

Expression of p53 as predictor for the development of esophageal cancer in achalasia patients

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SUMMARY. Patients with longstanding achalasia have an increased risk of developing esophageal cancer. Surveillance is hampered by chronic stasis. We investigated whether aberrant expressions of tumor suppressor gene p53 and proliferation marker ki67 are early predictors for progression to malignancy. In 399 achalasia patients, 4% died of esophageal cancer despite surveillance. We performed a cohort study, using surveillance biopsies from 18 patients (11 carcinoma, one high-grade dysplasia [HGD], and six low-grade dysplasia [LGD]) and 10 controls (achalasia patients without cancer or dysplasia development). One hundred sixty-four biopsies were re-evaluated and studied for p53 and ki67 expression using immunohistochemistry. Eighty-two percent of patients with cancer/HGD showed p53 overexpression in surveillance biopsies at a mean of 6 (1–11) years prior to cancer development. In 67% of patients with LGD and only in 10% of the controls p53 overexpression was present. The proportion of samples with p53 overexpression increased with increasing grades of dysplasia. We found no difference for ki67 overexpression. p53 overexpression may identify achalasia patients at increased risk of developing esophageal carcinoma. Further study is needed to determine if patients with p53 overexpression would benefit from intensive surveillance to detect esophageal neoplasia at a potential curable stage.

KEY WORDS: achalasia, esophageal cancer, surveillance, p53, immunohistochemistry.

INTRODUCTION

Achalasia is a motor disorder of the esophagus characterized by aperistalsis of the distal esophagus in combination with absent or impaired relaxation of the lower esophageal sphincter (LES). This causes symptoms of esophageal obstruction. It is an uncommon disorder with an annual incidence of one per 100 000 in the Western world and an equal prevalence in men and women with a peak incidence around 60 years of age.¹ Chronic food stasis (often present despite LES pressure lowering treatment) has been suggested to lead to chronic inflammation, epithelial hyperplasia, multifocal dysplasia, and squamous cell carcinoma (SCC).^{2–4} On the other hand, LES-pressure lowering therapy by balloon dilatation or

surgical myotomy may lead to chronic gastro-esophageal reflux, which in some cases is ultimately complicated by the development of Barrett's esophagus (BE) and esophageal adenocarcinoma (AC).^{5–8}

Achalasia is considered to be a premalignant disorder. The reported risks for esophageal carcinoma vary widely between different studies. Several autopsy studies have reported an esophageal neoplasia prevalence of over 20% in achalasia patients at the time of death.^{9–11} Wychulis *et al.* conducted the largest cohort follow-up study in 1318 patients with achalasia followed for a mean of 17 years. This study revealed a sevenfold increased risk for esophageal cancer compared with the general population.¹² Other follow-up studies reported esophageal cancer incidences varying from zero per 953 patient-years to 1 per 173 patient-years, which translates into relative risks varying from zero to 140 times increased compared with a sex- and age-adjusted population.^{13–15}

Surveillance should aim at detection of neoplastic transformation at an early, curable stage, as well as at

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the identification of patients at the highest risk of developing neoplasia. These are in particular those patients, who have persistent severe inflammation or food stasis despite LES pressure-lowering therapies and those with BE. Unfortunately, surveillance in achalasia is difficult to perform because of stasis and mucosal adherence of food, which compromise a careful inspection. Besides, most carcinomas develop in the middle and distal third of the esophagus and therefore, in contrast to BE, the whole length of the esophagus should be carefully inspected and sampled.¹⁶ Finally, histological evaluation of esophageal surveillance biopsy samples may be hampered by the persistent presence of chronic inflammation.

