EUROPEAN JOURNAL OF CANCER 45 (2009) 736–740

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Short Communication

Frequency of the mitochondrial DNA 4977bp deletion in oesophageal mucosa during the progression of Barrett's oesophagus

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ARTICLE INFO

Article history:
Received 17 November 2008
Received in revised form
6 January 2009
Accepted 13 January 2009
Available online 9 February 2009

Keywords:
Barrett's oesophagus
Oesophageal cancer
Mitochondrial DNA
Sequence deletion
Biomarker

ABSTRACT

Purpose: The mechanisms of the progression of Barrett's oesophagus (BO) to oesophageal adenocarcinoma (OA) are poorly understood. The frequency of the 4977bp deletion in mitochondrial DNA (mtDNA) was investigated in specimens ranging from normal oesophageal tissue to OA in order to investigate whether this deletion represents a useful biomarker of disease progression.

Methods: The presence of the 4977bp deletion was screened by PCR amplification from 70 specimens in total.

Results: The frequency of specimens with the 4977bp deletion increased in relation to the degree of dysplasia (8.3% in normal squamous epithelium; 15.4% in BO; 40% in low grade dysplasia (LGD); 69.2% in high-grade dysplasia and 90% in para-tumoural tissue). However, the frequency of the deletion reduced sharply in OA specimens (16.7%; $p < 0.001$).

Conclusion: The mtDNA 4977bp deletion may be useful as a biomarker to detect the severity of dysplasia but not the presence of OA.

1. Introduction

Cancer of the oesophagus is the eighth most common cancer in the world, being responsible for ~4% of new cancer cases every year.[1] Oesophageal adenocarcinoma (OA) has the fastest increasing incidence of any tumour in the Western world with a dismal 5-year survival rate of around 10%.[2]

The most common risk factor for OA is chronic gastro-oesophageal reflux disease (GORD), which is associated with an approximately 16-fold increased risk of OA, and occurs in as many as 60% of patients in whom this tumour is diagnosed.[3] Oesophageal squamous epithelium damaged by GORD may be replaced by Barrett's mucosa, a mosaic of metaplastic epithelium of gastric, intestinal, colonic or pancreatic types. The presence of Barrett's oesophagus (BO) confers a

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0959-8049/$ - see front matter
doi:10.1016/j.ejca.2009.01.013

0.5% per year risk of the development of OA.[4] The progression of BO to OA develops through established histologic changes: intestinal metaplasia (BO) to low grade dysplasia (LGD) to high-grade dysplasia (HGD) to OA. The presence of HGD confers a risk ranging from 25% to 80% of developing OA.[5–8]

The molecular and cellular mechanisms responsible for the development of BO and its conversion to neoplasia are not fully understood. If molecular markers of disease progression can be identified in oesophageal tissue obtained during endoscopic biopsy, this could lead to improved early detection and provide objective criteria for the selection of patients who might benefit from aggressive surgical management.

Alterations in mitochondria and in mitochondrial DNA (mtDNA) have been implicated in the modulation of the tumourigenic phenotype.[9,10] Various deletions of the mitochondrial genome have been reported of which the 4977bp deletion appears to be the most widespread. We hypothesised that the presence of the 4977bp deletion in mtDNA might represent an useful biomarker of oesophageal tissue that has been exposed to a potential mutagenic environment and is at the risk of progressive transformation towards malignancy. We therefore investigated the frequency of the deletion in a spectrum of specimens ranging from normal oesophageal tissue to OA.

2. Materials and methods

2.1. Tissue specimens

Formalin fixed, paraffin-embedded tissue sections of normal, metaplastic, dysplastic and malignant oesophageal tissue of anonymised patients who had undergone diagnostic endoscopic biopsies were obtained. All specimens were examined by a single consultant pathologist, and were classified according to the Vienna classification system.[11]

Fresh tissue samples from patients with histologically confirmed OA were obtained at the time of surgery. Following surgical resection, paired specimens of tumour and adjacent normal mucosa were identified and isolated by a consultant pathologist and were subsequently frozen.

All samples were obtained with appropriate local ethical committee approval, and informed consent was obtained from recruited patients.

2.2. DNA extraction

DNA was extracted from formalin fixed, paraffin-embedded tissue sections using the MagneSil Genomic, Fixed Tissue System (Promega, Southampton, UK) according to the manufacturers' instructions. The Wizard Genomic DNA purification kit (Promega, Southampton, UK) was used to extract DNA from fresh, frozen tissue samples. DNA concentration and quality was quantified by absorbance readings taken at 260 and 280 nm using an Ultrospec 2000 UV/visible spectrophotometer (Pharmacia Biotech, Bucks, UK).

