A search for molecular biomarkers in gastro-intestinal cancer

Davelaar, Akueni

Citation for published version (APA):

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
ABERRANT P53 DETECTED BY COMBINING IMMUNOHISTOCHEMISTRY AND DNA-FISH IMPROVES BARRETT’S ESOPHAGUS PROGRESSION PREDICTION: A PROSPECTIVE FOLLOW-UP STUDY

Akueni L. Davelaar\textsuperscript{1,2}, Silvia Calpe \textsuperscript{1,2}, Liana Lau \textsuperscript{1,2}, Margriet R. Timmer\textsuperscript{1,2}, Mike Visser\textsuperscript{3}, Fiebo J. ten Kate\textsuperscript{4}, Kaushal B. Parikh\textsuperscript{1,2}, Sybren L. Meijer\textsuperscript{1}, Jacques J. Bergman\textsuperscript{1}, Paul Fockens\textsuperscript{1}, Kausilia K. Krishnadath\textsuperscript{1,2}.

\textsuperscript{1}Department of Gastroenterology and Hepatology, Academic Medical Center, Amsterdam, The Netherlands. 
\textsuperscript{2}Center for Experimental and Molecular Medicine, Academic Medical Center, Amsterdam, The Netherlands. 
\textsuperscript{3}Department of Pathology, Academic Medical Center, Amsterdam, The Netherlands. 
\textsuperscript{4}Department of Pathology, University Medical Center, Utrecht, The Netherlands.

Accepted for publication in Genes, chromosomes and cancer
ABSTRACT

Objective: Barrett’s esophagus (BE) goes through a sequence of low grade dysplasia (LGD), high grade dysplasia (HGD) to esophageal adenocarcinoma (EAC). The current gold standard for BE outcome prediction, histopathological staging, can be unreliable. P53 abnormalities may serve as prognostic biomarkers. P53 protein accumulation detected by immunohistochemistry (IHC) indirectly assesses P53 mutations. DNA fluorescent in-situ hybridization (FISH) on brush cytology specimens directly evaluates gene locus loss. We evaluated if IHC and FISH are complementary tools to assess p53 abnormalities and tested their prognostic value in a long-term prospective follow-up of a BE cohort.

Materials and Methods: P53 IHC on tissue sections and FISH on brush cytology specimens were evaluated for 116 BE patients with respect to the different histological stages. The p53 abnormalities were further studied in a panel of cell lines representative of the Barrett’s carcinogenic sequence. For 91 patients the predictive value of p53 abnormalities with respect to progression to HGD/EAC was tested after long term follow-up.

Results: The frequency of IHC and FISH p53 abnormalities increased significantly with increasing histological stage (p < 0.001, Chi²-test). Combining the techniques detected p53 abnormalities in 100% of patients with LGD, HGD and EAC. Multivariate analysis showed that IHC (hazard ratio: 17, 95% CI: 3.2 – 96, p = 0.001) and FISH (hazard ratio: 7.3, 95% CI: 1.3 – 41, p = 0.02) were both independent significant predictors of progression.

Conclusion: Combining FISH and IHC in assessing p53 abnormalities leads to an increased detection rate of p53 aberrations and improved accuracy for predicting BE progression.
INTRODUCTION

