ORIGINAL ARTICLE

Aberrations in the mismatch repair genes and the clinical impact on oesophageal squamous carcinomas from a high incidence area in South Africa

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Aims: To investigate the incidence of genetic aberrations in the DNA repair genes in a cohort of oesophageal cancers.

Methods: One hundred oesophagectomy samples of squamous cell carcinoma were studied. Normal and tumour DNA were isolated using a standard phenol/chloroform extraction procedure. Six recommended microsatellite loci with high informativity were analysed. The following markers were used: D2S123 (2p), D3S659 (3p), D3S1255 (3p), Bat 25 (4q), Bat 26 (2p), and Bat 40 (1p). The results were analysed using software attached to an automated DNA sequencer. The molecular data were then correlated with clinicopathological parameters.

Results: The incidence of microsatellite instability and loss of heterozygosity was very low. There was no significant correlation between the clinicopathological and molecular data. However, D2S123 genetic abnormalities were seen more frequently in both moderately and well differentiated tumours than in poorly differentiated tumours ($p = 0.033$). Follow up data were available for only 67 of the 100 patients. Fifty patients were alive and 17 patients had died.

Conclusion: Low frequencies of genetic aberrations in these mismatch repair loci are found in squamous carcinomas of the oesophagus from a high incidence area in South Africa.

Oesophageal cancer is the most common malignancy in black South African men, accounting for 14.3% of all cancers in South Africa.¹ Over and above the various environmental factors that impact on the development of cancer in general, great advances have been made during the past decade in our understanding of the molecular mechanisms that occur in the transition from normal mucosa, through dysplasia, and finally to carcinoma.^{2–4} Several studies have revealed that an accumulation of genetic alterations gives rise to cancer. Furthermore, microsatellite instability (MSI) and loss of heterozygosity (LOH) at highly informative genetic loci at which tumour suppressor genes and oncogenes are located are thought to play a role in carcinogenesis.^{5–10} Genes investigated in recent studies include p53, Rb, DCC, APC, and MCC.¹¹ We recently showed that the occurrence of MSI in South African squamous carcinomas of the oesophagus was similar to other high incidence areas.⁷

"The mismatch repair genes play an important role in ensuring that errors occurring during DNA synthesis are corrected"

However, very little is known about the status of the mismatch repair genes and their impact on oesophageal cancer. The mismatch repair genes play an important role in ensuring that errors occurring during DNA synthesis are corrected. These genes were initially identified in bacterial systems and mutations in DNA mismatch repair genes have been associated with hereditary non-polyposis colorectal cancer.^{12–14} Five human DNA mismatch repair genes have been identified, which when mutated cause susceptibility to both sporadic and hereditary non-polyposis colorectal cancer. Mutational inactivation of both copies of a mismatch repair gene results in a profound repair defect and progressive accumulation of mutations throughout the genome. Our

study focuses on evaluating the incidence of genetic aberrations in the mismatch repair genes and its clinical impact on the development of oesophageal cancer in a high incidence area of South Africa.

MATERIALS AND METHODS

One hundred oesophagectomy specimens were obtained from the department of pathology, Nelson R Mandela School of Medicine, Durban, South Africa. The specimens were processed for routine histological examination after fixing in 10% buffered formal saline. Sections were stained with haematoxylin and eosin and all cases were reviewed by one of the authors (RC). Cases were graded as well, moderately, and poorly differentiated. Staging was performed using the TNM/UICC system, and clinical follow up was obtained from the department of cardiothoracic surgery. Normal and tumour sections were selected for DNA extraction after histological evaluation. Only sections containing more than 80% tumour tissue were used for extraction of tumour DNA. These areas were microdissected from the surrounding normal tissue.

DNA extraction

DNA was extracted from formalin fixed, paraffin wax embedded tissue blocks. Three sections (6 µm thick) were cut from the paired normal and tumour blocks. The procedure used was essentially as described by Naidoo *et al.*¹⁵ The quality of the isolated DNA was verified in a standard polymerase chain reaction (PCR) using primers targeting the ubiquitous insulin gene.¹⁶

Abbreviations: LOH, loss of heterozygosity; MSI, microsatellite instability; PCR, polymerase chain reaction

Table 1 Sequence data for the microsatellite markers

Marker	Primer sequence	Size range (bp)	Annealing temperature (°C)
D2S123	(F) 5'-AAACAGGATGCCTGCCTTA-3' (R) 5'-GAACTTCCACCTATGGGAC-3'	197–227	55
D3S659	(F) 5'-ATTCCAGGACAAGTTCCTCC-3' (R) 5'-CTGCAAGGTCTGTTAACAG-3'	103–140	55
D3S1255	(F) 5'-CTCACTCATGAACACAGATGC-3' (R) 5'-AACCCATCTGTATTCTGCAG-3'	145–160	55
Bat 25	(F) 5'-TCGCCTCCA AGAATGTAAGT-3' (R) 5'-TCTGCATTTTAACTATGGCTC-3'	± 90	55
Bat 26	(F) 5'-TGACTACTTTTGACTTCAGCC-3' (R) 5'-AACCATTCAACATTTTAACCC-3'	130–160	55
Bat 40	(F) 5'-ACAACCTGCTTTTGTTCCT-3' (R) 5'-GTAGAGCAAGACCACCTTG-3'	130–160	55

F, forward; R, reverse.

