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European Journal of Cancer 38 (2002) 832–841

European Journal of Cancer

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Alterations in p53 and pRb pathways and their prognostic significance in oesophageal cancer

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Received 14 June 2001; received in revised form 15 October 2001; accepted 24 December 2001

Abstract

The pRb (p16-pRb-cyclin D1) and p53 (p53-MDM2-p21) pathways play a critical role in tumorigenesis. To evaluate which of these cell cycle regulatory proteins are related to patients' prognosis, a comprehensive analysis of alterations in these components was carried out in 100 ESCCs (oesophageal squamous cell carcinoma) using immunohistochemistry and correlated with clinicopathological parameters by univariate analysis. Overexpression of p53, MDM2 and cyclin D1 proteins was observed in 73, 42 and 67% of the cases, respectively, while loss of expression of p21, p16 and pRb was observed in 36, 45 and 75% of the cases, respectively. Multiple logistic regression analysis revealed that loss of p16 immunoreactivity was a significant risk factor for tumour stage (pT) (Odds Ratio (OR) = 3.3), whereas the loss of pRb was a significant risk factor for nodal metastasis (pN) (OR = 8.8). MDM2 overexpression emerged as the most significant risk factor for distant organ metastasis (pM) (OR = 4.6). Of the ESCC patients who underwent oesophagectomy, 50 cases were followed-up for a maximum period of 44 months and median of 16 months. Survival analysis revealed that Cyclin D1 overexpression is an adverse prognosticator for disease-free survival, as well as overall survival, and tumour stage (pT) is an adverse prognosticator for disease-free survival. In conclusion, these data support a model of oesophageal cancer pathogenesis in which both the pRb and p53 pathways are inactivated and suggests an in-depth evaluation of the clinical utility of these putative markers is warranted. © 2002 Published by Elsevier Science Ltd.

Keywords: Prognostic markers; p53-MDM2 pathway; p16-pRb pathway; Oesophageal cancer; Cell cycle

1. Introduction

Despite advances in therapeutic strategies and post-operative management, prognosis for oesophageal cancer patients remains poor. Improvements in the prognosis and surveillance await a more comprehensive understanding of the molecular alterations and clinicopathological characteristics as predictors of a parsimonious model in tumour control. The clinical characterisation of oesophageal carcinoma remains inadequate using the conventional histological staging system. The TNM staging for oesophageal cancer [1] considered as the most important prognostic indicator does not differentiate between tumours confined to the mucosa and those involving the submucosa, a distinction that has been shown to be of considerable prognostic significance [2]. Several studies have demonstrated considerable heterogeneity in prognosis among oesophageal squamous cell carcinomas (ESCC) of various histological types. Submucosal oesophageal cancer has been shown to be associated with a significant risk of vascular invasion and lymph node metastasis [3]. Current inadequacy of clinicopathological parameters as prognosticators warrants a clearer understanding of the molecular alterations in predicting prognosis of the disease. Various molecular biological factors have been proposed as prognostic indicators of oesophageal cancer [4]. Cell cycle regulators of prognostic relevance in oesophageal cancer include, mutations in the tumour suppressor gene *TP53*, allelic loss at chromosomal loci encoding p16 and pRb and altered expression of p21,

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0959-8049/02/$ - see front matter © 2002 Published by Elsevier Science Ltd.
PII: S0959-8049(02)00007-2

cyclin D1 and MDM2 [5–7]. Nevertheless, nearly all of these studies have examined these markers in isolation or in dual combinations and therefore the overall conclusions from these studies are discordant and a composite picture of the interactions between p53-MDM2-p21 and p16-pRb-cyclin D1 pathways is currently lacking.

The aetiological factors implicated in the pathogenesis of ESCCs are markedly different in the Western population compared with that of developing countries such as India. In Western countries, alcohol consumption and tobacco smoking are the major predisposing factors for ESCCs while, in the developing countries such as India and China, tobacco chewing and a variety of dietary factors confounded by nutritional deficiency are causally associated with the disease [8–10]. The exposure of the gastric mucosa to a plethora of carcinogens present in betel and tobacco may provide a favourable milieu for neoplastic transformation. In light of the unique aetiological predisposition, it is worthwhile to investigate the prognostic profile of ESCC in the Indian population.