The incidence of esophageal adenocarcinoma (EAC) has been increasing at a rather high rate (1). EAC has a poor prognosis with a five-year survival rate of 10-20% (2,3). There are a variety of risk factors associated with EAC, such as male gender, obesity and smoking (4,5). One of the most recognized risk factors is Barrett’s esophagus (BE), a metaplastic change in which the normal squamous epithelium of the distal esophagus is replaced by a specialized columnar type of epithelium as a result of longstanding gastro-esophageal reflux (6). BE patients can have an 11-fold higher risk of developing EAC compared to the general population (7). Although BE is a major risk factor, the number of patients that actually progress to EAC, is estimated to be only 0.1 to 0.5% (7,8). Progression of BE is assumed to follow the histological sequence of intestinal metaplasia (IM), low grade dysplasia (LGD), high grade dysplasia (HGD), followed by EAC (6). The risk to progress to HGD/EAC increases once LGD has developed and varies between 0.4 to as high as 13.4 % (7,9,10). Histopathological staging is currently the gold standard when it comes to predicting the development of EAC in BE (5). Combined with endoscopic surveillance it is the main preventive strategy to reduce the cancer risk (11). Based on histological criteria, the surveillance frequency is determined, to improve the chance of early detection of neoplastic lesions and to initiate endoscopic treatment in case of HGD (12). This strategy is contested however, as histological grading is subject to significant intra- and inter-observer variation (13). Moreover, the progression from IM to EAC does not necessarily follow the assumed sequence of histopathological events (14). Researchers have been searching for biomarkers to strengthen or possibly replace histopathological staging. Abnormalities of the \( P53 \) gene and its associated protein are among the most frequently found aberrations in BE and EAC (14-18). \( P53 \) functions as a tumor suppressor through the stimulation of apoptosis and growth arrest upon experiencing cellular stress such as DNA damage. Its activity is often abrogated in neoplasms (19). This can occur through various mechanisms. The most common combination of events leading to complete inactivation of \( p53 \) in cancer is the functionally inactivating mutation of one allele in combination with the deletion of the other (i.e. loss of heterozygosity) (20). A clinically useful result of \( P53 \) mutation is that the mutant protein often has an increased stability, making it detectable by immunohistochemistry (IHC) as it accumulates in nuclei. Both loss of heterozygosity (LOH) of the \( P53 \) locus and \( P53 \) mutations have been shown as early events in BE progression (21,22). Previously, Reid et al, found that \( P53 \) LOH is a significant independent predictor of progression to EAC (23), while \( p53 \) protein accumulation frequencies have been shown to increase during the BE pathogenic sequence (15,18, 24-27). Recently, \( P53 \) IHC outperformed histopathological staging, showcasing a better sensitivity and specificity in HGD/EAC development prediction (28). Thus, \( p53 \) abnormalities as assessed by immunohistochemistry and LOH are potentially valuable as prognostic markers in BE surveillance programs. However, the sensitivity of these techniques for predicting progression is still generally poor (5,23,26,29). Nonetheless, IHC is a rather cheap and straightforward technique, routinely applied in pathology laboratories. The combination of IHC with another technique, which would detect larger gene aberrations, could potentially increase the accuracy of \( p53 \) as a clinical biomarker. One widely applicable method for determining gene loci losses and gains is DNA fluorescent in-situ hybridization (FISH) (30,31). Our group has shown that \( P53 \) locus loss detected by FISH on BE brush cytology
specimens is an event found in IM with a frequency that increases with successive dysplasia grades (31). Although the use of cytology alone for diagnosing dysplasia in BE is regarded to be less accurate as compared to histology (32), cytological material serves as a good source for the detection of genetic markers by DNA FISH (33). Other advantages of brush cytology include the lower cost and ability to sample a larger mucosal area when compared to taking random biopsies.

To assess whether FISH would complement IHC in detecting p53 abnormalities, we applied these two methods on a BE surveillance cohort and evaluated their combination as a novel prognostic tool in a prospective long-term follow-up study. The observed p53 aberrancies were validated with a panel of benign and malignant esophageal cell lines.

**MATERIALS AND METHODS**

**Study set-up.** The study investigated a total of 116 patients with BE who either were referred to the Academic Medical Center Amsterdam for treatment of HGD or EAC or were part of the routine surveillance program between 2002 and 2006. The Ethics Committee of the AMC approved the study and all patients signed informed consent. Only patients with proven IM in biopsies were included. All patients were on long-term proton pump inhibition of 40 to 80 mg daily to prevent reflux esophagitis. At study entry the brush cytology specimens were taken prior to the biopsies during endoscopy. Brushes of normal squamous epithelium were taken at least 3 cm above the BE segment for control purposes. Biopsies for routine histological examination were taken at least every 2 cm in 4 quadrants and from all suspected visible lesions as described by Reid et al (34).

For the follow-up study, patients underwent prospective surveillance endoscopy with random biopsies taken according to the latest ACG guidelines following the Seattle biopsy protocol (35). Only patients without a diagnosis of HGD or EAC were included in the prospective study. Endpoints of follow-up were development of HGD/EAC or reaching a follow-up of at least 3 years and all patients underwent at least one surveillance endoscopy. Patient’s clinical data were obtained from patient’s charts or via questionnaires.