2.3. Analysis of the mitochondrial 4977bp deletion

The method employed used two sets of primers, which span the common deletion described by Maximo and colleagues.[12] The MITIN primers (forward: 5′-ctgagccttttaccactccag-3′; reverse: 5′-ggtgattgatactcctgatgcg-3′) were used to amplify a 142bp fragment in a rarely deleted region as an internal control in each sample. The MITOUT primers (forward: 5′-cccaactaaatactaccgtatgg-3′; reverse: 5′-ggctcaggcgtttgtgtatgat-3′) were used to amplify a 214bp sequence created by the deletion (Fig. 1). Cycle conditions were as follows: an initial denaturation step of 95 °C for 15 mins; 40 cycles of 94 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s and a final extension step of 72 °C for 2 min. Products were resolved on a 1.6% agarose gel w/v, stained with ethidium bromide and visualised under UV illumination.

2.4. Statistics

Fisher's exact test was used to examine the association between the frequency of mitochondrial deletion and type of specimen. Logistic regression was used to determine hazard ratios for the 4977bp deletion in various specimen types compared with NSE. p values <0.05 were regarded as statistically significant. 95% confidence intervals were reported for hazard ratios. Statistical analysis was performed using SPSS v13.0 (Chicago, IL, United States of America (USA)).

3. Results

12 NSE, 13 BO, 10 LGD, 13 HGD and 2 OA formalin fixed, paraffin-embedded tissue sections were obtained along with 10 OA and 10 para-tumoural fresh frozen tissue specimens. Seventy specimens were analysed in total. The characteristic internal control PCR product of 142bp (using MITIN primers) was detected in all analysed samples.

An increase in frequency of the 4977bp deletion was observed in relation to the degree of dysplasia. The 4977bp deletion was observed in 8.3% (1/12) of NSE specimens, 15.4% (2/13) of BO specimens, 40% (4/10) of LGD specimens, 69.2% (9/13) of HGD specimens and 90% (9/10) of para-tumoural tissue specimens. However, the frequency of the 4977bp deletion

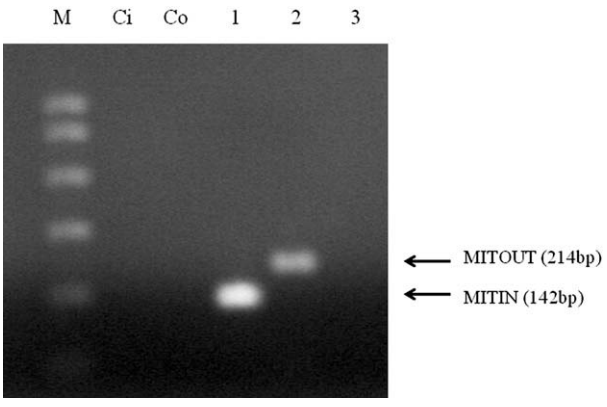

Fig. 1 – A typical gel. M: markers. Lanes Ci and Co are the negative control lanes for MITIN and MITOUT PCRs. Lane 1 shows the 142bp PCR products derived from using the MITIN internal control primers. Lanes 2 and 3 show the presence and absence of the mitochondrial deletion, respectively (214bp).

reduced sharply in adenocarcinoma specimens with only 16.7% (2/12) displaying the mutation (Fig. 2).

The association between the presence of the 4977bp deletion and the specimen type was statistically significant (Fisher's exact test, $p < 0.001$).

The hazard ratio of each specimen type for the presence of the 4977bp deletion was compared in relation with NSE. HGD and para-tumoural tissue were shown to have significantly greater odds of having the 4977bp deletion compared with NSE (Table 1).

4. Discussion

Carcinogenesis is a long-term, multistep process driven by genetic and epigenetic changes in susceptible cells, which gain a selective growth advantage and undergo clonal expression.[13] Somatic mutations in mtDNA have been implicated in this process in a variety of human tumours.[14–17] Interestingly, mtDNA alterations have been observed even at the earliest histologic stages of the multistep progression model of OA, namely, in Barrett's mucosa without evidence of dysplasia.[18]

mtDNA-deletion mutations have been shown to be implicated in the ageing of tissues especially the 4977bp deletion.[19,20] We observed that the 4977bp deletion increases in frequency in correlation with severity of dysplasia. However, in adenocarcinoma, the presence of the deletion was less frequent. Of note, similar phenomena have been observed in thyroid, renal, hepatocellular, breast and non-melanoma skin cancers, in which the mtDNA 4977bp deletion is less abundant and less frequent in tumour tissue compared with adjacent non-tumoural tissue.[21–24] This suggests that there may be an active selection pressure against the presence of mtDNA with the 4977bp deletion, and/or a population of tumour cells do not undergo the typical ageing response and subsequently form the tumour.