In a comprehensive analysis of molecular prognostic factors, Shimada and colleagues [4] reported that cyclin D1, E-cadherin, epidermal growth factor receptor (EGFR), pN, gender and cell growth capability are important predictors of patient survival and recurrence in the Japanese population. However, the relevance of the pRb protein, the central cog of the cell cycle regulation machinery as a molecular marker remains to be investigated. p53, MDM2, p21, p16, pRb and cyclin D1 constitute the most important proteins orchestrating the cell cycle regulation. To elucidate the prognostic significance of various cell cycle regulators, we examined the expression of six putative molecular markers: p53, MDM2, p21, p16, pRb and cyclin D1 by immunohistochemistry. The data were subjected to multivariate logistic regression analysis and the Kaplan–Meier method to identify predictors of prognosis for various clinicopathological parameters related to tumour progression.

2. Patients and methods

2.1. Tumour specimens

Tumour and matched normal oesophageal tissue specimens from a distal site were collected from 100 ESCC patients who underwent curative oesophagectomy in the Surgical Oncology Unit of the Institute Rotary Cancer Hospital, All India Institute of Medical Sciences, New Delhi, India, with the prior consent of the patients. Tissue samples were fixed in formalin and embedded in paraffin for immunohistochemical analysis. The clinical and pathological data were recorded in a predesigned proforma including age, gender, site of the lesion, histological differentiation and TNM staging. Each patient's clinical stage was classified according to pathological tumour node metastasis system (pTNM) [1]. The tumours were histologically graded as well, moderately or poorly differentiated. Radiation therapy (21 cases) or chemotherapy (12 cases) preceded the curative oesophagectomy in this cohort. The chemotherapy regime included a combination of cisplatin and 5-fluoroouracil (5-FU); cisplatin: 75–100 mg/m^2 intravenously (i.v.) day 1. 5-FU: 1000 mg/m^2 i.v. days 1–5. This dose was repeated every 21 days for three cycles, followed by surgery. Radiotherapy was given at a dose of 5 Gy/day for a period of 5 days (total dose 25 Gy) prior to surgery.

2.2. Follow-up studies

Of the ESCC patients who underwent oesophagectomy, 50 cases were followed-up for a maximum period of 44 months and median of 16 months (range: 0.07–44 months). The remaining cases were lost to follow-up due to poor compliance. Disease-free survival was expressed as the number of months from the date of surgery to the recurrence of the disease. During the follow-up period, 13 patients died from the disease.

2.3. Immunohistochemistry

Paraffin-embedded sections (5 μm thickness) of human ESCCs and matched normal tissues were used for histopathological analysis after antigen retrieval. Briefly, tissues were deparaffinised in xylene, hydrated and incubated with 0.5% H_2O_2 in methanol for 20 min to block the endogenous peroxidase activity. Slides were then washed with Tris-buffered saline (TBS) and heated for 15 min at 100 °C in 10 mM sodium citrate buffer (pH 6.0). The slides were cooled to 37 °C followed by incubation with 1% bovine serum albumin (BSA) for 1 h to block the non-specific binding. The primary antibodies were diluted to a final concentration of 0.1 μg/ml for p53, p21 and MDM2 and 0.2 μg/ml for p16, pRb and cyclin D1 and sections were incubated for 16 h at 4 °C. All the antibodies used in this study were purchased from Santa Cruz Biotechnology Inc., CA, USA: p53 (DO-1, monoclonal IgG antibody); MDM2 (SMP-14, monoclonal IgG antibody), p21 (187, monoclonal IgG antibody), p16 (C-20, polyclonal IgG antibody), pRb (IF8, monoclonal IgG antibody), cyclin D1 (SC 6281, monoclonal IgG antibody). The primary antibody was detected using biotinylated secondary antibody (rabbit anti-mouse IgG for monoclonal antibodies and goat anti-rabbit IgG for polyclonal antibodies) and avidin–biotin complex by the ABC method as previously described in Ref. [9] using Vectastain Elite kit and diaminobenzidine as a chromogen. Slides were washed

several times with phosphate-buffered solution (PBS) after each step. In negative controls, PBS replaced the primary antibody or non-immune mouse IgG of the same isotype to ensure specificity. Human oral squamous cell carcinoma tissue sections with known immunoreactivity for the respective protein were used as the positive or negative control in each batch of sections analysed (data not shown).