**Biopsy histological assessment.** Histopathological classification of the esophageal biopsies was done as per the Vienna classification (36-38). The samples were evaluated by two pathologists of whom at least one was an expert Barrett pathologist. Upon suspicion of dysplasia, cases were reviewed by a third GI pathologist to reach consensus. At least two pathologists had to agree on dysplasia stage.

**Brush cytology.** Brush cytology was performed on patients as described earlier (31). Briefly, after spraying with acetylcysteine (50 mg/ml) to dissolve the mucus layer, the BE mucosa was brushed using the Wilson-Cook brush type LCB-220-3-1.5-S (Cook Medical, Limerick, Ireland). Brush samples from Barrett’s and normal squamous mucosa were taken from each patient.

Brushes were placed in vials containing either a solution of 5% acetylcysteine in 0.9% NaCl or ThinPrep cytopreservation (PreservCyt, Hologic, Marlborough, MA, USA). The vials were carefully mixed to obtain a homogenous cell suspension. Using the Shandon Cytospin
4 Cytocentrifuge (Thermo, Waltham, MA, USA) separate spots of normal squamous and BE epithelium derived from the same patient were put on a single slide.

**Esophageal cell lines.** One normal squamous, four Barrett’s and four EAC cell lines were used to validate the patient data. The human hTERT immortalized squamous esophageal cell line, EPC2-hTERT, a kind gift from Prof. A. Rustgi (University of Pennsylvania, PA, USA) (39), was cultured in KSFM medium (Life technologies, Bleijswijk, Netherlands) supplemented with human recombinant epidermal growth factor and Bovine pituitary extract (BPE) (Life technologies). The Barrett cell lines CPA, CPB, CPC and CPD were kindly provided by Dr. R.C. Fitzgerald (University of Cambridge, Cambridge, UK) and used with the permission of Prof. P.S. Rabinovitch (University of Washington, WA, USA) (40). CPA is derived from nondysplastic BE. The other BE cell lines were initiated from HGD. These cell lines were grown in supplemented MCD8153 medium (Sigma, St Louis, MO, USA) as described previously (41). The EAC cell lines OE19 (commercially obtained from ATCC, Manassas, VA), OE33 (ATCC) and SKG-T4 (commercially obtained from Sigma Aldrich Chemie BV, Zwijndrecht, the Netherlands) were cultured in RPMI 1640 (Life technologies), while Flo-1 (ATCC) was cultured in DMEM (Life technologies), supplemented with L-glutamine, penicillin/streptomycin and 10% fetal bovine serum (Lonza, Basel, Switzerland). Cells in PBS solution were used to make the cytopsins for the FISH analysis and cell pellets for P53 IHC.

**Immunohistochemistry.** P53 IHC was performed by the Pathology department on the biopsy specimens and cell lines. With respect to the biopsy samples, 13 were stained immediately after isolation in the period 2002-2006. Of these 7 were IM/IND, 3 were LGD and 3 were HGD. P53 staining information, as per the standard pathology department approach, for these samples was retrieved from pathology reports. The remaining samples were stained consecutively in the period 2010-2011. A three-step polymer detection system was applied on the deparaffinated tissue slides using a P53 antibody mix of clones DO-7 and BP53-12 (Fisher scientific, Landsmeer, Netherlands) and a post antibody blocking step (15 min, room temperature) followed by an anti-rabbit/mouse/rat horseradish peroxidase (HRP)- conjugated polymer (Immunologic, Duiven, Netherlands). Diaminobenzidine was used to visualize HRP activity (Dako, Glostrup, Denmark).