Alternatively, Brandon and colleagues have hypothesised that mtDNA mutations involved in carcinogenesis fall into two main classes: (i) severe mutations that inhibit oxidative phosphorylation (OXPHOS), increase reactive oxygen species (ROS) production and promote tumour cell proliferation, and (ii) milder mutations that may permit tumours to adapt to new environments.[25] The former, or tumourigenic mutations, are thought to inhibit the electron transport chain (ETC) resulting in a marked increase in mitochondrial ROS. The mtDNA 4977bp deletion, which is known to disrupt mitochondrial complexes I, IV and V on the ETC resulting in significantly elevated mitochondrial ROS and depolarised mitochondrial membrane potential[26], is an example of such a mutation.

The increased mitochondrial ROS may facilitate cellular transformation by acting as both a tumour initiator, mutagenising proto-oncogenes into oncogenes, and as a tumour promoter, stimulating the cell to start replicating, which are advantageous in the initial phases of tumour growth. In the later phases of tumour development, when the tumour becomes vascularised and/or metastasised, it may be more advantageous for the established tumour cells to revert to a more oxidative metabolism thus leading to a selective loss of tumourigenic mutations[25] such as the mtDNA 4977bp deletion. As yet there is a little evidence to support these hypotheses.

Based on our understanding of the development of OA, current clinical practice advocates serial endoscopies and biopsies to identify dysplasia and cancer in patients with BO.[27] However, there is the potential of sampling error with

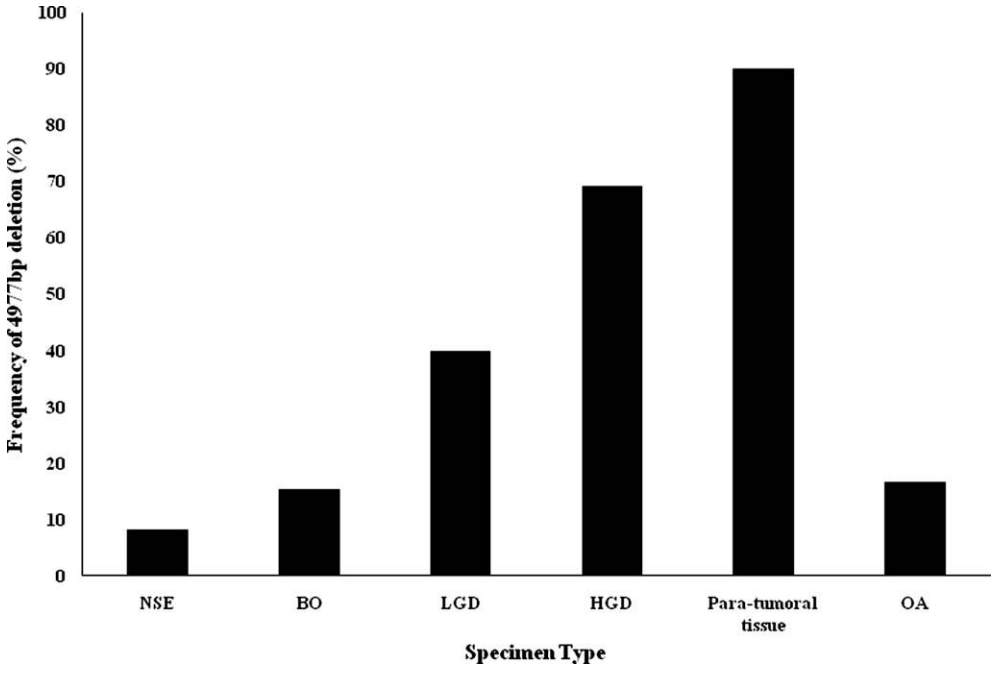

Fig. 2 – Frequency of the mitochondrial 4977bp deletion in normal squamous epithelium (NSE), Barrett's oesophagus (BO), low grade dysplasia (LSE), high-grade dysplasia (HSE), para-tumoural tissue and oesophageal adenocarcinoma (OA) specimens.

Table 1 – Hazard ratio for the presence of the mitochondrial 4977bp deletion.

	Hazard ratio	95% CI	P*
Barrett's oesophagus *versus* NSE	2.00	0.16–25.40	0.593
Low grade dysplasia *versus* NSE	7.33	0.66–81.37	0.105
High-grade dysplasia *versus* NSE	24.75	2.33–262.59	0.008
Para-tumoural tissue *versus* NSE	99.00	5.40–1814.47	0.002
Adenocarcinoma *versus* NSE	2.20	0.172–28.14	0.544

NSE = Normal squamous epithelium.
* Simple logistic regression.

endoscopic biopsies, and the interpretation of dysplasia is subject to interobserver variability.[28,29] Our study suggests that the mtDNA 4977bp deletion may be useful as a biomarker to detect the severity of dysplasia but not the presence of OA. Quantifying the 4977bp deletion using real-time PCR may be a more sensitive and specific method in determining the degree of dysplasia in oesophageal tissue samples and warrants further investigation.

Conflict of interest statement

None declared.

Acknowledgements

BHLT is supported by EC Grant LSHC-CT-2006-037777, RJES is supported by CSO Grant CZG/1/151 and NAS is supported by CRUK Grant C1128/A7309.

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