For these reasons, markers for the identification of patients at an increased risk of developing malignancy are needed. We therefore investigated whether expression of the tumor suppressor gene p53 and proliferation marker Ki67 are early predictors for progression to malignancy and are able to identify those patients with achalasia who are at highest risk of developing esophageal cancer. These markers were used earlier to predict malignant transformation in Barrett's metaplasia.^{17,18}

MATERIAL AND METHODS

Patients

Since 1975 all patients with achalasia referred to our hospital have been treated and followed according to a strict protocol. The total cohort consisted of 399 patients (male/female 198/201), who have been followed for a mean of 8.1 years (0–25.9) after diagnosis. The diagnosis and treatment protocol did not change over time and patients were taken care of by a selected number of physicians over the complete period of follow-up of the cohort. All patients were first treated with repeated pneumatic dilatation on three consecutive days using Rigiflex balloons (Boston Scientific, Natick, MA, USA) with increasing diameter (30/35/40 mm). Recurrent symptoms were treated by repeat dilatation. Patients with an early (within 3 months) or repeated recurrence (after three to four dilatation sessions) of symptoms were referred for Heller myotomy in combination with a Dor fundoplication.

Surveillance endoscopies were performed at least every 3 years. During surveillance a careful inspection of the esophagus was performed and random biopsy samples were taken from the distal part of the esophagus, 1–2 cm above the presumed LES. Suspicious areas were separately sampled. If food stasis compromised clear vision, patients were asked to return after a 3-day liquid diet. Patients who refused surveillance endoscopies or were considered too old to undergo surveillance were followed by phone calls at regular intervals.

Twelve achalasia patients (male/female, 7/5) who developed esophageal cancer or high-grade dysplasia (HGD) were included in the current study. In addition 10 achalasia patients matched for age, gender and duration of achalasia but who did not develop esophageal cancer served as controls. Six patients, who developed low-grade dysplasia (LGD, three in BE) during follow-up, were also included in this study.

Histology and immunohistochemistry

From the patients who developed esophageal carcinoma or dysplasia all available samples and from the control patients the five most recent biopsy samples were re-evaluated for the presence of BE, dysplastic changes (LGD or HGD) and carcinoma (AC or SCC) by an expert GI pathologist (H. van Dekken) who was blinded for the clinical information. The available paraffin blocks of these samples were retrieved from the archive and studied for p53 and ki67 expression using immunohistochemistry.

Four-µm tissue sections of the formalin-fixed, paraffin wax-embedded samples were sliced and mounted on adhesive slides, dried and deparaffinized with xylene and rehydration through graded ethanol. Antigen retrieval was performed by boiling these samples for 15 minutes in 10 mM monocitric acid buffer (pH 6.0) for ki67 staining and in 10 mM Tris/ethylenediaminetetraacetic acid buffer (pH 9.0) for p53 staining. Prior to immune staining, endogenous peroxidase activity was blocked by incubating the slides in a 0.5% solution of H₂O₂ in phosphate-buffered citric acid for 15 minutes at room temperature. Samples were 3 times washed with TRIS-buffered saline (TBS) with a pH of 7.4. The samples were incubated in TBS buffer containing 10% rabbit non-immune serum (DAKO, Glostrup, Denmark) and 10% normal human plasma (and 5% bovine serum albumin [BSA] for p53 staining) for 20 minutes. Sections were incubated with the primary antibody anti-human ki67 antigen (clone MIB-1, DAKO) in a 1 : 100 dilution or p53 antigen (clone DO-7, DAKO) in a 1 : 100 dilution for 14 hours at 4°C. Subsequently, biotin-labelled rabbit-anti-mouse antibody (DAKO) was used as second antibody followed by the addition of a streptavidin-horseradish peroxidase complex (DAKO). To detect ki67 and p53, 3-amino-9-ethylcarbazole was used as substrate. Two independent researchers who were blinded to the clinical data counted at least 300 nuclei in every sample. Only longitudinally sectioned squamous and Barrett's epithelium was evaluated. Cells were counted as positive when moderate to intense red nuclear staining was found. An isotype and a negative control staining were performed. Samples were considered p53 and ki67 positive if more than 15% of nuclei were stained (Fig. 1). When the counts of both

