PO Box 2345, Beijing 100023, China
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Cytoplasmic expression of p27^{kip1} is associated with a favourable prognosis in colorectal cancer patients

Nicholas FS Watson, Lindy G Durrant, John H Scholefield, Zahra Madjd, Duncan Scrimgeour, Ian Spendlove, Ian O Ellis, Poulam M Patel

Nicholas FS Watson, John H Scholefield, Section of Gastrointestinal Surgery, University of Nottingham, Queens' Medical Centre, Nottingham, United Kingdom
Lindy G Durrant, Zahra Madjd, Duncan Scrimgeour, Poulam M Patel, Academic Department of Clinical Oncology, University of Nottingham, Institute of Infections Immunity and Inflammation, City Hospital, Nottingham, United Kingdom
Ian O Ellis, Department of Histopathology, Nottingham City Hospital, and University of Nottingham, Nottingham, United Kingdom
Ian Spendlove, Academic Department of Clinical Oncology (with LGD, ZM, DS and PMP), University of Nottingham, Institute of Infections Immunity and Inflammation, City Hospital, Nottingham, United Kingdom
Supported by the Special Trustees of Nottingham Hospitals (grant No. STR 62/03/M)
Correspondence to: Mr NFS Watson, Section of Gastrointestinal Surgery, University of Nottingham, E Floor, West Block, Queens Medical Centre, Derby Road, Nottingham, NG7 2UH, United Kingdom. nicholas.watson@nottingham.ac.uk
Telephone: +44-115-9249924 Fax: +44-115-9249924
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Abstract

AIM: To evaluate the prognostic significance of p27^{kip1} in colorectal cancer patients.

METHODS: Cytoplasmic and nuclear p27^{kip1} expression was evaluated in 418 colorectal cancers using tissue microarrays. Data were associated with known patient and tumor variables and long-term patient outcomes, providing further insight into the mechanisms by which p27^{kip1} may influence tumor development.

RESULTS: Nuclear and cytoplasmic p27^{Kip1} expressions were detected in 59% and 19% of tumors respectively. Cytoplasmic p27^{Kip1} was almost invariably associated with positive nuclear p27^{Kip1} expression. Neither case correlated with known clinical or pathological variables, including tumor stage, grade or extramural vascular invasion. Furthermore, nuclear p27^{kip1} expression had no impact on survival. However, we identified a significant correlation between expression of cytoplasmic p27^{kip1} and longer disease-specific survival times. On multivariate analysis, TNM stage and extramural vascular invasion were highly significant independent prognostic factors, with positive cytoplasmic p27 expression showing a trend towards improved patient survival (P = 0.059).

CONCLUSION: These findings support the recent evidence that cytoplasmic p27^{kip1} has a distinct and important biological role that can influence tumor outcome.

Key words: Colorectal cancer; Tissue microarray; p27^{Kip1}; Prognostic factor

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INTRODUCTION

p27, also known as Kip1, is expressed in most cells and its role is to bind and inhibit cyclin/cyclin-dependent kinase (cyclin-CDK) complexes, thereby inhibiting cell cycle progression[1]. This role in the cell cycle makes p27^{kip1} a key player in multiple cell fate decisions including proliferation, motility, differentiation and apoptosis. Numerous studies have shown that p27^{kip1} is a tumor suppressor gene whose loss co-operates with mutations in several oncogenes and tumor suppressor genes in order to facilitate tumor growth. p27^{kip1} is rarely mutated, and is predominantly regulated at the post-transcriptional level by degradation in the ubiquitin-proteasome pathway[2]. However, in contrast to most tumor suppressor genes, which are recessive, tumor suppression by p27^{kip1} appears critically dependent upon on the absolute level of p27^{kip1}[3]. Several clinical studies have correlated absent or low p27^{kip1} expression with a poor prognosis in a range of malignancies, including breast, colorectal, gastric, ovary, prostate, bladder and oesophageal tumors[4-11]. Other investigators have failed to reproduce, or have only partially confirmed, the previously published results[12-14]. Some controversy also exists over the importance of cytoplasmic expression of p27^{kip1}, which was originally thought to represent a mechanism for inactivating p27^{kip1} by sequestering it away from its site of action within the nucleus[15]. Cytoplasmic expression of p27^{kip1} has been identified in colorectal cancer, ovarian cancer and Barrett's oesophagus[6,16-18], and recent evidence