**P53 immunohistochemistry scoring.** Scoring was performed by an expert pathologist in an expanded form of the standard approach. Staining score was divided into 5 categories: + (very weak nuclear staining), ++, +++, ++++ (dark nuclear staining) and a “null expression” category (Figure 1), characterized by Barrett’s areas completely lacking P53 staining paired with normal squamous areas within the same section showing wild type staining. The lack of expression is assumed to be the result of nonsense mutations or a quickly degraded mutant protein. The + to +++ categories are considered P53 negative according to the pathology department standards. Only the ++++ score and the null expression category are considered as abnormal P53 positive. Scoring was done without prior knowledge of histology or FISH results. For cases with heterogeneous staining, areas with the highest focal staining determined the final score. Pictures of the tissues were taken with the DFC500 camera mounted on a DMS5000B microscope.
P53 aberrancies in Barrett’s progression prediction

P53 DNA-FISH. FISH was performed on the cytology specimens with two probes that collectively assess the P53 gene and chromosome copy number. The locus specific probe LSI p53 specifically binds to the 17p13.1 (P53) region on chromosome 17 and was directly labelled with the Spectrum Orange fluorophore. CEP17 (Abbott Molecular, Chicago, IL, USA), labelled with Spectrum Green, binds to the centromeric region of chromosome 17. The probes were obtained from Abbott Molecular.

FISH scoring. After the FISH procedure at least 100 interphase BE cell nuclei were scored per patient sample by two experienced FISH scorers to obtain ‘per cell’ counts of LSIp53 and CEP17. For scoring, The BX51 mounted with the XM10 camera (Olympus) was used to score and take images. All cases were evaluated without prior knowledge of histology or
immunohistochemistry findings. From the per cell counts, absolute and relative $P53$ loss frequencies were determined. Absolute loss refers to nuclei with a loss of the $P53$ allele (LSIp53), but without CEP17 gains (Figure 1). Relative $P53$ loss refers to nuclei with gain of CEP17 and with a CEP17/LSIp53 ratio greater than 1. In a previous study similar $P53$ loss patterns were described in Barrett cytology samples.\(^{(42)}\) Cell nuclei with two signals for each probe were regarded as normal (Figure 1). The frequency cut-off value to determine absolute $P53$ loss status was obtained from counts of 30 normal squamous epithelium cytological specimens (100 nuclei evaluated per case) and calculated as the mean percentage of squamous nuclei with absolute loss plus 3x standard deviation, which proved to be 10%. The cut off for relative loss was determined as the approximate probability of a concurrent gain of CEP17 with gain of $P53$ or wt $P53$ as calculated in the normal samples, which was 4%. A patient/cell line was considered positive for $p53$ FISH abnormality if either their absolute or relative loss frequencies were above their respective cut-offs.

**Statistical analysis.** Correlation between $p53$ locus loss and protein accumulation and association of abnormalities frequencies with histology were tested by Chi^2^-test (IBM SPSS statistics version 20.0; IBM, Armonk, New York, USA). P-values < 0.05 were considered as statistically significant. For the follow-up cohort, Kaplan Meier curves and log-rank statistics were calculated with GraphPad Prism (version 5.01, GraphPad software inc.). Spearman rank test, logistic regression and Cox proportional–hazards regression analyses were also performed with SPSS statistics software.

**RESULTS**

**Patient characteristics.** In total 116 BE patients were analyzed for IHC and FISH $p53$ abnormalities. Of these 95 were male and 21 female. The mean age was 64 (± 13) years. Median maximal length of the BE segment was 3 cm (interquartile range, IQR, 2-5 cm). The BE population included 80 IM, 13 indefinite for dysplasia (IND), 7 LGD, 6 HGD and 10 EAC cases. Other characteristics are depicted in table 1.

**$P53$ abnormalities correlate with histological stage.** The $p53$ IHC and FISH stainings (Figure 1) were overall rather heterogeneous. Of the IHC positive cases 22 (81%) had ++++ level staining and 5 (19%) showed ‘null expression’. Of the $P53$ FISH positive cases, 8 (36%) showed only absolute loss and 12 (55%) had only relative loss. Two cases were positive for both types of abnormalities. For both IHC and FISH the number of cases with $p53$ abnormalities, upon dichotomous scoring, significantly increased with histological stage (Chi^2^-trend test, p < 0.001). However, a large number of patients was detected by only one technique (Figure 2). From a total of 40 patients with $p53$ abnormalities, only 9 tested positive for both FISH and IHC. By combining the two techniques the detection rate of $p53$ abnormalities increased. Combining the two techniques led to a $p53$ aberrancy detection rate of 100% for patients with LGD, HGD and EAC (Figure 2).