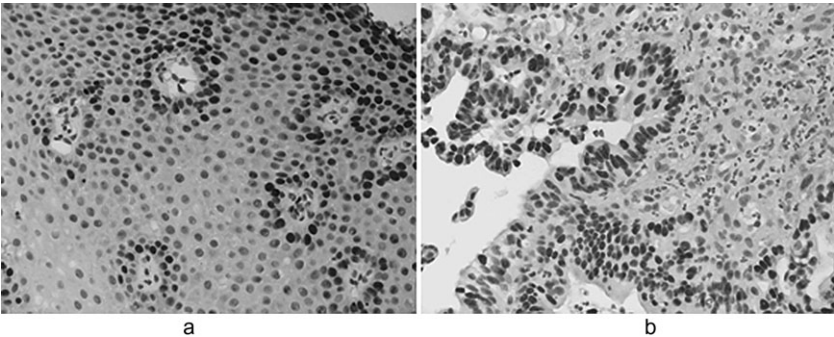


Fig. 1 Examples of ki 67 (a) p53 (b) overexpression in esophageal biopsy samples of patients with achalasia.

researchers on a specific sample differed more than 10%, both researchers counted the sample again. When there was still at least a 10% difference a third researcher counted the sample for a final classification (negative/positive).

Statistics

Statistical analyses were conducted using SPSS software (SPSS version 13.0, Chicago, IL, USA). Chi-square tests and *t*-tests were used to analyse the results of ki67 and p53 expression. The hazard ratio to develop carcinoma or HGD was calculated using Cox regression analysis, with and without adjustment for histology, using age as the time-axis and p53 status as a time dependent variable. The time course of p53 expression of the carcinoma patients in time was visualised using cubic spline functions in S-plus 6.0 software (Insightful Inc., Seattle, WA, USA).

RESULTS

Patients

From 12 cancer cases (eight SCCs, three ACs, and one HGD), 65 follow-up biopsy samples, obtained prior to the development of malignancy, were available. The mean age of these patients at achalasia diagnosis was 62.5 years (range 33–80 years) and they were followed-up for a mean of 22 years (range

9–33 years) after the onset of achalasia symptoms. One patient who developed HGD during follow-up and underwent esophageal resection was included in this group. In addition six patients (mean age at diagnosis 50 years [range 28–62 years] and followed-up for a mean of 20 years [range 5–44 years] who developed LGD during follow-up) were also selected and included. Ten patients with a mean age of 46 years (range 17–75 years) and a mean follow-up of 21 years (range 14–28 years) after start of achalasia symptoms and at least five available samples served as controls. Patient characteristics are listed in Table 1.

Histology

One hundred sixty-four samples from 28 patients were re-evaluated. All three patients with AC who had undergone an average of 5 (range 2–8) endoscopies with biopsy sampling during follow-up displayed BE in biopsies taken prior to cancer development, whereas only one patient showed LGD and HGD prior to cancer development.

From eight patients who had undergone an average of 5.4 (range 3–10) endoscopies with biopsy sampling during follow-up, who developed SCC, only three patients were diagnosed with LGD or HGD in the prior biopsies. One patient already displayed LGD, one also HGD and in one patient some of previous biopsies showed BE and/or LGD.

Table 1 Patient characteristics

	Esophageal cancer/high grade dysplasia patients (<i>n</i> = 12)	Low grade dysplasia patients (<i>n</i> = 6)	Control patients (<i>n</i> = 10)
Squamous cell carcinoma	8		
Adenocarcinoma	3		
Low-grade dysplasia		6	
High-grade dysplasia	1		
Barrett's metaplasia	4	3	0
Male/female	7/5	4/2	6/4
Age of diagnosis	63 (33–80)	50 (28–62)	46 (17–75)
Duration of symptoms till	23 (9–33)	Dysplasia: 11 (4–23)	21 (14–28)
carc/dysplasia/end FU		End FU: 20 (5–44)	
Age of carcinoma/end FU	74 (38–88)	65 (53–76)	63 (30–84)

FU, follow up.

