Aberrant P53 Expression Lacks Prognostic or Predictive Significance in Colorectal Cancer: Results from the VICTOR Trial

MEGAN J. MCGREGOR[1*], WAKKAS FADHIL[2*], ROSE WHARTON[3], YOKO YANAGISAWA[1], MICHAEL PRESZ[1], ALISON PRITCHARD[4], CHRIS WOMACK[4], SUSAN DUTTON[3], RACHEL S. KERR[1], DAVID J. KERR[5], ELAINE C. JOHNSTONE[1] and MOHAMMAD ILYAS[2]

[1]*Department of Oncology, Old Road Campus Research Building, ad* [3]*Centre for Statistics in Medicine, University of Oxford, Oxford, U.K.;*
[2]*Queen's Medical Centre, School of Medicine, University of Nottingham, Nottingham, U.K.;*
[4]*AstraZeneca Oncology Innovative Medicines, Mereside, Macclesfield, Cheshire, U.K.;*
[5]*Nuffield Department of Clinical Laboratory Sciences, John Radcliffe Hospital, University of Oxford, Oxford, U.K.*

Abstract. *Aim: Biomarkers with prognostic and predictive value can help stratify patients with colorectal cancer (CRC) into appropriate treatment groups. We sought to evaluate the clinical utility of P53 protein expression as a biomarker in VICTOR, a large phase III trial of rofecoxib in stage II and III CRC. Patients and Methods: Tissue microarrays were constructed from 884 tumors and the expression of P53 was examined by immunohistochemistry. Tumors were dichotomised as either P53-positive (nuclear expression in >10% of cells or the 'absent' pattern, both representing TP53 mutation) or P53-negative (nuclear expression in <10% of cells). Results: Aberrant P53 expression was found in 65% (482/740) of patients. It was associated with distal location (p<0.001) and stage III disease (p<0.001). No effect was observed on disease-free or overall survival, and there was no interaction with chemotherapy or radiotherapy. Conclusion: Analysis of P53 expression in the patients recruited to the VICTOR trial confirmed that P53 expression is associated with site and stage of CRC. However, independently, this biomarker has neither prognostic nor predictive utility in this cohort of patients.*

*These Authors contributed equally to this study.

Correspondence to: Professor Mohammad Ilyas, School of Medicine, Queen's Medical Centre, Nottingham University, Nottingham. U.K. E-mail: mohammad.ilyas@nottingham.ac.uk

Key Words: Colorectal cancer, P53, immunohistochemistry, prognosis, biomarkers.

Colorectal cancer (CRC) is the second most common cause of cancer-related death in the Western countries (1, 2). Management of patients with CRC is dependent on the staging of the resected tumor by a pathologist. Patients with early-stage CRC (*i.e.* stage I) have a good prognosis and will not receive adjuvant therapy (3). In cases with more advanced-stage disease (*i.e.* stage II and III), the prognosis is more variable and consequently the management is more complex. Treating all cases of stage II disease with adjuvant chemotherapy gives a net benefit of around 4% improvement in survival (4). This is most probably due to the fact that around 70% of patient with stage II CRC will be disease-free five years later and thus will not benefit from adjuvant therapy. It would be expedient to target only the 30% of stage II cases who are at a high risk of recurrent disease. However, discriminating between the two groups is not easy, although certain pathological features (such as extramural vascular invasion) are associated with poorer outcome, pathological evaluation is operator-dependent and can be a highly variable method of gathering prognostic information (5). Furthermore, pathological staging does not provide any information on tumor biology.

Almost all patients with stage III disease are given adjuvant chemotherapy, as the overall prognosis is quite poor (6). However, not all tumors are the same and it is not known which tumors will respond to which specific therapy. There is, thus, a need for robust predictive and prognostic biomarkers which can add to current standard pathological analysis (7).