**The follow-up cohort.** To investigate the predictive value of the $p53$ aberrancies, we performed prospective follow-up of the cohort from 2004 to 2011. Only patients with a diagnosis of IM/IND or LGD were included (Table 1). Of the 100 eligible patients, nine patients were lost to
Follow up (Figure 3). For the remaining 91 patients the median follow-up period was 71 (IQR 47-84) months. Eleven patients progressed during a total of 505 patient-years of endoscopic surveillance, leading to a progression incidence of 2.18 per 100 patient-years for the complete follow-up cohort. For the IM/IND group seven patients progressed to HGD/EAC during 498 years of follow up (incidence = 1.4 per 100 patient-years), of which three progressed to EAC (incidence = 0.6 per 100 patient-years). Furthermore, with respect to the complete follow-up cohort, three patients progressed from LGD to HGD and one from LGD to EAC. The EAC incidence in the complete follow-up cohort was thus 0.79 per 100 patient-years. The median time to progression for the complete follow-up cohort was 12 (IQR = 4-41) months.

Table 1. Patient characteristics for research cohorts.

<table>
<thead>
<tr>
<th>Patient characteristic</th>
<th>Complete cohort N = 116</th>
<th>Follow-up cohort n = 91</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age</td>
<td>63 y (± 13y)</td>
<td>62 y (± 13y)</td>
</tr>
<tr>
<td>Sex</td>
<td>95 (82%) male</td>
<td>77 (85%) male</td>
</tr>
<tr>
<td></td>
<td>21 (18%) female</td>
<td>14 (15%) female</td>
</tr>
<tr>
<td>Histology</td>
<td>80 (69%) IM</td>
<td>73 (80%) IM</td>
</tr>
<tr>
<td></td>
<td>13 (11%) IND</td>
<td>11 (12%) IND</td>
</tr>
<tr>
<td></td>
<td>7 (6%) LGD</td>
<td>7 (8%) LGD</td>
</tr>
<tr>
<td></td>
<td>6 (5%) HGD</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10 (9%) EAC</td>
<td></td>
</tr>
<tr>
<td>Median (maximal)</td>
<td>3 cm (IQR = 2 - 5 cm) a)SSB = 69 patients</td>
<td>3 cm (IQR = 2 - 5 cm) SSB = 53 patients</td>
</tr>
<tr>
<td>Barrett length</td>
<td>a)LSB = 47 patients</td>
<td>a)LSB = 38 patients</td>
</tr>
<tr>
<td>a)Smoking</td>
<td>56 (52%) smokers</td>
<td>47 (53%) smokers</td>
</tr>
<tr>
<td></td>
<td>52 (48%) non-smokers</td>
<td>42 (47%) non-smokers</td>
</tr>
</tbody>
</table>

a) Short segment Barrett’s (SSB) = maximal Barrett length ≤ 3cm. b) Long segment Barrett’s (LSB) = maximal Barrett length > 3cm. c) non-smokers = never smoked or stopped smoking > 15 years ago. For 8 patients information on smoking could not be recovered.

Figure 2. Patient frequency distributions of p53 abnormalities as detected by FISH and IHC per Barrett’s histological categories.
Combining FISH and IHC to predict progression. The number of patients positive for p53 aberrancies was significantly higher in the progressors than in non-progressors for both IHC (63.6 vs 7.5%, Chi2-test, p<0.001) and FISH (36.4 vs 7.5%, p = 0.02, Table 2). Also when IHC and FISH were combined, this result was observed (Table 2). However, there was no correlation between patients detected by IHC and FISH (Spearman rank test, p = 0.59).

Univariate logistic regression analyses showed that both IHC positivity (odds ratio (OR) = 21.6, 95% CI: 4.90 - 95.2, p < 0.001) and FISH (OR = 7.05, 95% CI: 1.60 - 31.1, p = 0.01) were
significantly associated with progression to HGD/EAC. Combining P53 FISH with P53 IHC positivity was associated with an OR of 25.5 (95% CI: 4.90 – 133, p < 0.001) and had a sensitivity and specificity of 81.8 and 85.0, respectively for predicting progression.

Multivariate Cox regression analysis controlling for clinical variables including age, sex, and BE length pointed out that p53 abnormalities as detected by IHC (hazard ratio, HR 17, 95% CI:3.2 – 96) and FISH (HR 7.3, 95% CI:1.3 – 41) are both independent significant predictors of progression (Table 3).

