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Original Paper

TP53 Gene Mutation Status in Pretreatment Biopsies of Oesophageal Adenocarcinoma Has No Prognostic Value

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Identification of markers which help to predict response to treatment and overall survival at the time of diagnosis would assist in the management of patients with oesophageal adenocarcinoma. In the present study we investigated the prognostic significance of mutations to the *TP53* tumour suppressor gene in a large, consecutive series of oesophageal adenocarcinomas. The incidence of *TP53* mutation determined by molecular analysis of endoscopic biopsy specimens was 36% (49/135). No statistically significant difference was observed in patient survival according to the *TP53* status of the tumour biopsy. The median survival time for patients with mutation was 12 ± 1 months compared with 14 ± 2 months for patients with *TP53*. These results demonstrate that mutation of the *TP53* gene is not a useful predictive marker for patient survival in oesophageal adenocarcinoma. © **1999 Elsevier Science Ltd. All rights reserved.**

Key words: oesophageal adenocarcinoma, *TP53* gene, prognostic marker, biopsy, patient outcome, adjuvant therapy
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INTRODUCTION

OESOPHAGEAL CARCINOMA is one of the more aggressive cancers and has a 5-year survival of less than 10%. It is the sixth most common cancer worldwide [1] and is responsible for more than 600 deaths per year in Australia alone. Adenocarcinoma is the predominant subtype in Western countries where the incidence and mortality from this cancer have been increasing [2]. Definitive diagnosis is made by endoscopic biopsy. Because most patients present with late stage disease, surgical resection alone does not greatly extend patient survival. Various pre- and postoperative adjuvant treatment protocols have, therefore, been tried in an attempt to reduce local recurrence and improve survival, although the results remain controversial [3–6].

The identification of markers which predict oesophageal tumour aggressiveness and response to therapy would be of immense help for the management of these cancer patients. More accurate prognostic information obtained by analysis of endoscopic biopsy specimens at the time of diagnosis would be especially useful in determining further treatment.

The discovery of mutations to oncogenes and tumour suppressor genes has opened the possibility of using these as novel prognostic markers. Because of its central role in DNA damage repair, the most promising marker so far has been aberration of the *TP53* gene [7]. This can be observed using immunohistochemical (IHC) techniques to detect abnormal accumulation of the *TP53* protein in tumour cells, although IHC does not always reflect the presence of an underlying gene mutation. Polymerase chain reaction (PCR)-based techniques such as single strand conformation polymorphism (SSCP) and denaturing gradient gel electrophoresis (DGGE) have also been widely used to screen for *TP53* gene mutation. Relatively few studies have used DNA sequencing to examine the prognostic significance of *TP53* mutation because of the tedious nature and expense of this technique. An additional problem with sequencing is the masking of mutant *TP53* allele by the wild-type arising from contaminating normal tissue present within all solid tumours. In spite of the variety of techniques used, it is clear that in some tumour types, most notably breast [8, 9] and gastric cancers [10], *TP53* alteration

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is an independent risk factor for shortened patient survival and may become a marker of clinical value. For other tumour types, however, including colorectal carcinoma and gliomas, the prognostic significance of *TP53* aberration remains unclear [7].

Both IHC [11–15] and molecular techniques [16–19] have been used to investigate the association between *TP53* alteration and patient outcome for oesophageal carcinoma. Conflicting findings have been reported with both methods. Major shortfalls in these studies include the small number of tumours examined, the analysis of post-treatment tissue for *TP53* mutation, and the use of mixed series of squamous and adenocarcinoma cell subtypes. In the present investigation, these problems were avoided by examining a large, consecutive series of pretreatment oesophageal adenocarcinoma biopsies. Furthermore using a similar SSCP-based screening technique, we have previously observed a strong association between *TP53* mutation and poor prognosis in breast [9] and gastric [10] carcinomas, thus allowing comparison with other tumour types.

