or close to the cutoff value of 3%.

Table 2: Overall comparison of FISH and ICDA results. FISH –fluorescent in situ hybridization, ICDA –image cytometry DNA analysis.

<table>
<thead>
<tr>
<th>Ploidy classification</th>
<th>FISH (CEP 7 or /and 17)</th>
<th>ICDA</th>
<th>Both Methods (concordance)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>53/90 (58%)</td>
<td>71/90 (79%)</td>
<td>51/90 (56%)</td>
</tr>
<tr>
<td>Abnormal</td>
<td>37/90 (41%)</td>
<td>19/90 (21%)</td>
<td>17/90 (19%)</td>
</tr>
</tbody>
</table>

Correlation of cytology, FISH and ICDA results with histology

Figure 2 compares the frequencies of gains of chromosome 7 and/or 17 detected by FISH with ICDA results in the no-dysplasia–dysplasia-adenocarcinoma stages of BE. In general, FISH detected chromosomal gains in 13% (5/38) of the ND and 21% (4/19) of the IND/LGD cases, which then increased to 83% (15/18) in the HGD and 87% (13/15) in the EAC cases (p<0.0001). ICDA showed aneuploidy in 3% of ND (1/38), in none of the IND/LGD, in 50% (9/18) of the HGD and in 60% (9/15) of the EAC cases (p<0.0001; Figure 2)

We further compared results of cytology, FISH and ICDA to the histological classification of BE as assessed in the biopsy specimens. Table 4 compares the different approaches for assessing the dysplasia stage in BE using cytological diagnosis and the abnormalities as found by FISH and ICDA. In the first approach, the sensitivity to detect any dysplasia or EAC was 77%, 62% and 35% for cytology, FISH and ICDA, respectively. In the second approach, the accuracy of the assays to distinguish IND/LGD from ND was compared. The sensitivity hereto was 50%, 21% and 0 for cytology, FISH and ICDA, respectively. In these two approaches, the specificity for all three methods was comparably high varying from 87% to 97%. In the third approach, the accuracy of the assays to distinguish HGD/EAC from IND/LGD and ND was determined. The sensitivity to detect EAC or HGD was 60%, 85% and 54% for cytology, FISH and ICDA, respectively with corresponding specificities of 77%, 84% and 98%. Interestingly,
FISH and ICDA detected abnormalities in 11 and 9 cases out of 13 cases with HGD/EAC, respectively, which were misdiagnosed by cytology. Finally, since FISH and cytology results were the most sensitive assays, we combined these two methodologies and found even higher sensitivities of 85% to detect any dysplasia, 75% to distinguish IND/LGD from ND, and 90% to detect HGD or EAC. However, the specificity of this combined assay decreased to 76%, 76% and 60%, respectively (Table 4).

Table 3: Discordant cases between FISH and ICDA in the detection of aneuploidy. Gain – more than two copies of chromosome 7 and/or 17 (the number of signal gain varied from 3 to 6 signals per cell), DI –DNA index, D – diploid (DI 0.9–1.1), A – aneuploidy (DI >1.1). FISH –fluorescent in situ hybridization, ICDA –image cytometry DNA analysis, ND- no dysplasia, IND – indefinite for dysplasia, LGD- low grade dysplasia, HGD- high grade dysplasia, EAC- esophageal adenocarcinoma.
### Table 4: ICDA, FISH and conventional cytology in the identification of different stages of dysplasia in BE.