Microsatellite PCR

CY5 labelled primers

The microsatellite primers were purchased from Roche Diagnostics, Penzberg, Germany. Table 1 lists the primer sequences for the mismatch repair markers used in our study. These markers were chosen because of the recommendation of the American Association for Cancer Research.¹⁷ They are also deemed to be highly informative and significant when investigating mismatch repair abnormalities.

PCR reaction

PCR was carried out in 200 µl thin walled PCR tubes. The PCR core kit (Roche Diagnostics) was used for this procedure. The kit consisted of: 10× reaction buffer (containing 1.5mM MgCl₂), dNTP mix, and Taq DNA polymerase. The CY5 labelled primers (10 pmoles) were used in the PCR in a total reaction volume of 25 µl, containing 5 µl template DNA, 200µM dNTPs, 50mM PCR buffer containing 1.5mM MgCl₂, and 0.75 U Taq DNA polymerase. The PCR amplification was performed using a Techne Progene Thermocycler. The PCR reaction mixture was initially denatured at 95°C for five minutes. Thirty five cycles were performed, consisting of 30 seconds at 94°C, 30 seconds at 55°C, and 40 seconds at 72°C. This was followed by a final extension step for 10 minutes at 72°C.

Sample preparation and DNA fragment analysis

The PCR product (3 µl) was added to 3 µl of loading buffer (98% formamide, 5% dextran blue 2000). The samples were heat denatured at 96°C for three minutes before separation on a 6% Longranger sequencing gel (FMC Bioproducts, Rockland, Maine, USA). The electrophoretic running conditions were 1500 V, 60 mA, and 15 W at a constant temperature of 55°C. The raw data were analysed using the Fragment Manager software (Pharmacia Biotech, Uppsala, Sweden). The preparation of the size marker and the assessment of MSI and LOH were carried out as reported previously.¹⁵

Table 2 Summary table showing microsatellite data

Marker	NLOH	H	LOH	MSI	Percentage informativity
D2S123	27	61	7	5	39
D2S1255	25	52	18	5	48
D3S659	43	35	21	1	65
Bat 25	12	85	3	0	15
Bat 26	8	83	7	2	17
Bat 40	8	83	5	4	17

H, homozygous with no change; LOH, loss of heterozygosity; MSI, microsatellite instability; NLOH, no loss of heterozygosity.

RESULTS

Table 2 shows the microsatellite data and table 3 outlines the composite clinical and pathological data.

Molecular analysis

MSI of D2S123 was found in five cases (5%) and LOH was seen in seven of 39 cases. For the D3S659 locus, MSI (fig 1A) was found in only one case (1%), whereas LOH was seen in 21 of the 65 informative cases. MSI of the D3S1255 locus was seen in five cases (5%) and LOH in 18 of 48 informative cases. For the Bat loci (Bat 25, Bat 26, and Bat 40), MSI ranged from 0% to 4%, whereas LOH (fig 1B) ranged from 20% to 41% in the informative cases.

DISCUSSION

Several risk factors have been identified that are thought to influence the pathogenesis of oesophageal cancer. Among these are nutritional deficiencies, cigarette smoking, excessive alcohol consumption, and exposure to environmental toxins. However, the role of genetic factors in the development of this cancer in this high incidence area have only recently been investigated.^{7 18–21}

MSI is a reflection of the mismatch repair gene status, which is dependent on mutations in human mismatch repair

Table 3 Clinical and pathological features

Characteristic	
Number of patients	100
Age range (years)	24–92
Mean age (years)	56
Males	53
Females	45
Number of patients followed up	67
Follow up range (weeks)	0–284
Mean follow up (weeks)	28
Number of patients alive	50
Number of patients dead	17
Pathological data	
Stage	
I	1
IIA	44
IIB	10
III	40
Unknown	5
Tumour grade	
Well differentiated	26
Moderately differentiated	59
Poorly differentiated	12
Unknown	3
Lymph node metastases	
No	41
Yes	47
No record	12

Figure 1 Representative electropherogram showing microsatellite instability (MSI) and loss of heterozygosity (LOH). (A) MSI for marker D3S659. The normal DNA (lane 6) shows a single peak (homozygous, non-informative case), whereas tumour DNA (lane 7) contains a novel peak (122.6 bp). (B) Electropherogram showing LOH for Bat 25: the middle trace (lane 22) shows normal DNA and the lower trace (lane 23) shows tumour DNA with loss of the larger allele. Lane 1 represents the external marker that was run with all samples.