suggests that it may have an active role, as it has been shown to bind to RhoA and inhibit its activity[19]. Members of the Rho family include Cdc42, Rac and RhoA. These act as molecular switches in signalling pathways affecting gene transcription and cytoskeletal rearrangements. In particular, the Rho proteins regulate and co-ordinate the cytoskeletal remodelling that underlies changes in cell adhesion and migration[20]. Consistent with these studies, p27's ability to regulate motility is distinct from its ability to inhibit cellular proliferation. Indeed, the region of $p27^{kip1}$ that binds to RhoA and is required for migration is different from the region that binds cyclin-CDKs[21].

Using high-throughput Tissue Microarray (TMA) technology[22] we have investigated the expression of both cytoplasmic and nuclear $p27^{kip1}$ in a series of over 400 paraffin wax-embedded colorectal tumor specimens. Data derived from this analysis was then associated with known patient and tumor variables, and with long-term patient outcome data, in order to gain further insight into the mechanisms by which p27 may influence tumor development.

MATERIALS AND METHODS

Patients

Four hundred and sixty-two patients were included in this study. A detailed description of these cases has been provided previously[23,24]. Briefly, all patients included in the TMA underwent elective resection of a histologically proven primary colorectal cancer between 1st January 1993 and 31st December 2000 at the University Hospital Nottingham, with prospective collection of associated clinical and pathological data including tumor site, TNM stage, histological tumor type and grade, and the presence of extramural vascular invasion. Histological factors more recently identified as having potential prognostic value in colorectal cancer, such as tumor budding and the tumor border configuration, were not recorded routinely at our institution and hence were not considered in the data analysis. Patients with lymph node positive disease were characteristically treated with adjuvant chemotherapy, consisting of 5-flurouracil and folinic acid. The UK Office for National Statistics has provided comprehensive follow-up regarding the date and cause of death for this cohort of patients. The length of follow-up was determined from the date of primary tumor resection, with surviving cases censored for analysis on the 31st December 2003. Disease specific survival was used as the primary end-point of the study.

Immunohistochemical procedures

Arrayed tumors were analysed for the expression of $p27^{kip1}$ using a murine monoclonal anti-human $p27^{kip1}$ antibody (clone SX53G8; Dako Ltd, Ely, UK) and standard immunohistochemistry with an avidin-biotin/horseradish peroxidase development system. Five microns formalin-fixed, wax-embedded tissue array sections were dewaxed in an incubator for 20 min at 60℃, deparaffinised with xylene, rehydrated through graded alcohol and immersed in 0.3% hydrogen peroxide-methanol solution to block endogenous peroxidase activity. Antigen retrieval was achieved by microwaving slides for 20 min in pH 9.0 EDTA buffer. Endogenous avidin/biotin activity was blocked using an avidin/biotin blocking kit (Vector Labs, USA). One hundred microliters of normal swine serum (NSS) was added to the sections for 10 min to block non-specific binding of the primary antibody. 100 μL of anti-$p27^{kip1}$ antibody diluted 1:25 (v/v) in NSS/TBS was then applied to the test sections for 1hr at room temperature. Positive controls consisted of multi-tissue sections containing human kidney and tonsil, with omission of the primary antibody from negative control sections. After washing with TBS, sections were incubated with 100 μL of biotinylated goat anti-mouse/rabbit immunoglobulin (Dako Ltd, Ely, UK) diluted 1:100 in NSS for 30 min. One hundred microliters of pre-formed streptavidin-biotin/horseradish peroxidase (HRP) complex (Dako Ltd, Ely, UK) was then applied for 60 min at room temperature. Finally, bound antibody visualisation was accomplished using 3, 3'-Diaminobenzidine tetrahydrochloride (Dab, Dako Ltd, Ely, UK).