2.4. Positive criteria for immunohistochemical staining

The intensity of immunohistochemical staining was evaluated in five areas of the slide sections for correlation and confirmation of the tissue analysis. For scoring p53 immunoreactivity, we adopted the criteria by Barbatis and colleagues [11]. p53 and cyclin D1 were considered to be overexpressed when more than 10% positive nuclear staining was observed, given that normal oesophageal squamous epithelial cells do not show cyclin D1 immunoreactivity [12]. To score p16 and pRb immunoreactivity, we have followed the criteria by Geradts and Wilson [13]. For p21 protein, less than 50% of nuclear staining was considered as loss of expression [14]. Lack of immunoreactivity in the tumour nests with surrounding stromal elements clearly showing immunoreactivity has been scored as loss of expression for the tumour suppressor proteins. For the MDM2 protein, more than 30% of nuclear or cytoplasmic staining was considered as positive staining [15–17]. The sections were counter-stained with haematoxylin and the tumour differentiation status was graded as Well (W), Moderate (M), or Poor (P). The immunostained slides were graded on a four-point scale by four of us independently, one of whom is a Professor of Pathology.

2.5. Statistical analysis

The immunohistochemical data have been subjected to statistical analysis for their predictive utility. Derivative variables of protein combinations were derived by transformation in order to assess the association of alterations in the biomarkers with clinicopathological outcome. Variables were dichotomised and the associations between the protein expression and clinicopathological parameters of oesophageal cancer patients were statistically evaluated using the Chi-square test or Fisher's Exact test (Epistat). A P value $\leqslant 0.05$ has been defined as the criterion for statistical significance. Stepwise logistic regression analysis and Survival analysis has been carried out by the Statistical Package for the Social Sciences (SPSS) (Version 6.0) or STATA 6.0. Overall and disease-free survival was estimated according to the Kaplan–Meier method. The univariate association between patient outcome and variables was assessed by the log rank test.

3. Results

3.1. Alterations in the p53-MDM2-p21 pathway in human oesophageal squamous cell carcinoma

The results of the immunohistochemical analysis of the cell cycle regulatory proteins, p53, mdm2, p21, p16, pRb and cyclin D1 in 100 ESCC patients in relation to clinicopathological parameters are summarised in Table 1. The photomicrographs in Fig. 1 illustrate representative immunostaining patterns for p53, MDM2 and p21 proteins in normal oesophageal tissues (Fig. 1a, c and e, respectively). In ESCCs, p53 protein was localised predominantly in the nuclei of tumour cells (Fig. 1b). Interestingly, in addition to the nuclear localisation of MDM2, we observed MDM2 immunoreactivity in the cytoplasm and plasma membrane of tumour cells in a subset of ESCCs (Fig. 1d). Fig. 1f shows the lack of detectable p21 immunoreactivity in the nuclei of tumour cells, while the surrounding stromal elements showed positive staining.

The alterations in the expression of cell cycle regulatory proteins were correlated with various clinicopathological parameters such as pT, pN, pM, tumour differentiation, treatment (chemotherapy or radiotherapy) and tumour site by univariate analysis. We observed an inverse correlation between p53 and MDM2 immunoreactivities ($r = -0.2127$; $P = 0.034$). Both $p53^+$ ($P = 0.05$) and $MDM2^+$ ($P = 0.018$) showed significant association with distant organ metastasis by univariate analysis. MDM2 overexpression was significantly associated with distant organ metastasis. Multivariate analysis (stepwise logistic regression model) revealed MDM2 overexpression as the most significant independent risk factor for distant organ metastasis ($P = 0.05$; Odds Ratio (OR) = 4.6).

3.2. Alterations in the p16-pRb-cyclin D1 pathway in human oesophageal squamous cell carcinoma

Representative photomicrographs of cyclin D1, p16 and pRb immunostained sections of normal oesophageal tissues are shown in Fig. 1g, i and k, respectively. Cyclin D1 accumulation was observed in the tumour nuclei in ESCCs (Fig. 1h). p16 or pRb immunoreactivity was observed in stromal elements surrounding the tumour nests, while the tumour nuclei were predominantly negative (Fig. 1j and l, respectively). The alterations in the expression of p16, pRb and cyclin D1 proteins were correlated with clinicopathological parameters by univariate analysis, which revealed that the loss of p16 ($p16^-$) and preoperative chemotherapy were significantly associated with tumour stage (pT). Chemotherapy showed an inverse correlation with tumour stage (pT) ($r = -0.2543$; $P = 0.03$), while the loss of p16 was positively associated with pT ($r = 0.2853$;