TP53 is a tumor-suppressor gene and it is mutated in 60-70% of CRCs (8). It can be inactivated by truncating mutations or by missense mutations and these lead to aberrant P53 protein expression. In the former case, there is complete loss of expression of P53 protein, whilst in the latter case,

Table I. *Clinicopathological features and outcomes associated with P53 expression in patients with colorectal cancer.*

No of patients		All	P53−	P53+	*p*-Value
Sex, n (%)	M	474 (64.0)	164 (63.6)	310 (64.3)	0.840
	F	266 (36.0)	94 (36.4)	172 (35.7)	
Mean (SD) age, years		64.08 (10.17)	64.58 (11.18)	64.24 (9.41)	0.327
Tumor location, n (%)	Proximal	500 (67.6)	204 (79.1)	296 (61.4)	<0.001
	Distal	240 (32.4)	54 (20.9)	186 (38.6)	
Tumor differentiation, n (%)	Well	55 (7.6)	21 (8.3)	34 (7.2)	0.561
	Moderate	589 (81.0)	200 (78.7)	389 (82.2)	
	Poor	82 (11.3)	33 (13.0)	49 (10.4)	
	Unknown	1 (0.1)	0 (0.00)	1 (0.2)	
T-Stage, n (%)	pT1/2	66 (9.1)	16 (6.3)	50 (10.6)	0.299
	pT3	512 (70.4)	185 (72.8)	327 (69.1)	
	pT4	146 (20.1)	52 (20.5)	94 (19.9)	
	Unknown	3 (0.4)	1 (0.4)	2 (0.4)	
Vascular invasion, n (%)	Yes	136 (18.7)	38 (15.0)	98 (20.7)	0.161
	No	581 (79.9)	212 (83.5)	369 (78.0)	
	Unknown	10 (1.4)	4 (1.6)	6 (1.3)	
Lymphatic invasion, n (%)	Yes	68 (9.3)	23 (9.0)	45 (9.5)	0.928
	No	649 (89.3)	227 (89.4)	422 (89.2)	
	Unknown	10 (1.4)	4 (1.6)	6 (1.3)	
Clinical stage, n (%)	II	334 (45.1)	139 (53.9)	195 (40.5)	<0.001
	III	406 (54.9)	119 (46.1)	287 (59.5)	
Chemotherapy n (%)	Yes	482 (65.1)	155 (60.1)	327 (67.8)	0.035
	No	258 (34.9)	103 (39.9)	155 (32.2)	
Radiotherapy n (%)	Yes	77 (10.4)	14 (5.4)	63 (13.1)	0.001
	No	663 (89.6)	244 (94.6)	419 (86.9)	
Recurrence n (%)	Yes	208 (28.1)	64 (24.8)	144 (29.9)	0.144
	No	532 (71.9)	194 (75.2)	338 (70.1)	
Deaths, n (%)	Yes	146 (19.7)	45 (17.4)	101 (20.9)	0.253
	No	594 (80.3)	213 (82.6)	381 (79.1)	

P53+ includes both tumors with strong expression and those with the 'absent' pattern (*i.e.* all tumors with mutation).

there is post-translational stabilisation leading to gross overexpression of the protein (9). These changes can be detected by immunohistochemistry (IHC), thereby allowing tumors containing mutant *TP53* to be distinguished from those which are wild-type for *TP53* (10).

We aimed to test the clinical utility of aberrant P53 expression as a prognostic and predictive marker in CRC. Although there exist previous studies investigating this biomarker, published data are not completely conclusive. Studies have been confounded by a variety of factors such as pooling data from multiple trials and technical variation in the laboratory methods. This study was performed on tissue obtained from patients recruited to the VICTOR trial (a single large randomised phase III trial run in the UK) (11) and all the IHC was performed in a single, fully accredited, diagnostic laboratory.

Patients and Methods

Patients. The VICTOR trial was a phase III randomised, placebo-controlled double-blind trial of rofecoxib (VIOXX®) in patients with stage II or III CRC who had undergone potentially curative surgery and completion of adjuvant therapy (if it was given). The trial was terminated prematurely in 2004 but up to that point, it had recruited 2434 patients from 151 hospitals in the UK. The rofecoxib-treated population comprised 1167 patients and there were 1160 placebo control patients. The trial showed no survival benefit of rofecoxib in the overall population and a lack of prognostic or predictive significance of Cycolo-oxygenase 2 expression (12).