Evaluation of immunohistochemical staining

Immunohistochemical staining patterns were interpreted by two observers (DSGS and NFSW) blinded to the associated clinicopathological data. Nuclear $p27^{kip1}$ expression was scored as follows: cases with < 10% tumor cell nuclei stained = 0, 10%-20% tumor cell nuclei stained = 1 and > 20% tumor cell nuclei stained = 2, irrespective of the staining intensity. Cytoplasmic staining was scored: complete absence of cytoplasmic staining or cytoplasmic staining of any intensity in < 10% tumor cells = 0, weak/moderate intensity cytoplasmic staining in > 10% tumor cells = 1, and intense cytoplasmic staining in > 10% tumor cells = 2. These cutoffs were based on previously published reports investigating the prognostic significance of $p27^{Kip1}$ expression in colorectal tumors[16,25]. For the purposes of statistical analysis, these scores were re-categorized, with tumors considered either positive (score 1 or 2) or negative (score 0) for both nuclear and cytoplasmic $p27^{kip1}$ expression. It has previously been established that in colorectal cancers, no significant differences in p27 immunoreactivity are usually seen between the centre and the invasive edge of the tumors[26].

Statistical analysis

All calculations were performed using SPSS software (version 11.5 for Windows, SPSS Inc., Chicago, IL). Associations between categorical variables were examined using crosstabulation and the Pearson chi-square test. Kaplan-Meier curves were plotted in order to assess correlations with disease-specific survival and the significance of differences in disease-specific survival between groups was calculated using the log-rank test. Patients whose deaths related to their colorectal cancer, including early deaths from post-operative complications, were considered in the disease-specific survival calculations. Deaths resulting from non-colorectal cancer related causes were censored at the time of death. Multivariate analysis was conducted using the Cox proportional-hazards model to determine hazard ratios, and to identify variables with independent prognostic significance in this cohort. In

Table 1 Patient and tumor characteristics (n = 462)

Variable	Category	n (%)
Age (yr)	Median	72
	Range	57-89
Gender	Male	266 (58)
	Female	196 (42)
Status	Alive	169 (37)
	Dead (cancer related)	228 (49)
	Dead (unrelated causes)	64 (14)
	Unknown	1
Histological type	Adenocarcinoma	392 (85)
	Adenocarcinoma with Mucinous differentiation	51 (11)
	Adenocarcinoma with Signet ring differentiation	7 (1)
	Other	4 (1)
	Unknown	8 (2)
Histological grade	Well differentiated	29 (6)
	Moderately differentiated	353 (77)
	Poorly differentiated	71 (15)
	Unknown	9 (2)
Tumor site	Colon	238 (52)
	Rectal	181 (39)
	Unknown	43 (9)
TNM stage	0 (T_{is})	3 (1)
	1	69 (15)
	2	174 (38)
	3	155 (33)
	4	54 (12)
	Unknown	7 (2)
Extramural vascular invasion	Negative	224 (48)
	Positive	128 (28)
	Unknown	110 (24)

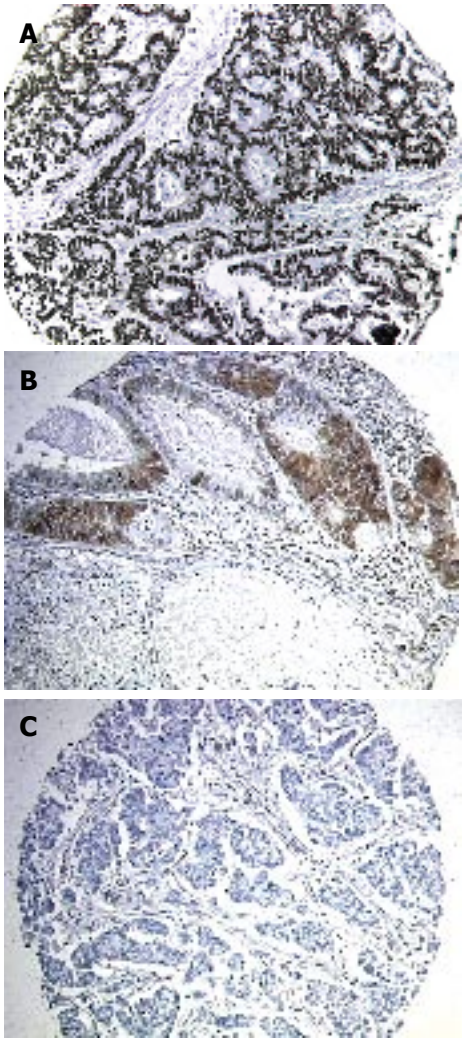

Figure 1 p27^{Kip1} expression in representative tumor samples. Tumors displayed either nuclear p27^{Kip1} alone (**A**), both nuclear and cytoplasmic p27^{Kip1} (**B**), or absent p27^{Kip1} expression (**C**).