<table>
<thead>
<tr>
<th>Histology</th>
<th>ICDA</th>
<th></th>
<th></th>
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<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>N</td>
<td>A</td>
<td>N</td>
<td>A</td>
<td>N</td>
<td>A</td>
</tr>
<tr>
<td>Any dysplasia or EAC</td>
<td>18 (35%)</td>
<td>34</td>
<td>32 (62%)</td>
<td>20</td>
<td>37 (77%)</td>
<td>11</td>
<td>41 (85%)</td>
</tr>
<tr>
<td>ND</td>
<td>1</td>
<td>37 (97%)</td>
<td>5</td>
<td>33 (87%)</td>
<td>3</td>
<td>34 (91%)</td>
<td>9</td>
</tr>
<tr>
<td>IND/LGD</td>
<td>0</td>
<td>19</td>
<td>4 (21%)</td>
<td>15</td>
<td>8 (50%)</td>
<td>8</td>
<td>12 (75%)</td>
</tr>
<tr>
<td>HGD or EAC</td>
<td>18 (54%)</td>
<td>15</td>
<td>28 (85%)</td>
<td>5</td>
<td>19 (60%)</td>
<td>13</td>
<td>29 (90%)</td>
</tr>
<tr>
<td>IND/LGD or ND</td>
<td>1</td>
<td>56 (98%)</td>
<td>9</td>
<td>48 (84%)</td>
<td>12</td>
<td>41 (77%)</td>
<td>21</td>
</tr>
</tbody>
</table>

*the analysis was performed for 85 cases with known cytology diagnosis.

FISH - fluorescent in situ hybridization,
ICDA - image cytometry DNA analysis, ND - no dysplasia, IND – indefinite for dysplasia, LGD- low grade dysplasia,
HGD- high grade dysplasia, EAC- esophageal adenocarcinoma
DISCUSSION

In this study, we compared FISH to image cytometry DNA analysis (ICDA) in the assessment of DNA content changes in BE brush cytology specimens. We demonstrated that FISH is a more sensitive method to detect abnormal fractions of cells with single chromosomal gains compared to ICDA. Moreover, we showed that detection of chromosomal gains by FISH alone or combined with cytology diagnosis is a very sensitive assay to detect dysplasia in BE patients.

We first compared FISH and ICDA analysis in assessing the ploidy abnormalities in BE brush cytology specimens and found an overall concordance of 76%. FISH, however, detected abnormalities in a higher number of BE cases as compared to ICDA (41% vs. 22%). Further, the majority of discordant cases (20 of 22) were diploid by ICDA but showed chromosomal gains by FISH indicating that this method is a more sensitive assay than ICDA to detect DNA content abnormalities. In nine of the 22 discordant cases FISH detected gains in a small fraction of cells of 3 to 5%, which was close to the cutoff value. Thus, at least part of the discrepant results may be a reflection of ICDA limitation to detect small populations of aneuploid cells. Remarkably, in most of the discordant cases (19 out of 22), gain of only one out of two investigated chromosomes was detected by FISH. In three of these cases this single gain was
Chromosomal gains in a BE

even confined to significant fraction of cells of 20-41%. Although we assume that the chromosomal gains are a reflection of a general aneuploid status of the specimen, it is possible that, in certain BE cases, gains of chromosome 7 and 17 are selective events that are independent of gross ploidy changes. Our results suggest also that gains of single chromosomes may result in a too small DNA content change to be detected by ICDA. A similar observation has been previously described in a study comparing ploidy results between FISH, flow cytometry and ICDA on fresh touch preparation and paraffin-embedded samples of prostate tumors. This study showed a case having 40% of cells with gain of a single chromosome (chromosome 8) by FISH, which was still assessed as diploid by ICDA and flow cytometry, supporting our conclusion that FISH is more sensitive to detect these subtle DNA content changes. It should be noted, however, that the limitation of the sensitivity of FISH depends on the selection of appropriate and informative centromeric probes, especially in cases when a limited number of chromosomes may be polysomic. Overall DNA content aneuploidy is generally related to genetic instability, while this may not be true in case of single chromosomal gains. Therefore, one can also argue that the abnormal count of FISH signal for a single chromosome in an overall ‘diploid’ cell is not comparable to a general aneuploid state and should be considered as a separate entity.