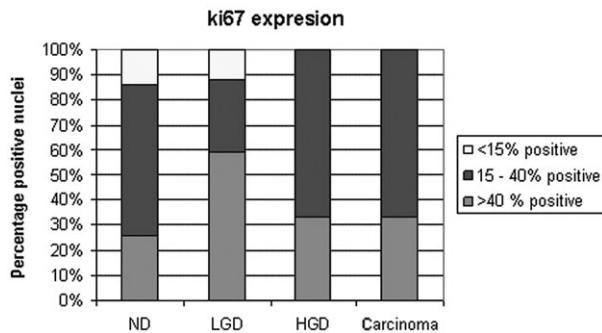


Fig. 2 Number of ki 67 positive nuclei in esophageal biopsies without dysplasia (ND), with low-grade dysplasia (LGD), with high-grade dysplasia (HGD), and with carcinoma.

In the patient with HGD, this dysplasia developed in previous noticed BE with already LGD in two earlier samples.

Six LGD patients had undergone an average of 6.5 (range 3–10 per patient) endoscopies with biopsy sampling during follow-up. Three of them also had BE in the biopsy samples prior to and together with dysplasia development. In three patients LGD was present in some of the consecutive samples, but this was no longer found in samples taken at a later time point during follow-up. The other three patients showed LGD in the more recent samples, obtained at the end of follow-up.

Ten controls had undergone biopsy sampling during their follow-up with an average 5.6 (range 5–7 per patient) endoscopies. Dysplasia and metaplasia were not present in any of these samples upon blinded revision.

Ki67 expression

Ki67 expression was assessed in 135 samples from 28 achalasia patients (Fig. 2). Eighteen paraffin blocks were missing in the archive and 14 slides were empty or not assessable because of background staining. Ki67 expression was positive in 45/51 (88.2%) control patient samples, 26/30 (86.7%) samples from dysplasia patients, and 47/54 (87.0%) esophageal cancer or HGD patient samples. Chi-square testing showed no statistically significant differences between these groups ($P = 0.97$). In addition the proportions of positive nuclei were not significantly different between the three groups (87% in cancer and HGD cases, 86.7% in LGD cases and 88.2% in controls) ($P = 0.38$ cancer compared with controls; $P = 0.69$ LGD compared with controls).

p53 expression

p53 expression was determined in 133 samples from all 28 achalasia patients (Fig. 3). In the control group, 2/48 (4%) biopsy samples (both from one patient)

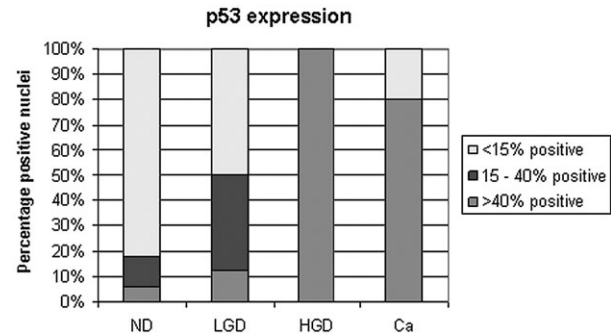


Fig. 3 Number of p53 positive nuclei in esophageal biopsies without dysplasia (ND), with low-grade dysplasia (LGD), with high-grade dysplasia (HGD), and with carcinoma.

showed p53 overexpression. These biopsies had been taken during the last follow-up endoscopy in this patient. In patients who developed LGD 11/37 (29.7%) biopsy samples were positive (in 67% of patients). In patients with esophageal cancer or HGD, 25/48 (52.1%) biopsy samples were positive (in 82% of patients). The first positive sample had been obtained at a mean of 6 years (range 1–11 years) prior to cancer development (Fig. 4). The proportion of biopsy samples with p53 overexpression increased with increasing grades of dysplasia (19/103 [18%] in no dysplasia), 8/16 (50%) in LGD, 3/3 (100%) in HGD, and 8/10 (80%) in AC and SSC ($P < 0.001$).

Of all 37 p53 positive samples, 18 (49%) also showed dysplasia or carcinoma.