all cases P values < 0.05 were considered statistically significant.

RESULTS

Clinicopathological data

The characteristics of the 462 patients included in this study are summarized in Table 1. Male patients comprised 58% of the cohort, and the median patient age at the time of surgery was 72 years. At the time of censoring for data analysis 49% of patients had died from their colorectal cancer, with a further 14% deceased from non-colorectal cancer causes. The remaining 37% were still alive. The majority of tumors were of a moderately differentiated histological grade (77%). Similarly, 85% of tumors were adenocarcinomas, with a further 11% showing mucinous differentiation. Surviving patients had a median length follow-up of 75 (range 36-116) mo. Of the conventional clinicopathological variables, strong correlations were observed between tumor stage and disease specific survival (DSS) (Log rank = 207.33, P < 0.001) and between the presence of extramural vascular invasion and DSS (Log rank = 44.30, P < 0.001).

Of the 462 tumor specimens analyzed, 44 (9.5%) were subsequently uninterpretable due to loss of tissue from the TMA during the Immunohistochemical procedure. Among the remaining 418 tumors, three distinct patterns of p27^{kip1} expression were observed (Figure 1A-1C). These comprised staining of the tumor cell nuclei alone, staining of both tumor cell nuclei and cytoplasm, and absent staining of tumor elements. In contrast, no tumor cell membrane or stromal expression of p27^{kip1} was detected in any specimen.

Nuclear p27^{kip1} expression

Variable expression of nuclear p27^{kip1} was observed in 217/418 (51.9%) tumors (Table 2). In 29 of these cases nuclear p27^{kip1} was present in 10%-20% of tumor cells within the core, and in the remainder, nuclear p27^{kip1} was present in > 20% of tumor cells. There were no significant associations detected between any level of nuclear p27^{kip1} expression and known clinicopathological variables, including tumor grade, stage and the presence of extramural vascular invasion. Furthermore, on Kaplan-Meier analysis, no association was found between nuclear p27^{kip1} expression and DSS (Figure 2A, Log rank = 1.701, P = 0.1815).

Cytoplasmic p27^{kip1} expression

Expression of cytoplasmic p27^{kip1} was detected in 79/418

Table 2 $p27^{kip1}$ localisation within 418 colorectal tumor specimens

p27 localisation	Positive *n* (%)	Negative *n* (%)	Missing *n* (%)
Nuclear	217 (51.9)	201 (48.1)	44 (9.5)
Cytoplasmic	79 (18.9)	339 (81.1)	44 (9.5)

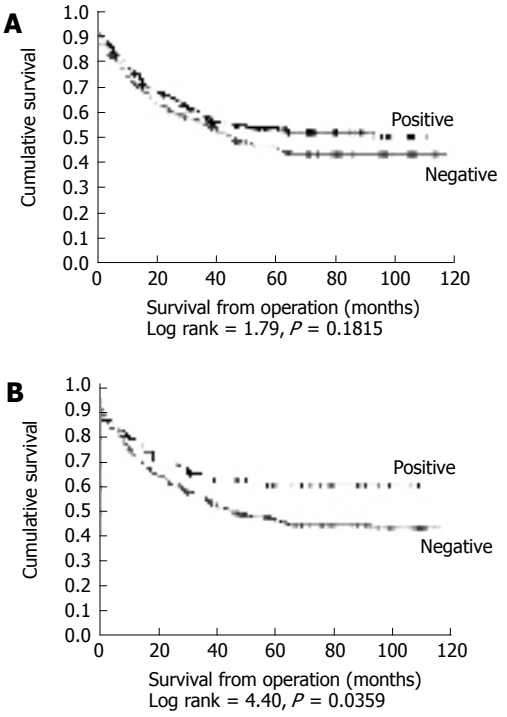

Figure 2 Kaplan-Meier plots for disease specific survival in relation to expression of nuclear (**A**) and cytoplasmic (**B**) $p27^{Kip1}$.