Tissue microarrays (TMAs) and IHC. Formalin-fixed paraffin-embedded (FFPE) tumor samples were collected (with full consent for research use) from 1,006 patients. Of these, 884 were suitable for use in TMAs and the characteristics of this population were typical of the whole VICTOR population. Three cores of tumor (plus, if available, one core from adjacent normal tissue) were taken from each block and a total of 29 TMAs were assembled at the Astra-Zeneca Oncology Molecular Pathology Laboratory (Alderley Park, Cheshire, UK) and Oxford Radcliffe Bio-bank Oxford University using standard techniques. All IHC was performed using compact polymer technology on an automated Bond-Max (Leica Microsystems, Milton Keynes, Buckinghamshire, UK) using consumables provided by the manufacturer (Leica Bond Refine Detection kit; DS9800).

Sections (3 μm-thick) were de-waxed using Leica Dewax solution (AR9222) for 30 s at 72˚C followed by the antigen retrieval

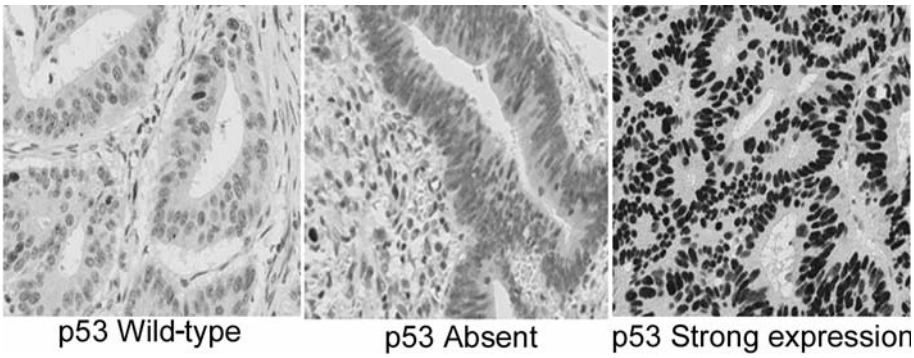

Figure 1. *Immunohistochemical staining for P53. Ordinarily tumors with wild-type P53 would be expected to exhibit weak staining in the epithelium and stroma (left panel). With truncating mutations, staining of the epithelium is negative but that of the stroma is weakly positive (i.e. the 'absent' pattern, central panel). With misense mutations, the epithelium is strongly positively-stained (right panel). The patterns of strong expression and the absent pattern were grouped together and scored as P53+ (as this is indicative of TP53 mutation), whilst the weak expression was scored as P53−.*

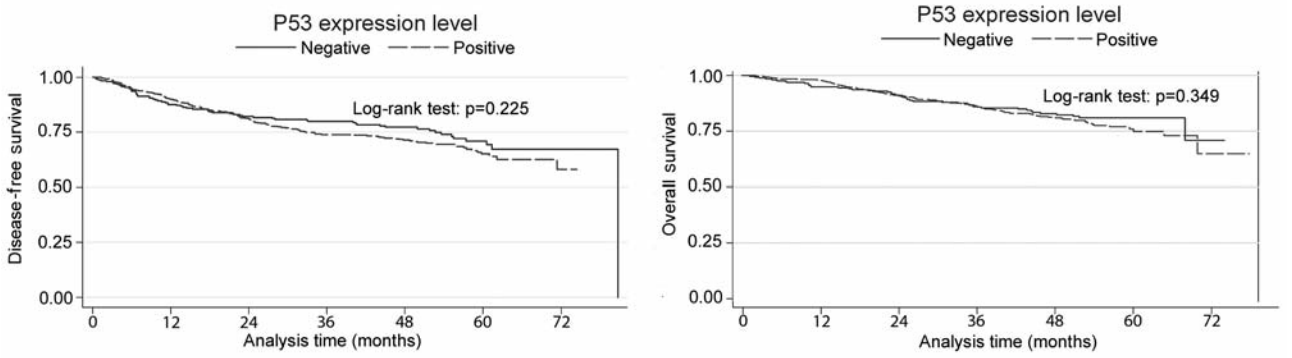

Figure 2. *Kaplan-Meier survival graphs showing that there was no difference in 5-year disease-free survival (DFS) nor overall survival (OS) when tumors were stratified according to TP53 mutational status.*