PATIENTS AND METHODS

Characteristics of patients and tumours

The study population consisted of 135 consecutive patients under the care of surgeons and physicians from the Sir Charles Gairdner Hospital, Nedlands, who were diagnosed with adenocarcinoma of the oesophagus between February 1985 and December 1996. These comprised 110 males and 25 females with a median age of 69 years (range 41–95). These cases were identified by review of the hospital pathology records. Follow-up information was obtained from hospital records and from the Death Register, Health Department of Western Australia. Median duration of follow-up was 80 months (range 19–164 months). By the end of the study period (February 1998), 110 patients had died as a result of their disease, one from surgical complications and two from unrelated causes.

42 patients underwent surgical resection of their tumour, with 4 of these receiving neoadjuvant chemoradiotherapy. Of the other patients, 10 received combined chemotherapy and radiotherapy [6], 2 received chemotherapy alone and 6 received radiotherapy as a main treatment. 20 received palliative treatment (stenting, dilatation and laser therapy) and 10 palliative care alone. Clinical information was not available for 45 cases due to patient management being performed outside of Sir Charles Gairdner Hospital. Patients were staged according to the International Union Against Cancer (UICC) TNM staging system [20]. The staging information was complete in 72 cases. The 45 patients with unavailable data, together with 17 patients having incomplete UICC staging information and 1 patient who died postoperatively, were excluded from the subgroup analysis of prognostic factors. Reasons for incomplete UICC staging information were failure to evaluate the surgical specimen due to aborted surgery after the identification of metastases.

PCR–SSCP screening for TP53 *gene mutation*

Archival paraffin blocks of biopsy specimens were selected by a pathologist for maximal tumour cell content. In all cases these contained at least 25% neoplastic cells. DNA for PCR–SSCP analysis of *TP53* gene mutation was obtained following incubation of one 10 μm section of formalin-fixed, paraffin-embedded biopsy tumour sample with proteinase-K enzyme (1 mg/ml final concentration) in 200 μl of digestion buffer (10 mM Tris–HCl, pH 8.3; 1 mM EDTA) for 3 days at 50°C. The enzyme was then inactivated by heating the digest at 95°C for 10 min, followed by centrifugation for 10 min at 12 000*g*. No further purification of the DNA was carried out. PCR amplifications of exons 5–8 were performed in the presence of [^{32}P]dCTP and using primers and conditions identical to those described earlier [21]. Final reaction volumes for PCR were 13 μl and included 1 μl of the DNA sample and 0.2 μCi of [^{32}P]dCTP. Isotopic SSCP of single-stranded PCR product was carried out using previously optimised gel conditions [21]. Briefly, this involved the use of 12% acrylamide/10% glycerol non-denaturing gels run in a BioRad Sequigen electrophoretic apparatus at room temperature for 16 h and at 1200 V. 5 μl of radio-labelled PCR product was added to 5 μl of formamide loading buffer (95% formamide, 10 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol) and denatured by heating at 95°C for 5 min prior to loading on to the gel. Equal volumes of PCR product from exons 5 and 7 were combined prior to SSCP in order to allow simultaneous analysis in the same gel run. Under the gel conditions used, mutant *TP53* alleles can be detected even when diluted by up to 95% with the normal *TP53* allele [21]. At the end of each run, gels were exposed without drying for 1-3 days with X-ray film (Fuji, Australia) prior to development. Mutations were visible as additional, aberrantly migrating bands compared with the normal *TP53* gene migration pattern. All mutations were confirmed at least once by separate PCR and SSCP runs.

Statistical analysis

Associations between clinicopathological features of oesophageal adenocarcinomas and *TP53* mutation were evaluated using the chi-squared test. The Mantel–Haenszel test for linear association was used to determine the correlation with histological grade and UICC staging, both of which were treated as continuous variables. All other variables were treated as dichotomous or categorical. Using overall survival as the endpoint, Cox's regression analysis was performed to determine the prognostic significance of several clinicopathological features. Univariate analyses were performed using the method of Kaplan and Meier and the difference in survival between each group was evaluated by the log-rank test. All tests were two-tailed, and a $P<0.05$ was considered to be significant. Analyses were conducted using the Statistical Package for Social Sciences (SPSS) software package.