Correcting for the number of samples per patient and age this indicates a hazard ratio (HR) of 8.0 (95% confidence interval [CI] 1.6–41) for p53 positive patients to develop carcinoma in comparison with p53 negative patients ($P = 0.013$). The HR for patients with LGD to develop carcinoma compared with patients without LGD was 0.98 (95% CI 0.3–0.4) ($P = 0.97$).

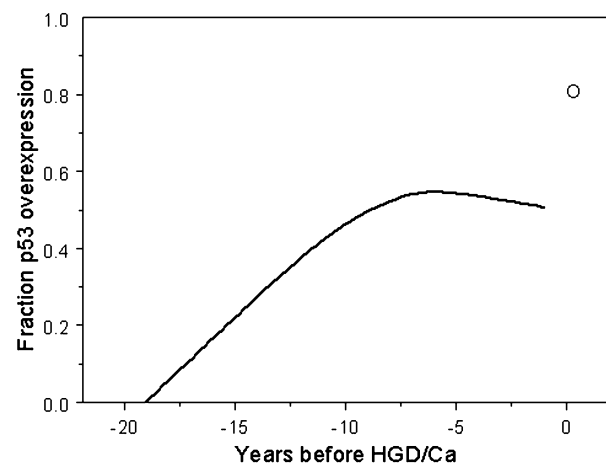


Fig. 4 Fraction of patients with a positive p53 sample in the years prior to high-grade dysplasia (HGD) or cancer development.

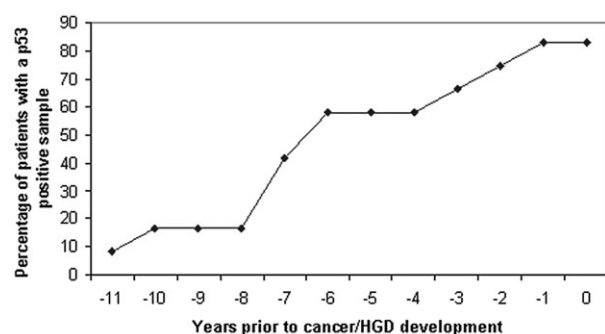


Fig. 5 Overexpression of p53 in esophageal biopsy samples from achalasia patients taken some years prior to high-grade dysplasia (HGD) or cancer development. At $t = 0$ (presence of HGD or esophageal cancer) the fraction of p53 overexpression is 83%.

The gradual increase over time in p53 overexpression is shown in Figure 5. From this, it can be seen that p53 overexpression increased at time points closer to the development of esophageal cancer. Moreover, p53 overexpression was already present at a mean of 6 years prior to cancer development at a time when there were no endoscopic signs of progression towards neoplasia.

DISCUSSION

Patients with achalasia have a significantly increased risk of developing of esophageal cancer, both SCC and adenocarcinoma. This is thought to be related to chronic food stasis and impaired esophageal clearance, which often persists after dilatation treatment. In this study we therefore focused on both tumor types. Achalasia patients with esophageal cancer often have an even more dismal prognosis than other esophageal cancer patients. This is at least partly because of the fact that these patients are used to symptoms of impaired food passage and often report worsening of their symptoms at a stage of advanced malignancy.¹⁹ This warrants the consideration for surveillance endoscopies in these patients. However the benefit of surveillance endoscopies in achalasia is disputed because a diagnosis of neoplasia in achalasia at an early and therefore presumably curable stage is often difficult. The findings of this longitudinal analysis show that easily to apply biomarkers such as p53 can be used for the detection of patients at an increased risk of neoplastic progression.

Almost all patients with achalasia, with or without future development of HGD or esophageal cancer, showed overexpression of ki67 in biopsies taken from the esophagus at time points before macroscopically or microscopically presence of neoplastic changes. Consequently, this marker was found to be not discriminative between patients at risk or those not at risk for the development of esophageal cancer in achalasia.