Kaplan-Meier analyses (data not shown), showed that the progression free survival was significantly poorer for patients positive for p53 IHC or FISH and for the combination of FISH/IHC (logrank test p-value were <0.001, 0.002, and <0.001, respectively).

**Esophageal cell line validation.** Our data suggest that FISH and IHC might detect different types of p53 abnormalities. To validate these observations, we analyzed p53 protein and gene expression status by IHC, and FISH, in 9 esophageal cell lines representative of the Barrett sequence with known P53 gene status (Table 4). All the cell lines analyzed, except for EPC2-hTERT and CPA, contain p53 abnormalities as indicated by the literature (Table 4). Using IHC, light to moderate P53 expression was observed in some of the EPC2-hTERT and CPA nuclei.

**Table 2.** Sensitivity, specificity and positive and negative predictive values of p53 IHC and FISH.

<table>
<thead>
<tr>
<th>P53 abnormality</th>
<th>Test neg (%)</th>
<th>Test pos (%)</th>
<th>Chi² (p-value)</th>
<th>Sens/ Spec</th>
<th>PPV/ NPV</th>
</tr>
</thead>
<tbody>
<tr>
<td>IHC non-progressor</td>
<td>74 (92.5)</td>
<td>6 (7.5)</td>
<td>&lt; 0.001</td>
<td>63.6/92.5</td>
<td>53.8/94.9</td>
</tr>
<tr>
<td>IHC progressor</td>
<td>4 (36.4)</td>
<td>7 (63.6)</td>
<td>0.02</td>
<td>36.4/92.5</td>
<td>40.0/91.4</td>
</tr>
<tr>
<td>FISH non-progressor</td>
<td>74 (92.5)</td>
<td>6 (7.5)</td>
<td>0.02</td>
<td>36.4/92.5</td>
<td>40.0/91.4</td>
</tr>
<tr>
<td>FISH progressor</td>
<td>7 (63.6)</td>
<td>4 (36.4)</td>
<td>0.02</td>
<td>36.4/92.5</td>
<td>40.0/91.4</td>
</tr>
<tr>
<td>FISH + IHC non-progressor</td>
<td>68 (85.0)</td>
<td>12 (15.0)</td>
<td>&lt; 0.001</td>
<td>81.8/85.0</td>
<td>42.9/97.1</td>
</tr>
<tr>
<td>FISH + IHC progressor</td>
<td>2 (18.1)</td>
<td>9 (81.8)</td>
<td>0.02</td>
<td>36.4/92.5</td>
<td>40.0/91.4</td>
</tr>
</tbody>
</table>

Sens = sensitivity. Spec = specificity. PPV = positive predictive value. NPV = negative predictive value.

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Significance (p-value)</th>
<th>Hazard ratio (eβ)</th>
<th>95% CI for eβ</th>
</tr>
</thead>
<tbody>
<tr>
<td>P53 IHC abnormalities</td>
<td>0.001</td>
<td>17</td>
<td>3.2 – 96</td>
</tr>
<tr>
<td>P53 FISH abnormalities</td>
<td>0.024</td>
<td>7.3</td>
<td>1.3 – 41</td>
</tr>
<tr>
<td>Male sex</td>
<td>0.30</td>
<td>0.41</td>
<td>0.075 – 2.2</td>
</tr>
<tr>
<td>age (&gt; 65 yrs)</td>
<td>0.79</td>
<td>0.82</td>
<td>0.18 – 3.6</td>
</tr>
<tr>
<td>Barrett length (&gt; 3cm)</td>
<td>0.11</td>
<td>5.8</td>
<td>0.67 – 50</td>
</tr>
</tbody>
</table>
These cells were scored negative for P53 IHC (Figure 4 A). CPB, CPC, CPD, OE33 and Flo-1 had strong nuclear P53 staining (Figure 4 B+C), while SKGT-4 showed an absence of staining reminiscent of the patient ‘null expression’ category (Figure 4D). These cells, scored positive for P53 IHC, have known mutations (Table 4). OE19 had an intermediate nuclear P53 staining pattern (Figure 4 A) and was scored as negative. It presented a mixture of different patterns of stainings, whose two separate scorings by a pathologist led to differing results. Literature showed that it contained a frame shift mutation. These data indicate that P53 mutations result in diverse patterns of staining, which could make IHC difficult to interpret.