RESULTS

The overall incidence of *TP53* mutations observed by SSCP was 36% (49/135). Representative SSCP results are shown in Figure 1. Nineteen tumours had a mutation in exon 5, 3 in exon 6, 12 in exon 7 and 16 in exon 8. One tumour had a mutation in both exons 6 and 8. No significant associations were observed between the presence of the *TP53* mutation and any of the standard clinicopathological features of oesophageal adenocarcinoma (Table 1). Trends were observed for an increased prevalence of *TP53* mutations in patients having positive nodes and metastases, suggesting an association with a more aggressive phenotype. In 19 of 38 cases which underwent surgery alone, the *TP53* status of the biopsy specimen was compared with that of the corresponding surgical specimen. A perfect concordance was observed in each case (seven mutations, 12 wild-type), thus validating the

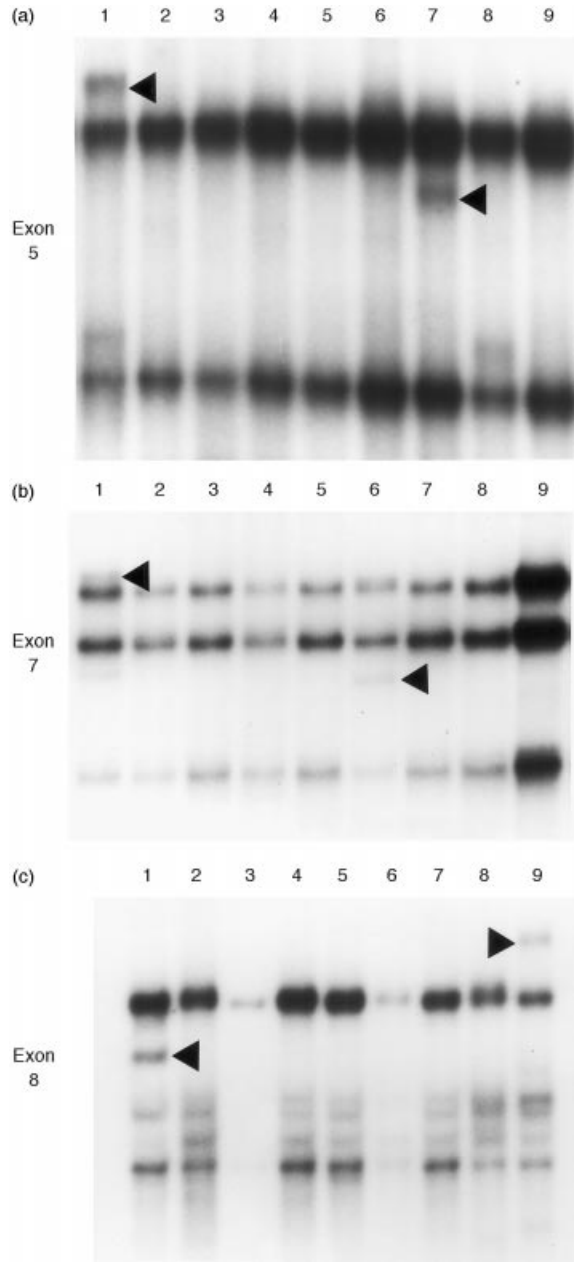

Figure 1. PCR–SSCP screening for mutations in exons 5, 7 and 8 of the *TP53* gene in oesophageal adenocarcinoma. Mutations are indicated by arrows in lanes 1 and 7 for exon 5 (a), lanes 1 and 6 for exon 7 (b) and lanes 1 and 9 for exon 8 (c).

use of biopsy samples for the determination of *TP53* status in oesophageal cancer.