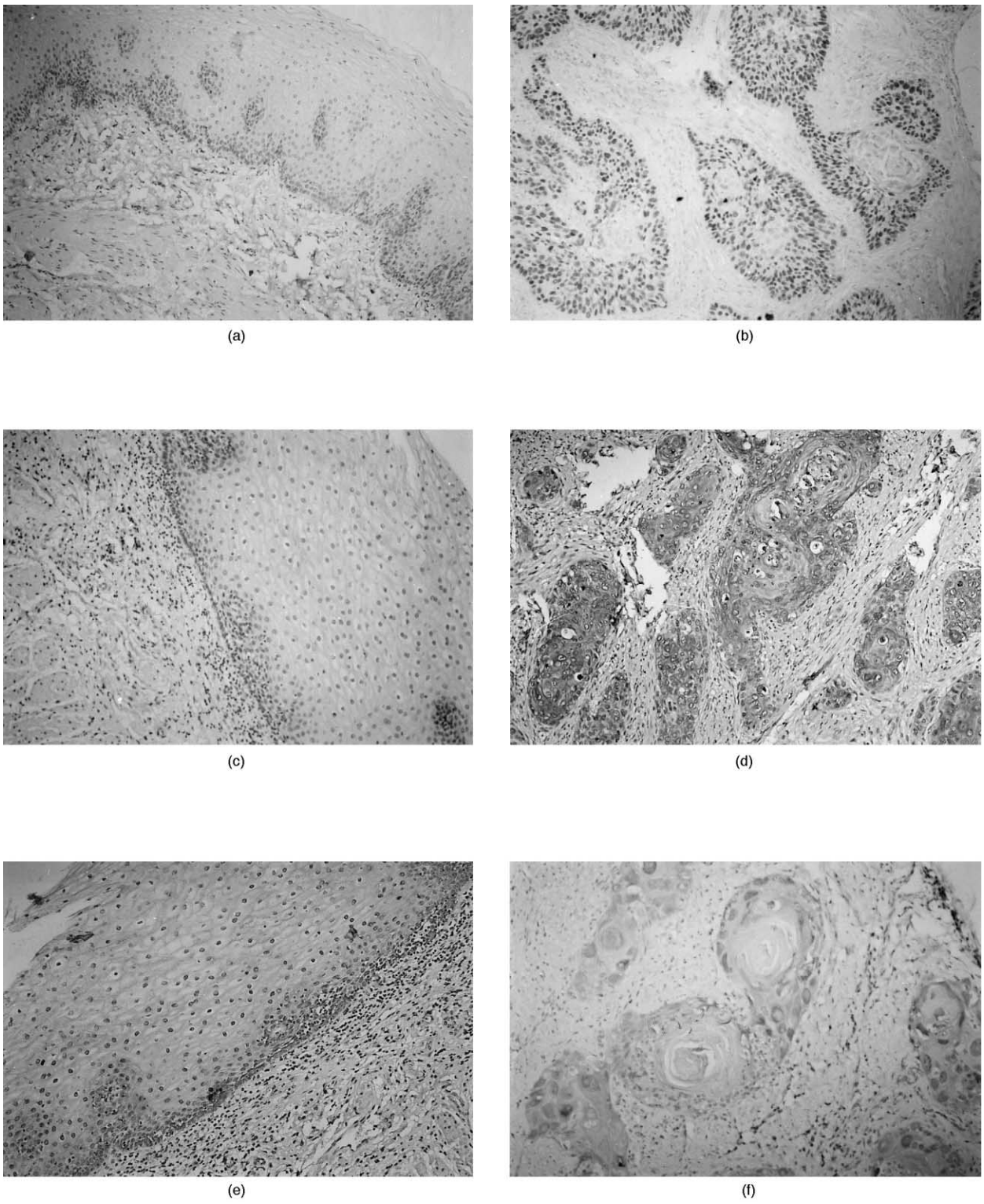

Fig. 1. Immunohistochemical detection of p53, MDM2, p21, p16, pRb and cyclin D1: (a) photomicrograph of normal epithelium showing lack of p53 immunoreactivity; (b) moderately differentiated oesophageal squamous cell carcinomas (ESCC) showing overexpression of p53 immunoreactivity; (c) photomicrograph of normal epithelium showing lack of MDM2 immunoreactivity; (d) moderately well differentiated ESCC showing overexpression of MDM2 immunoreactivity; (e) photomicrograph showing basal expression of p21 protein in the normal epithelium; (f) moderately differentiated ESCC showing lack of expression of p21 