The human ki67 protein is present during all active phases of the cell cycle (G1, S, G2, M), but is absent in resting cells (G0). Although some of its features have been characterized, such as phosphorylation and transport to the cell nucleus for expression to become evident, the exact function of the ki67 protein is still largely unknown. Expression of the ki67 protein is strictly associated with cell proliferation. The fraction of ki67 positive cells has been demonstrated to correlate with the clinical course of the neoplastic disorder.²⁰ Our results confirm the previously reported presence of hyperproliferation in the esophageal mucosa of achalasia patients.³ It can be assumed that this hyperproliferation is one of the risk factors for development of LGD, HGD and esophageal cancer in these patients with achalasia. The hyperproliferation is likely caused by the commonly observed food stasis and fermentation in the distal part of the esophagus in these patients. To test this hypothesis, biopsies taken at higher levels in the esophagus should be examined assuming that distally more stasis and therefore, more hyperproliferation is present. In the current study, surveillance biopsies were always taken 1–2 cm above the GE-junction. Another problem with ki67 expression is the presence of inflammation in esophageal samples of achalasia patients. Inflammation causes to extend ki67 expression to upper layers of the epithelium. This can be another explanation for the high number of ki67 positive samples.

Overexpression of p53 was more frequently observed in achalasia patients who developed esophageal cancer at a later time point. Of the 12 investigated esophageal cancer or HGD patients, only five displayed histological evidence of dysplasia prior to cancer/HGD development, whereas 10 patients already showed p53 overexpression at an earlier stage (Fig. 5) Most importantly, this overexpression was already noted at a mean of 6 years prior to cancer development and was a significant predictor for progression towards neoplasia (HR 8.0, 95% CI 1.6–41). The hazard ratio was corrected for the fact that the number of samples differed between patients and that the patients in the control group were younger. It is important to stress that the presence of LGD did not affect this ratio.

The p53 tumor suppressor gene is located on the 17p13 chromosome and is involved in controlling cell proliferation. Normally, cells contain low levels of wild-type p53 that regulates two common responses to oncogenic stress, i.e., cell cycle arrest/DNA repair and apoptosis. In cells that are early in the G1-phase, p53 triggers a checkpoint blocking further progression through the cell cycle, allowing the damaged DNA to be repaired before the cell enters the S-phase.²¹ If the DNA damage cannot be repaired, p53 induces apoptosis.²² This suggests that failure of p53 to respond to DNA damage will increase the susceptibility to oncogenic changes.

Mutated p53 is dominantly negative and it overwhelms the wild-type protein and prevents it from functioning.²³ These p53 mutations are associated with an increased half-life of the p53 protein, resulting in the accumulation in the cell nucleus to levels that can be detected by immunohistochemistry.²⁴ In contrast, wild-type p53 has a short half-life and, as a consequence, these proteins do not accumulate and are mostly below the threshold of being detected by immunohistochemistry.²⁵ Approximately 90% of p53 mutations are point mutations.²⁶

Although immunohistochemistry for detecting mutated p53 is cheap, quick and easy to apply compared with other techniques, there are some additional limitations that are important to consider. The p53 antibodies that are commonly used do not only stain the mutant p53 mutation but probably also the wild-type.²⁵ A second limitation of p53-based immunohistochemistry is that mutations for this tumor suppressor gene may exist without protein overexpression, and therefore will not be detected by immunohistochemistry.^{25–27} As with ki67 expression, presence of inflammation can influence p53 evaluation. It is important to stress that only intense red cells were considered p53 positive.

Despite these limitations, our results suggest that p53 is an early marker of neoplastic progression in achalasia.

In the current study, we controlled for duration of disease; however, the three patient groups differed with respect to age, with esophageal cancer patients being the oldest at the time of achalasia development and the control patients being the youngest. The most likely explanation for this is that we selected on outcome (cancer, LGD, no dysplasia), which is related to age. In the reported hazard ratio of 8.0 a correction for age was made.

In conclusion, this longitudinal cohort study suggests that p53 but not ki67 can well be used to identify achalasia patients at the highest risk of developing malignancy. Consequently, achalasia patients with p53 overexpression may benefit from a more intensive surveillance interval and possibly the use of newer imaging techniques such as high resolution endoscopy or chrome endoscopy to detect neoplastic changes at an earlier and therefore potentially curable stage. This should, however, be studied in a prospective follow-up study.

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