Table 3 Multivariate analysis of Cytoplasmic $p27^{kip}$ expression in relation to known clinical and pathological variables (*n* = 418)

Variable	Category	Hazard ratio (HR)	95% CI for HR	*P*
Gender	Female	1		
	Male	1.055	0.787-1.415	0.719
Patient age (yr)	< 65	1		
	65-79	1.262	0.843-1.889	
	80+	1.609	0.961-2.694	0.194
Tumor site	Colon	1		
	Rectal	1.117	0.813-1.535	
	Unknown	1.452	0.870-2.423	0.353
Tumor histological type	Adenocarcinoma	1		
	Non-adenocarcinoma	0.956	0.621-1.472	0.838
Tumor grade	Well differentiated	1		
	Moderately differentiated	1.167	0.582-2.337	
	Poorly differentiated	1.139	0.528-2.457	
	Unknown	0.869	0.245-3.079	0.926
Vascular invasion	Negative	1		
	Positive	1.884	1.344-2.641	
	Unknown	1.308	0.876-1.954	0.001
Tumor (TNM) stage	0/ I	1		
	II	2.021	1.102-3.708	
	III	3.741	2.066-6.774	
	IV	16.977	8.829-32.645	
	Unknown	4.211	1.049-16.902	< 0.001
Cytoplasmic $p27^{Kip1}$ expression	Negative	1		
	Positive	0.681	0.458-1.015	0.059

(18.9%) tumors (Table 2). These tumors appeared to form a distinct subset of the nuclear $p27^{kip1}$ positive tumors, as co-expression of nuclear and cytoplasmic $p27^{kip1}$ was noted in 74/79 cases (93.6%). In 55 of the cytoplasmic $p27^{kip1}$ positive tumors, expression was of weak or moderate intensity and in the remaining 24 cases strong expression was noted. As with nuclear $p27^{kip1}$, no statistically significant associations were found between the presence of cytoplasmic $p27^{kip1}$ within the tumor and clinicopathological variables for the patient cohort. However, a significant relationship was identified between the presence of cytoplasmic $p27^{kip1}$ and DSS. Kaplan-Meier analysis revealed that patients with cytoplasmic p27(+) tumors, had a significantly longer mean DSS of 72 (95% CI 62-83) mo, compared with a mean DSS of 62 (95% CI 57-68) mo in patients with cytoplasmic $p27^{kip1}$(-) tumors (Figure 2B, Log rank = 4.40, $P = 0.0359$).

Multivariate analysis

A multivariate analysis of factors influencing survival in 418 available cases was performed using the Cox proportional hazards model (Table 3). Of the conventional clinicopathological variables analysed, tumor stage ($P < 0.001$) and extramural vascular invasion status ($P = 0.001$) were demonstrated to confer independently significant prognostic information. In this model expression of cytoplasmic $p27^{kip1}$ within the tumor samples was associated with a strong trend towards improved DSS (HR for death in cytoplasmic $p27^{kip1}$ positive tumors = 0.681, 95% CI 0.458-1.015), which approached statistical significance ($P = 0.059$).

DISCUSSION

$p27^{kip1}$ is a universal CDK inhibitor that acts in G_0 and early G_1 to inhibit cyclin E/CDK2 and thereby prevents entry into the S phase of the cell cycle. It can also bind other cyclin-CDK complexes, including those involving the D-cyclins, and complexes of cyclin A/cdk2. Mitogenic growth factor signalling causes a decrease of $p27^{kip1}$ levels and/or activity and, conversely, $p27^{kip1}$ levels and/or activity increase in response to differentiation signals, loss of adhesion to extracellular matrix, or signalling by growth-regulatory factors such as TGF-β, c-AMP and IFN-γ. Studies in animals have shown that loss of $p27^{kip1}$ increases the formation of tumors and also increases tumor associated deaths rates. Several studies have shown that loss of nuclear $p27^{kip1}$ is an independent predictor of poor prognosis in colorectal cancer[6,27,28], although other studies have failed to confirm this observation[29,30]. Loss of nuclear

p27^{kip1} was observed in 48% of tumors in our study. However, this showed no significant associations with known clinical or pathological variables. Additionally, on Kaplan-Meier analysis, no association was found between nuclear p27^{kip1} expression and survival.