Cox's univariate analysis revealed that only the presence of metastases at diagnosis was a significant prognostic indicator for overall survival of oesophageal adenocarcinoma patients (Table 2). In the 52 patients without metastasis, there was also a strong trend for worse survival in cases with more advanced stage. Kaplan–Meier analysis revealed that the presence of *TP53* mutation was not associated with significantly altered survival (Figure 2). The median survival of patients with a mutation was 12 ± 1 months compared with 14 ± 2 months for those without apparent mutation. The association of *TP53* mutation with a patient survival was also

Table 1. *Association between* TP53 *mutation and clinicopathological features in oesophageal adenocarcinomas (*n = 72*)*

	TP53 mutation		
	Per cent positive	χ^2-value	*P* value
Histological grade			
Well differentiated ($n = 5$)	25		
Moderately differentiated ($n = 34$)	48		
Poorly differentiated ($n = 33$)	34	3.1	0.21
Node status			
Negative ($n = 20$)	21		
Positive ($n = 34$)	39	1.7	0.19
Metastases			
Absent ($n = 51$)	34		
Present ($n = 21$)	45	0.70	0.40
Age			
<69 years ($n = 34$)	33		
≥69 years ($n = 38$)	40	0.69	0.41
Sex			
Male ($n = 60$)	37		
Female ($n = 12$)	32	0.24	0.82

Table 2. *Univariate analyses of overall survival in oesophageal adenocarcinoma patients with complete* UICC *staging information*

Feature	Relative risk	95% CI	*P*
Stage IIB/III versus I/IIA*	1.35	0.98–1.86	0.06
Histological grade*	1.28	0.84–1.99	0.25
Node status*	1.42	0.70–2.88	0.32
TP53 mutation*	1.15	0.52–2.51	0.73
Metastases†	6.9	3.57–13.33	<0.0001

*51 patients with complete UICC staging information and with no metastasis; †72 patients with complete UICC staging information. CI, confidence intervals.

examined in treatment subgroups. No significant associations were observed in any of the surgery alone or non-surgical groups (results not shown), although it should be cautioned that patient numbers in each group were relatively small.

DISCUSSION

In the present investigation we evaluated the clinical significance of *TP53* gene mutation in a large series of oesophageal adenocarcinomas in terms of its association with overall patient survival. In agreement with previous findings [17, 19, 22, 23], our results confirm the relatively high incidence of *TP53* mutation in this cancer type. The frequency of mutation observed here in oesophageal adenocarcinoma (36%) compares with our previous observations of 13% in endometrial [24], 19% in breast [9], 26% in gastric [10] and 36% in colorectal [25] carcinomas. In these earlier studies, we used either identical or very similar SSCP screening techniques, thus allowing direct comparison with the present findings on oesophageal adenocarcinoma. The presence of *TP53* mutation in oesophageal adenocarcinoma was not associated with significantly shortened patient survival. This contrasts with the findings in breast [9] and gastric [10] carcinomas but is similar to our observation in proximal colon

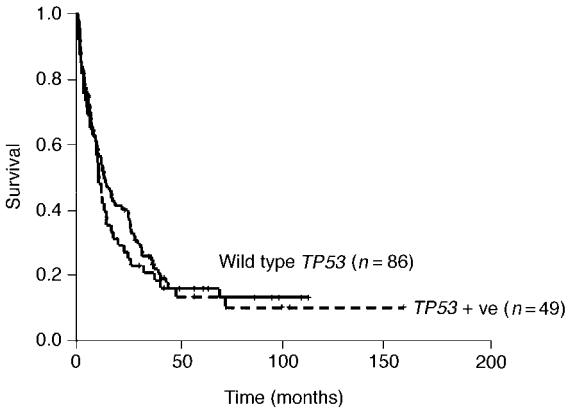

Figure 2. Kaplan–Meier analysis of the overall survival of oesophageal adenocarcinoma patients with or without *TP53* gene mutation.

carcinoma [25]. Our results suggest that the impact of *TP53* aberration on the overall survival of cancer patients is dependent upon tumour histogenesis, although the reasons for this remain to be determined.