step by incubating for 30 min at 90˚C with Epitope Retrieval solution 1 (AR9961). After washing in Leica Bond wash solution (AR9590) sections were immersed in peroxide block solution (Kit DS9800) to block endogenous peroxidase for 5 min at room temperature (RT). The sections were incubated with a 1:100 dilution of primary antibody (Clone NCL-L-P53-D07; Leica Microsystems) for 15 min at RT. The post primary and polymer detection system were incubated each for 8 min at RT, before addition of 3,3'-Diamino-benzidine (DAB) for 10 min at RT followed by the DAB enhancer (AR9432) for 5 min at RT. The sections were counterstained with haematoxylin for 5 min at RT. Finally, sections were removed from the Bond staining machine, dehydrated in three baths of 100% Industrial methylated spirits (Genta Medical, York, Yorkshire, UK), cleared in Xylene (Genta Medical) and permanently mounted under glass coverslips using Pertex (Histolab, Hemel Hempstead, London UK). The stained TMA sections were scanned (at ×40 magnification) using a NanoZoomer Digital slide scanner (Hamamatsu, Hamamatsu City, Shizuoka Pref., Japan) and the digital images were uploaded into the SlidePath digital pathology system (SlidePath, Dublin, Ireland) and scored using Distiller software (SlidePath). Blinded scoring for P53 was performed by two independent observers (W.F and M.M).

Criteria for the scoring of the IHC. Scoring for P53 expression was based on the intensity, pattern and percentage of stained tumor nuclei and was scored dichotomously as either P53+ or P53−. Non-neoplastic colonic mucosa, inflammatory and stromal cells adjacent to neoplastic cells exhibited weak staining and served as positive internal controls. Using these as a reference, two patterns of aberrant P53 expression were seen. The more common pattern was strong nuclear expression in >10% of the tumor cells and this has been shown to be indicative of missense mutations of *TP53* (10). In addition, we and others have described a pattern characterised by a complete loss of P53 expression in the tumor cells, which we term the 'absent' pattern and this has been correlated with truncating *TP53* mutations (13, 14). Either of the aberrant patterns was classed as P53+, whilst other patterns were classed as P53− (Figure 1). There were tumors where neither the tumor cells nor the stromal cells exhibited any P53 expression and these cases were considered as technical failures and deemed unclassifiable.

Statistical analysis methods. Cases exhibiting the unclassifiable pattern were excluded from further analysis. The $P53^{absent}$ and P53+ cases were grouped together (as they all represent mutant P53) and called P53+ and these were compared against P53− cases. Overall

survival (OS) was the primary endpoint and disease-free survival (DFS) was the secondary endpoint. OS and DFS are displayed using Kaplan-Meier plots by expression category together with the associated log-rank *p*-value. Univariate and multivariate analyses were undertaken using the Cox proportional hazards model adjusting for treatment and for clinical factors including gender, tumor site (proximal, defined as proximal to the sigmoid colon; distal, defined as within the sigmoid and rectum), tumor stage, age and prior chemotherapy/radiotherapy. Hazard ratios and 95% confidence intervals (CI) are presented and analysis was carried out using STATA version 11.0 (StataCorp, College Station, Texas, USA).

Results

Clinicopathological features and outcomes associated with P53 expression. Scoring for P53 expression was dichotomised as either P53+ (including both patterns associated with *TP53* mutation) or P53− (associated with wild-type *TP53*, Figure 1). Overall, 65% (482/740) of the tumors were P53+ (Table I). Compared to P53− tumors, the P53+ tumors were associated with distal location ($p<0.001$) and advanced tumor stage ($p=0.009$). There was no association with gender, age of patient at presentation, pathological T stage, vascular/lymphatic invasion or lymph node recovery.

The hazard ratio for DFS for the P53+ group *versus* the P53− group was 1.08 (95% CI=0.79-1.47; $p=0.634$). The 5-year DFS rates were 70.9% (95% CI=63.6-77.0%) for the P53− group and 65.0% (95% CI=59.5-70.0%) for the P53+ group (Figure 2). The hazard ratio for OS for the P53+ group *versus* the P53− group was 1.12 (95% CI=0.77-1.62, $p=0.55$). The 5-year OS rates were 81.0 (95% CI=75.2-85.6%) for P53− and 77.6% (95% CI=73.2-81.3%) for P53+ (Figure 2). The patients with P53+ tumors were more likely to have received chemotherapy and radiotherapy than those with P53− tumors ($p<0.001$) which probably reflects their clinical staging and distal location. However, analysis according to chemotherapy sub-group did not reveal any significant difference in outcome for those with P53+ tumors in either the chemotherapy-treated or chemotherapy-naive groups.