Several clinical studies have detected cytoplasmic expression of p27^{kip1}, and found an inverse correlation with disease free survival[17,28,31]. In contrast, in this large study of 418 colorectal tumors, cytoplasmic expression of p27^{kip1} was significantly associated with a good prognosis. This may be related to the findings from recent studies showing that p27^{kip1} can bind and inactivate Rho proteins. For tumors to metastasise, cells must alter their connections to both their neighbours and their substrate, and then migrate. Efficient migration requires a tightly balanced activation and deactivation of Cdc42, Rac and RhoA in both time and space. Indeed, two modes of tumor cell motility have been described in 3D matrices. Rho signalling through ROCK promotes a rounded bleb-associated mode of motility that does not require pericellular proteolysis. In contrast, elongated cell motility is associated with Rac-dependent F-actin-rich protrusions and does not require Rho or ROCK[32]. Consistent with this observation are other studies that have shown inhibition of ROCK reduces the invasive behaviour of tumor cells *in vivo*[33,34]. Sequestrations of RhoA by cytoplamsic p27^{kip1} may inhibit RhoA, resulting in inhibition of ROCK and less aggressive tumors. In the current study, cytoplasmic expression of p27^{kip1} was almost invariably associated with nuclear expression. These tumors may therefore have relatively controlled cell proliferation together with a reduced capacity for migration, resulting in a less aggressive tumor and a good prognosis. In contrast, loss of both cytoplasmic and nuclear p27^{kip1} may result in uncontrolled proliferation and increased invasion, leading to an aggressive tumor and poor prognosis. Tumor cells expressing only nuclear p27^{kip1} would have reduced proliferation but may still be invasive, resulting in an intermediate prognosis. This may explain the inconsistency of previous studies linking p27^{kip1} expression with prognosis, as studies considering nuclear p27^{kip1} expression alone have not identified the subgroup of tumors with additional cytoplasmic p27^{kip1} expression. It is anticipated that the role of cytoplasmic p27^{kip1} in promoting tumor progression may also depend upon the site and mechanism of invasion, as in a study of 61 patients with pancreatic cancer in which those with exclusively nuclear p27^{kip1} expression were shown to have a better prognosis than those with both nuclear and cytoplasmic p27^{kip1} expression[31].

Recently, it has been suggested that the presence of high numbers of detached clusters of tumor cells in adjacent stroma (termed tumor budding) may serve as an adverse histopathological prognostic feature in colorectal cancer[35]. As this tumor budding is the morphological counterpart of epithelial-mesenchymal transition, microinvasion, and acquisition of individual cell motility, it would be of interest to correlate p27 immuno-expression patterns with colorectal tumor budding activity. However, this would be best performed using conventional whole tissue section analysis rather than TMA's. Similarly, as the size of this array does not provide us with the statistical power to co-analyze two rare events, we have not performed analysis of DNA mismatch repair gene status and therefore cannot comment on whether cytoplasmic p27 expression may have a diverse role in colorectal tumors of differing microsatelite instability status. Finally, although out of the scope of this manuscript, it would be of interest to compare disease specific survival between p27$^{Kip1\ -/-}$ and wild-type mouse in a colorectal cancer mouse model.

The identification of novel molecular and genetic markers of prognosis will eventually allow us to provide a cancer patient with individually tailored therapy based upon on the specific molecular fingerprint of his or her tumor. Our findings in a large cohort of unselected patients with colorectal cancer suggest that cytoplasmic p27^{Kip1} expression deserves further consideration as a marker of prognosis, as patients with colorectal tumors showing cytoplasmic expression of p27^{kip1} appear to have a more favourable disease specific survival.

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