Next, we analyzed the same benign and malignant cell lines for chromosome 17 and P53 status by FISH (Figure 5). EPC2-hTERT and CPA were considered as having normal P53 status as abnormalities of the two FISH probes were below cut-off in these lines. All other cell lines were positive for P53 FISH abnormalities (Figure 5). Of interest is that the Barrett cell lines CPB, CPC and CPD showed comparatively higher absolute loss percentages while relative loss was higher in most of the EAC cell lines, reaching the highest level (94%) in OE33. Thus, relative loss is associated with a more malignant phenotype. Here, FISH was able to identify cells bearing p53 abnormalities (OE19) that were missed by IHC.

**DISCUSSION**

To judge the ability of p53 IHC and FISH as disease outcome predictors for BE, we first investigated the frequencies of these abnormalities within the Barrett’s pathogenic sequence.

We found that FISH and IHC detected similar rates of p53 abnormalities that increased as histology worsened (Figure 2). With respect to P53 IHC, varying frequencies of 0-39% for IM/IND, 0-62% for LGD, 77-100% for HGD and 69-100% for EAC have been reported (15, 18, 26, 28, 44). Even when taking into account differences in scoring systems and histochemical
P53 aberrancies in Barrett’s progression prediction

Figure 4. Immunohistochemistry analysis of esophageal cell lines. All images were taken with the 40x objective. (a) EPC2-hTERT squamous (Sq), CPA intestinal metaplasia (IM) and OE19 cancer cell lines were scored negative for p53 IHC. (b) Esophageal high grade dysplasia (HGD) cell lines CPB, CPC and CPD were scored positive for P53 IHC. (c) OE33 and Flo-1 esophageal adenocarcinoma (EAC) cell lines were scored positive for P53 IHC. (d) SKGT-4 cancer cell line with a complete lack of p53 staining was also scored as positive for P53 IHC (null expression).

procedures, our results are in line with this trend of a low percentage of abnormalities in early BE and a high percentage at the later HGD/EAC stages. In our study all LGD patients were found to have aberrant P53 IHC. At our institute, cases for which the pathologist doubts between a diagnosis of reactive and neoplastic changes, P53 immunostaining might be used to come to a definite diagnosis, which might have contributed to a scoring bias. Contrary to IHC, few studies have applied DNA- FISH for assessing P53 aberrations. One study showed P53 loss by FISH in 76% of 21 HGD/EAC and 0% of 19 IM cases (42). The results are comparable to the overall FISH loss that we found in our HGD and EAC cases. Our IM category contained a far larger amount of patients, which may explain the higher frequency of abnormal FISH in our cohort.

Interestingly, we found little overlap between the abnormalities found by IHC and FISH (Figure 2). A large percentage of patients with HGD and EAC (> 65%) are detected by each
single technique, but combining the techniques increased this frequency up to 100% for these stages. This indicated that the techniques are complementary and merits combining both to detect more p53 aberrant cases at earlier stages and as such improve the risk stratification performance of p53.

To test the actual predictive value of p53 FISH and IHC abnormalities for progression to HGD/EAC, a prospective follow-up study was performed. In our study we found a HGD/EAC incidence of 2.18 per 100 patient-years or 2.2% for the complete follow-up cohort. The incidence of HGD/EAC in Barrett’s reported in the literature ranges from 0.26 to 1.0% per year (7, 45). The higher incidence in our cohort is likely due to the institutional referral bias which led to a higher number of increased risk LGD cases in our cohort. However, the incidence of EAC in the complete follow-up cohort was 0.79%, which is only slightly higher than the 0.63% as calculated in a recent meta-analysis (45). The results indicated that both p53 IHC and FISH positivity are independent predictors of progression (Table 3). The sensitivities for p53 FISH and IHC of 36.4 and 63.6% respectively, were rather low (Table 2). Here, the non-redundancy and complementary effect between FISH and IHC to detect p53 abnormalities was reflected.