Discussion

The present study sought to investigate the clinical utility of aberrant P53 expression as a prognostic and predictive biomarker in CRC. We had a large study population (n=824) which was drawn only from the VICTOR trial. We ensured technical excellence by performing the IHC in a fully accredited diagnostic lab and having two reviewers for the immunostaining. Under these conditions we failed to find any prognostic or predictive value for aberrant P53 expression.

The published data regarding the effect of P53 in CRC are heterogeneous (2, 15-22). In this study we defined two patterns of aberrant expression which are associated with *TP53* mutation and grouped these patterns together as P53+. As the "absent" pattern has only recently been described, it has not been included in previous studies. Since both the absent pattern and the pattern of strong over-expression are associated with *TP53* mutation, we felt is was justified to group the two patterns together. Tumors with wild-type pattern of P53 expression were designated as P53−. Consistent with many studies, our data showed that 65% of the tumors were P53+. We found that aberrant P53 expression was associated with advanced stage ($p<0.001$). This finding runs contrary to the Fearon and Vogelstein model which postulates that *TP53* mutation presages the acquisition of invasive tendencies in an adenoma (23). However, other studies have also reported this association (24-26), possibly suggesting that a diathesis for lymph node metastasis may follow *TP53* mutation. However despite this association, analysis of DFS and OS showed no difference between the P53+ and the P53− tumors either in the group overall or in stage-specific analysis. Furthermore, there was no effect of aberrant P53 expression on outcome of patients who received either chemotherapy or radiotherapy.

In summary, this was a meticulous study of the prognostic and predictive value of aberrant P53 expression conducted in the context of the VICTOR clinical trial for stage II and III CRC. Whilst many of the previously reported clinicopathological associations were confirmed, there was no prognostic or predictive information to be derived from evaluation of this biomarker.

Acknowledgements

The Authors would like to thank Neil Hand, Anne Kane, Dr Andrew Green, Haitao Wang and Joy Winter for their contribution to sample handling, tissue processing, immunostaining and slide scanning.

References

1 CR-UK. Bowel cancer statistics-UK 2012. Available from: http://info.cancerresearchuk.org/cancerstats/types/bowel/.

2 Bell SM, Scott N, Cross D, Sagar P, Lewis FA, Blair GE *et al*: Prognostic value of P53 overexpression and c-Ki-ras gene mutations in colorectal cancer. Gastroenterology 1993 Jan; 104(1): 57-64.

3 Day DW, Jass JR, Price AB, Shepherd NA, Sloan JM, Talbot IC *et al*: Morson and Dawson's Gastrointestinal Pathology 4th ed. Oxford: Blackwell Sciences Ltd.; 2003.

4 Midgley R and Kerr DJ: Adjuvant chemotherapy for stage II colorectal cancer: the time is right! Nat Clin Pract Oncol 2005 Jul; *2(7)*: 364-9.

5 Morris EJ, Maughan NJ, Forman D and Quirke P: Who to treat with adjuvant therapy in Dukes B/stage II colorectal cancer? The need for high quality pathology. Gut 2007 Oct *56(10)*: 1419-25. PubMed PMID: 17494107.

6 Poston GJ, Tait D, O'Connell S, Bennett A and Berendse S: Diagnosis and management of colorectal cancer: summary of NICE guidance. BMJ 2012 *343*: d6751.