protein; (g) photomicrograph of normal epithelium showing basal level expression of cyclin D1 immunoreactivity; (h) Poorly differentiated ESCC showing cyclin D1 overexpression; (i) photomicrograph of normal epithelium showing basal p16 immunoreactivity; (j) moderately differentiated ESCC showing the loss of p16 immunoreactivity; (k) photomicrograph of normal epithelium showing basal level pRb immunoreactivity; (l) moderately differentiated ESCC showing loss of pRb immunoreactivity. a, b, c, d, e, g, h, i: original magnification $\times 100$. f, j, k, l; original magnification $\times 200$.

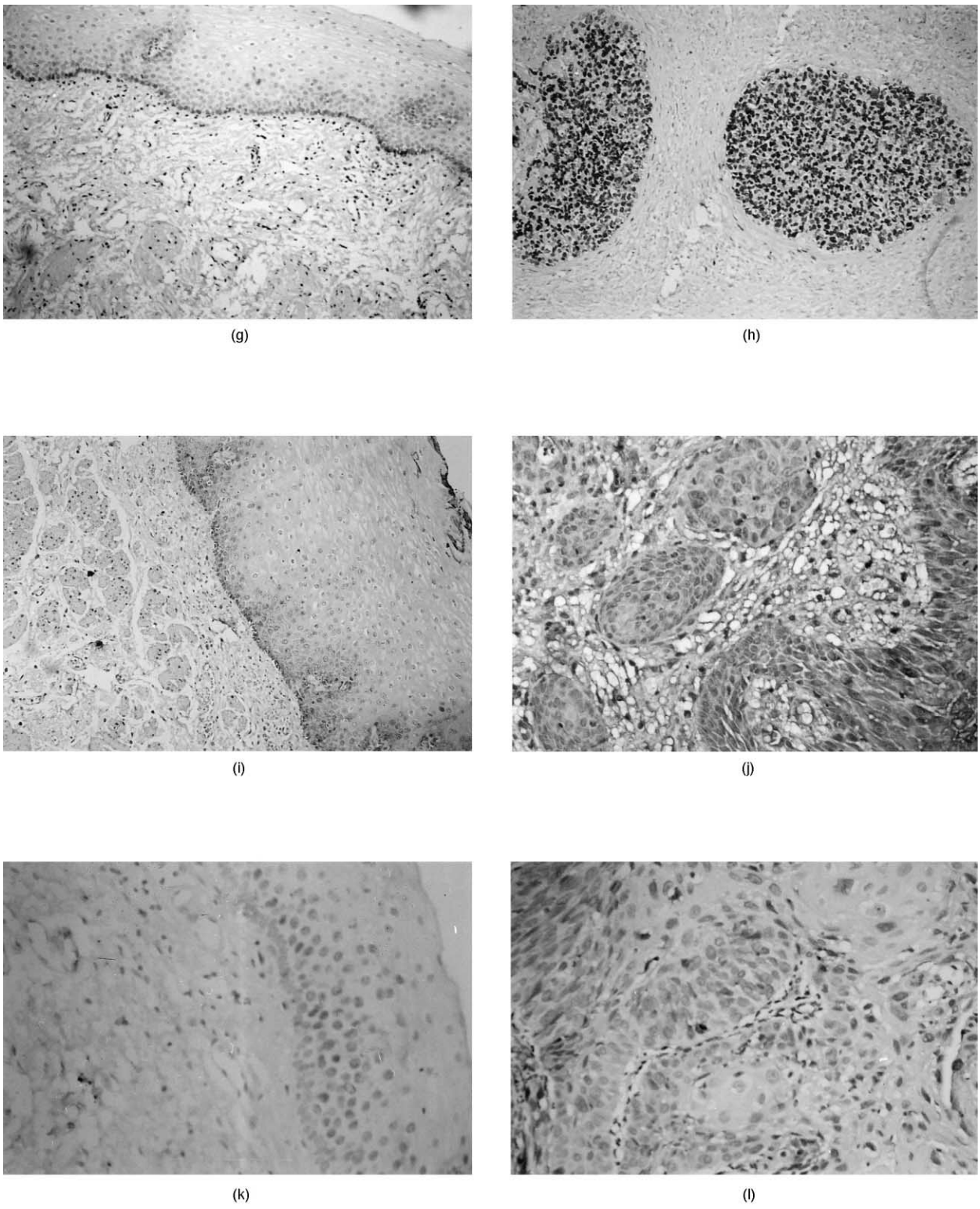

Fig. 1. *(continued)*.