It has been shown that DNA content abnormalities, as assessed by flow cytometry, identifies BE patients at higher risk for developing HGD or EAC. Therefore, in our study, we correlated chromosomal gains and overall DNA ploidy results as detected by FISH and ICDA with histological grade of dysplasia and EAC. We showed that the abnormalities as detected by FISH using centromeric probes for chromosome 7 and 17, is already present in 13% of ND cases and that its frequency significantly increases with increasing dysplasia reaching 21% in IND/LGD, 83% in HGD and 87% in EAC cases (p<0.0001). This observation is similar to that of a study by Doak et al. showing that aneuploidy of other chromosomes (chromosome 4 and 8) as detected by FISH on brush cytology, are early changes present in the non-dysplastic BE correlating with increasing grade of dysplasia. In our study, however, ICDA detected aneuploidy only in 3% of ND cases, in none of IND or LGD cases and in only 50% of HGD and 60% of EAC cases. This finding does not correlate well with the results of a recent ICDA study on paraffin BE biopsy sections, which showed aneuploidy in 13% of ND, 60-73% of LGD/HGD and 100% of EAC cases. This discrepancy could be due to differences of standardization of the image cytometry methods to determine aneuploidy (i.e. different cutoff values to define aneuploidy, different image cytometry systems), the use of tissue section (overlapping and truncated nuclei) instead of brush cytology or an observer variation when classifying the grade of dysplasia in BE.
Brush cytology has potential advantages over endoscopic biopsy for diagnosing BE and monitoring for dysplasia since it has ability to sample a greater area of the BE mucosa surface, and may therefore detect lesions that are missed by biopsy protocols. Indeed, in our study, cytology diagnosed IND/LGD in 3 cases and in 1 case HGD, which were classified as ND and IND/LGD by histology, respectively. On the other hand, cytology misclassified 13 cases, which were regarded as HGD/EAC by histology. (Table 1) In general, we found that conventional cytology has a good sensitivity to detect any dysplasia (77%) and moderate sensitivity to detect IND/LGD (50%) or HGD/EAC (60%) with specificities of 91%, 91% and 77%, respectively. The sensitivity of the cytology to detect HGD/EAC in our study is however lower than demonstrated in other reports.33-35 Although these studies suggest that cytology may be as accurate as histology to detect HGD or EAC, the small number of cases investigated in these studies makes it difficult to draw definitive conclusion. The sensitivity of routine cytology to detect IND or LGD, in our study, was higher than in the study by Saad et al. where cytology detected only 20% of cases with LGD.35 This discrepancy may be due to the interobserver and intraobserver variations and the expertise of the pathologist for scoring dysplasia in cytological specimens. Still, for cytology to become a useful surveillance tool to identify BE patients at higher risk for cancer development, its sensitivity and specificity for dysplasia detection lesions has to be improved36, which might be accomplished by adding biomarkers i.e. DNA ploidy status to routine cytology diagnosis. Therefore, in this study, we compared the sensitivity of the abnormalities as determined by FISH and image ICDA in cytology samples with routine cytology diagnosis in the detection of dysplasia or EAC in BE. The histology diagnosis was considered as the gold standard. The sensitivity to detect any dysplasia or EAC by FISH or ICDA was lower compared to routine cytology (62% and 35% vs. 77%, respectively). Cytology was also more sensitive to detect IND or LGD cases compared to FISH and ICDA assays (50% vs. 21% and 0%, respectively). In these analyses, the specificity for all three methods was comparably high varying from 87% to 97%. We found, however, that chromosomal gains assessed by FISH have a higher sensitivity and specificity to detect HGD or EAC as compared to routine cytology (85% vs. 60% and 84% vs. 77 %, respectively). Moreover, FISH detected gains in 11 of 13 HGD/EAC cases that were misdiagnosed by cytology. Upon combining results of FISH and cytology diagnosis we observed a further increase in sensitivities up to 90% to discriminate between the different stages of dysplasia in BE (Table 4). In case histology is truly the ‘gold standard’, the combination of these two assays to detect HGD/EAC show a specificity of 60%, yielding a considerable number of false positives. On the other hand the false positive cases may reflect a population of BE patients that are at higher risk for developing dysplasia. Future follow up of these cases will enlighten us on this matter. At present we advocate that FISH combined with cytology is a highly promising method for the identification of dysplasia and/or EAC in BE.
In summary, we showed that FISH is more sensitive technique than ICDA to detect chromosomal changes in early stages of BE and therefore might be of value to identify BE groups at high risk for EAC development. Additionally, we demonstrated that assessment of chromosomal gains by FISH represents a valuable adjunct to conventional cytology to detect dysplasia and/or EAC in BE.
REFERENCES

Chromosomal gains in a BE