7 Locker GY, Hamilton S, Harris J, Jessup JM, Kemeny N, Macdonald JS *et al*: ASCO 2006 update of recommendations for the use of tumor markers in gastrointestinal cancer. J Clin Oncol 2006 Nov 20 *24(33)*: 5313-27.
8 Hollstein M, Sidransky D, Vogelstein B and Harris CC: P53 mutations in human cancers. Science 1991 Jul 5 *253(5015)*: 49-53.
9 Lane DP: Cancer. P53, guardian of the genome. Nature 1992 Jul 2 *358(6381)*: 15-6.
10 Baas IO, Mulder JW, Offerhaus GJ, Vogelstein B, Hamilton SR: An evaluation of six antibodies for immunohistochemistry of mutant P53 gene product in archival colorectal neoplasms. J Pathol 1994 Jan *172(1)*: 5-12.
11 Midgley RS, McConkey CC, Johnstone EC, Dunn JA, Smith JL, Grumett SA *et al*: Phase III randomized trial assessing rofecoxib in the adjuvant setting of colorectal cancer: final results of the VICTOR trial. J Clin Oncol 2010 Oct 20 *28(30)*: 4575-80.
12 Midgley RS, McConkey CC, Johnstone EC, Dunn JA, Smith JL, Grumett SA *et al*: Phase III randomized trial Assessing Rofecoxib in the adjuvant setting of colorectal cancer: final results of the VICTOR Trial. Journal of Clinical Oncology 2010 October 20, 2010 *28(30)*: 4575-80.
13 Kaye PV, Haider SA, James PD, Soomro I, Catton J, Parsons SL *et al*: Novel staining pattern of P53 in Barrett's dysplasia--the absent pattern. Histopathology 2010 Dec *57(6)*: 933-5.
14 Kobel M, Reuss A, Bois A, Kommoss S, Kommoss F, Gao D *et al*: The biological and clinical value of P53 expression in pelvic high-grade serous carcinomas. J Pathol 2010 Oct *222(2)*: 191-8.
15 Hamelin R, Laurent-Puig P, Olschwang S, Jego N, Asselain B, Remvikos Y *et al*: Association of P53 mutations with short survival in colorectal cancer. Gastroenterology 1994 Jan *106(1)*: 42-8.
16 Ilyas M, Hao XP, Wilkinson K, Tomlinson IP, Abbasi AM, Forbes A *et al*: Loss of Bcl-2 expression correlates with tumour recurrence in colorectal cancer. Gut 1998 Sep *43(3)*: 383-7.
17 Khine K, Smith DR and Goh HS: High frequency of allelic deletion on chromosome 17p in advanced colorectal cancer. Cancer. 1994 Jan 1 *73(1)*: 28-35.
18 Morikawa T, Kuchiba A, Liao X, Imamura Y, Yamauchi M, Qian ZR *et al*: Tumor TP53 expression status, body mass index and prognosis in colorectal cancer. Int J Cancer 2011 Oct 31.
19 Morrin M, Kelly M, Barrett N and Delaney P: Mutations of Ki-ras and P53 genes in colorectal cancer and their prognostic significance. Gut 1994 Nov *35(11)*: 1627-31.
20 Remvikos Y, Tominaga O, Hammel P, Laurent-Puig P, Salmon RJ, Dutrillaux B *et al*: Increased P53 protein content of colorectal tumours correlates with poor survival. Br J Cancer 1992 Oct *66(4)*: 758-64.
21 Sun XF, Carstensen JM, Zhang H, Stal O, Wingren S, Hatschek T *et al*: Prognostic significance of cytoplasmic P53 oncoprotein in colorectal adenocarcinoma. Lancet 1992 Dec 5 340(8832): 1369-73.
22 Tornillo L, Lugli A, Zlobec I, Willi N, Glatz K, Lehmann F *et al*: Prognostic value of cell cycle and apoptosis regulatory proteins in mismatch repair-proficient colorectal cancer: a tissue microarray-based approach. Am J Clin Pathol 2007 Jan *127(1)*: 114-23.
23 Fearon ER and Vogelstein B: A genetic model for colorectal tumorigenesis. Cell 1990 Jun 1 *61(5)*: 759-67.
24 Kimura O, Sugamura K, Kijima T, Makino M, Shirai H, Tatebe S *et al*: Flow cytometric examination of P53 protein in primary tumors and metastases to the liver and lymph nodes of colorectal cancer. Dis Colon Rectum 1996 Dec *39(12)*: 1428-33.
25 Theodoropoulos GE, Karafoka E, Papailiou JG, Stamopoulos P, Zambirinis CP, Bramis K *et al*: P53 and EGFR expression in colorectal cancer: a reappraisal of 'old' tissue markers in patients with long follow-up. Anticancer Res 2009 Feb *29(2)*: 785-91.
26 Tomoda Hand Kakeji Y: Immunohistochemical analysis of P53 in colorectal cancer regarding clinicopathological correlation and prognostic significance. J Surg Oncol 1995 Feb *58(2)*: 125-8.

Received November 12, 2014
Revised December 15, 2014
Accepted December 17, 2014