**Figure 5.** Frequencies of P53 FISH abnormalities as detected in esophageal cell lines. CEP17 gains, P53 absolute and P53 relative losses were assessed. P53 absolute losses are higher in high grade dysplasia cell lines. P53 relative losses are higher in esophageal adenocarcinoma cell lines.
Figure 6. Proposed p53 sequence of abnormalities that associate with malignant Barrett’s disease.

PS3 mutations occur in the malignant Barrett’s cells and result in mutant forms of the protein that can be detected by IHC as either an accumulating long-lived or truncated non-staining ‘null expression’ form. Genetic instability resulting from these PS3 mutations leads to a loss of PS3 alleles. The resulting cells can be characterized by FISH as cells carrying absolute losses of PS3. Depending on which chromosome has endured the loss, one type of cell will carry a single wild type PS3 and the other a single mutated version of the allele. Further genetic instabilities can lead to aneuploidy, as measured by CEP17 gains. These cells will be characterized by FISH as PS3 relative losses. Only cells carrying the mutated form of the PS3 gene will also be identified by IHC.

in an improved combined Barrett’s progression prognostic test as observed in the increase of the sensitivity to 81.8 % with respect to the individual methods. The combination also led to a slight reduction of the specificity to 85% compared to the separate techniques (Table 2). The complementary effect of the techniques was also highlighted upon closer analysis of the 11 progressors. Of these, 5 (45%) were detected solely with IHC, 2 (18%) solely by FISH and 2 (18%) by both techniques. Two progressors were missed by both techniques. These two patients did not share any overt specific patient characteristics that might explain them being missed by both techniques. Recently, Kastelein et al, found that BE patients with PS3 IHC abnormalities had a relative risk of 6.4 (95% CI: 3.6-11.3) to progress to HGD/EAC. This relative risk further increased when they combined a LGD diagnosis with PS3 IHC, which is similar to our data, although in their study PS3 IHC had a lower sensitivity (28). Most studies reporting on the predictive value of PS3 abnormalities in BE, applied a single methodology, which may have led to an underestimation of the number of aberrant cases (23-25,28). This approach is likely insufficient for accurate risk stratification and different techniques should
be combined (5,46). This is reflected within our results by the improvement of the test statistics upon combining IHC and FISH.

We used the esophageal cell lines to further investigate the additive value of p53 IHC and FISH with respect to Barrett’s progression prediction. These studies showed that some mutations, such as in OE19 (Table 4), can lead to truncated mutant P53 that could be missed by IHC if one is not familiar with the staining pattern, which corresponds with the literature (47,48). The genetic instability that results from P53 mutations in turn may lead to loss of P53 alleles and thus the high percentage of LOH that is present in the more malignant Barrett’s stages (21,23). This loss of P53 loci, missed by IHC, can be detected by FISH, as exemplified with our cell line results. Inversely, P53 mutations detected by IHC can be missed by FISH if they do not lead to gross chromosomal aberrancies. Interestingly, whilst Barrett dysplastic cells were enriched for P53 absolute losses, EAC cell lines contained a higher percentage of relative losses (Figure 5). This indicated an increasing genetic instability, i.e. the chromosomal gains detected through relative losses, with increasing histological malignancy. A model as to how p53 aberrations might develop during Barrett’s disease and how FISH and IHC might detect or miss these abnormalities is depicted in figure 6.

Our findings undermine the use of P53 IHC as a single progression prediction technique. Several other methods could be used to compliment IHC. In additional cell line experiments we observed that Western blot analysis is able to differentiate between wt or mutant P53 (data not shown). However, this technique is less likely to be utilized in clinical practice as it is rather labour intensive. Our “two pronged” P53 FISH analysis can be more easily and quickly applied to routine diagnostic practice. By combining two probes, CEP17 and LSIp53, we are able to detect P53 relative in addition to absolute losses. A high percentage of relative losses was observed in malignant tissues and cells indicating a high risk prediction potential.

Concluding, we set out to compare P53 IHC, an established candidate tool in Barrett’s disease progression prediction, with a FISH method to detect P53 loss. We discovered that the two tests were complementary prognostic tools. The combination of both techniques, leads to an improvement of their independent sensitivities and a more accurate predictive test that can be used for risk stratification of Barrett’s patient.

REFERENCE LIST

3. P53 aberrancies in Barrett’s progression prediction