Previous studies using the IHC technique to examine the prognostic significance of p53 protein accumulation in mixed squamous cell and adenocarcinoma series of oesophageal cancer have reported conflicting findings. Some reported no association with patient survival [11, 15, 26], others found an association with reduced survival [12, 18] and two an association with prolonged survival [13, 14]. Apart from possible differences related to the diverse geographical origins of these tumour series, the discrepant results are also likely to be due to interlaboratory variability in IHC procedures. These include the use of antigen retrieval methods, the type of primary antibody used and the criteria used to score tumours as positive for p53 accumulation. False-negatives are also a concern with IHC because mutations which give rise to truncated proteins are often not detected with this method. The lack of a standardised IHC protocol has made it difficult to compare results between laboratories and consequently we decided against the investigation of p53 accumulation in the present study.

Four studies have examined the prognostic significance of *TP53* mutation in oesophageal carcinoma using molecular techniques [16–19]. Mixed series of between 42 and 82 cases of squamous cell and adenocarcinomas were investigated by either SSCP [16–18] or sequence analysis [19]. The present study differs from each of the above in two important aspects. Firstly the tumours comprised a consecutive series of adenocarcinomas in which patients had not been selected specifically to undergo surgery or chemoradiotherapy. Secondly, pretreatment biopsy tumour tissue was analysed for mutation as opposed to the surgical tumour specimens used in each of the above studies. This avoids the possibility of preoperative chemotherapy/radiotherapy altering the tumours' *TP53* status prior to analysis. Our finding of a lack of prognostic significance for *TP53* mutation concurs with two of the previous studies [17, 18] but differs from the other two [16, 19]. The different result obtained by Uchino and colleagues [16] could be because their Japanese-derived tumour series comprised almost exclusively squamous cell carcinomas. *TP53* mutation might be prognostically significant in squamous cell carcinomas but not in adenocarcinomas, although further work will be required to verify this.

The study by Ribeiro and colleagues [19] comprised 42 cases of which 31 were adenocarcinomas. These workers observed a similar incidence of *TP53* mutation (40%) to the current study but in contrast to the present work found mutation to be associated with significantly reduced patient survival. A major difference between the two studies is that all patients in the series analysed by Ribeiro and colleagues were selected for potential surgery and received preoperative chemotherapy/radiotherapy, whereas the current series was not selected and only a small proportion received adjuvant therapy. Although patient numbers were small in this treatment subgroup, we did not find evidence of worse survival for cases with *TP53* mutation. It remains possible that *TP53* mutation is a predictive factor for response to preoperative chemotherapy and radiotherapy and this should be tested in large clinical trials such as those being run by the Irish [5] and Trans Tasman Radiation Oncology [6] groups.

Despite our failure to observe shorter survival times for patients having *TP53* mutation, trends for more frequent mutation in tumours with positive nodal status and metastases were seen (Table 1). Although there was no detectable impact on overall survival, the presence of *TP53* mutation appeared to be weakly associated with more advanced stages of disease. Significant associations between *TP53* mutation, higher TNM stage and response to treatment were reported by Ribeiro and colleagues [19].

To date, there is still no effective treatment for carcinoma of the oesophagus due to the late presentation of patients with this disease and to the proximity of vital organs which restricts the efficacy of local treatment such as surgery or radiotherapy. Palliation can be achieved by several methods including chemoradiotherapy, brachytherapy, stenting and laser therapy which, in high-risk patients, may be more suitable than surgery.

Identification of high-risk patients at the time of diagnosis is important in determining the prognosis and deciding upon the type of treatment. Our results indicate that the presence of metastases and the UICC staging are currently the only useful prognostic factors at diagnosis (Table 2). These factors require imaging methods such as computed tomography scan or magnetic resonance imaging for evaluation, or more accurate endoscopic ultrasound, each of which can provide information on the extent of tumour invasion and on the presence of nodal metastases. Our results also indicate that, in contrast to several other tumour types, *TP53* mutation status does not have prognostic value for the overall survival of unselected oesophageal adenocarcinoma patients.

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