genes hMLH, hPMS1, hPMS2, hMSH2, hMSH3, and hMSH6.^{13–14} Very few reports have looked at the role of the mismatch repair genes in oesophageal cancer, although several studies have investigated MSI using a range of microsatellite markers located within tumour suppressor genes and oncogenes. Because there are no clear guidelines for the investigation of MSI in oesophageal cancer we adopted the criteria suggested by Boland *et al* for our study.¹⁷ A recent study showed 80% LOH within the TP53 gene in oesophageal cancer.²² Kagawa and co-workers²³ investigating MSI in 41 resected oesophageal carcinomas showed that the incidence of MSI was 42%, which was substantially lower than that reported by Wang *et al*.⁵ Another study investigating DNA replication error in 30 oesophageal squamous cell carcinomas found that LOH was very high (73%). That study was carried out at seven microsatellite markers in the 2p, 3p, and 16q loci in a cohort of patients from the Indian population.²⁴ Thus, there are large discrepancies in the findings from these studies, probably as a result of the different technologies used in the various studies, the number of cases and microsatellite markers investigated, and also the specific microsatellite markers used in the studies. Another important factor that cannot be ignored is the fact that these findings are a true reflection of the population genetics of the population group being investigated.

“Our results suggest that the molecular pathway for the development of oesophageal cancer is different to mismatch repair gene deficient colorectal cancers”

We found that MSI occurred at a much lower level than that reported in other studies. In contrast, LOH was much higher than MSI and was similar to other published reports, particularly at the 3p region, which showed LOH in 40% of cases. These observations in the 3p region are similar to

Take home messages

- We found a low frequency of genetic aberrations in six microsatellite markers located on mismatch repair loci in squamous carcinomas of the oesophagus from a high incidence area in South Africa
- No genetic marker at these mismatch repair gene loci predisposed to the onset of oesophageal cancer in our population group
- Loss of heterozygosity was much more frequent at these loci and the 3p region seemed to be an important “hot spot” with regard to abnormalities in oesophageal cancer, although this requires further investigation because of the low informativity rate of these markers in our population group

previous studies,^{5, 25–27} although two other studies^{28, 29} found LOH in the 3p region to be just 10% and 9%, respectively. LOH at the 3p loci was frequently associated with carcinomas that had spread to lymph nodes.²⁶ We found that MSI in the mismatch repair loci ranged from 0% to 5%. These figures are much lower than previous reports.

In conclusion, we found a low rate of MSI at the mismatch repair loci in oesophageal cancer in South Africa, whereas LOH was much more frequent at these loci. Furthermore, the 3p region seems to be an important “hot spot” with regard to abnormalities in oesophageal cancer, although this requires further investigation because of the low informativity rate of these markers in our population group. This variation in the informativity rate may result from population genetics. The Bat regions showed minimal instability and are markers of low informativity in our population group. Because of the low informativity of the Bat markers, no clear conclusions can be made with regard to the LOH data at these microsatellite loci.

Our results clearly indicate that there is no genetic marker at these mismatch repair gene loci that predisposes to the onset of oesophageal cancer in our population group. A combination of environmental factors, and/or other genetic factors may have a stronger influence on the onset of the disease. Furthermore, our results suggest that the molecular pathway for the development of oesophageal cancer is different to mismatch repair gene deficient colorectal cancers.

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ECHO

Mesenchymal changes mirror epithelial changes in gastric metaplasia

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A histological study has helped our understanding of gastric cancer by showing that changes in the mesenchyme occur in tandem with those of the gastric epithelium in the progression to cancer.

The mesenchymal sheath—called the pericryptal fibroblast sheath (PCFS)—underlies the epithelium and is closely associated with it, enveloping the normal intestine. Together the epithelium and PCFS act as a unit whose cell turnover seems to be regulated in parallel to ensure normal structure, maintenance, and function of the crypts of Lieberkühn.

This arrangement was found to be confined to gastrointestinal metaplasia in the stomach of humans and the Cdx2 transgenic mouse but did not appear in human intestinal-type gastric adenocarcinoma, nor normal human or mouse gastric mucosa, stained histochemically for α -smooth muscle actin to locate PCFS. It was present in normal human and Cdx2 mouse large and small intestinal mucosa.

The precursors to human intestinal-type gastric cancer are gastric atrophy and transformation of normal gastric mucosa to intestinal metaplasia, mainly owing to chronic gastric infection with *Helicobacter pylori*. Human gastric intestinal metaplastic cells express Cdx2—a transcription factor specific to intestinal cells. Transgenic Cdx2 mice have gastric metaplasia throughout their stomachs and are therefore a suitable model for this form of human cancer.

Until now, changes in only gastric epithelial cells had been reported, and it was useful to know whether these changes also affected the mesenchymal sheath.

▲ Mutoh H, et al. *Gut* 2005;**54**:33–39.