$P=0.014$). Concomitant loss of p16 and pRb was also significantly associated with pT ($r=0.3304$; $P=0.004$). We also observed significant association between pRb^- ($r=0.4310$; $P=0.0007$), $p16^-/pRb^-$ ($r=0.2542$; $P=0.03$), $p53^+/pRb^-$ ($r=0.3482$; $P=0.003$), cyclin $D1^+/p16^-$ ($r=0.2817$; $P=0.016$) and cyclin $D1^+/pRb^-$ ($r=0.3202$; $P=0.006$) phenotypes and nodal metastasis. Logistic regression analysis revealed that loss of pRb was the most significant independent risk factor for nodal metastasis ($P=0.001$; OR = 8.2). In univariate

analysis, cyclin $D1^+/p16^-$ ($P=0.047$), cyclin$D1^+/pRb^-$ ($P=0.05$), phenotypes were significantly associated with distant organ metastasis.

3.3. Concomitant alterations in p53 and pRb pathways

Correlation of alterations in p53 and Rb pathways with clinicopathological parameters yielded interesting insights suggestive of a cooperative synergistic effect as prognosticators in ESCCs. $p53^+/pRb^-$ phenotype showed significant correlation with nodal metastasis ($r=0.3482$; $P=0.003$) and $MDM2^+/pRb^-$ showed significant association with distant organ metastasis ($P=0.046$). The most intriguing feature of our study was the significant association of concomitant alterations in p53, p16 and pRb ($p53^+/p16^-/pRb^-$ phenotype) with pT ($r=0.2432$; $P=0.038$). However, when adjusted for confounding variables and interactions, using a multiple logistic regression model, only loss of p16 emerged as the independent risk factor for tumour size (pT) ($P=0.03$; OR = 3.3).

3.4. Prognostic analysis of molecular alterations in p53 and pRb pathways

Prognostic analysis was carried out using six molecular markers (p53, MDM2, p21, pRb, p16 and cyclin D1), seven clinicopathological parameters (age, gender, site of tumour, histological grade, tumour stage, nodal status and distant organ metastasis) and two therapeutic variables (chemotherapy and radiation therapy). All patients underwent surgical resection with curative intent as their primary treatment. 50 patients were followed-up for disease-free survival, as well as the overall survival, for a maximum period of 44 months. During the follow-up period, 13 (26%) patients died from their disease. Stratified univariate analysis was carried out by the Kaplan–Meier method to determine the predictors of prognosis. Univariate analyses revealed that cyclin $D1^+$ ($P=0.05$), cyclin $D1^+/p16^-$ ($P=0.023$), $pRb^-/p16^-/p21^-$ ($P=0.03$) and $p53^+/p16^-/pRb^-$ ($P=0.02$) were adverse prognosticators for overall survival (Fig. 2a–d, respectively). The univariate analysis for

Table 1
Expression of p53, MDM2, p21, pRb, p16 and cyclin D1 proteins in human ESCCs

Tissue type	Total cases	p53-positive *n* (%)	MDM2-positive *n* (%)	p21-negative *n* (%)	pRb-negative *n* (%)	p16-negative *n* (%)	Cyclin D1-positive *n* (%)
ESCC	100	73 (73)	42 (42)	36 (36)	75 (75)	45 (45)	67 (67)
Age (years)							
⩽40	16	14 (88)	7 (44)	3 (19)	13 (81)	8 (50)	10 (63)
>40	84	59 (70)	35 (42)	33 (39)	62 (74)	37 (44)	57 (68)
Gender							
Male	60	43 (72)	24 (40)	22 (37)	40 (67)	31 (52)	41 (68)
Female	40	30 (75)	18 (45)	14 (35)	35 (88)	14 (35)	26 (65)
Site							
Lower 1/3rd	55	41 (75)	17 (31)	19 (35)	44 (80)	26 (47)	36 (65)
Upper and middle 1/3rd	43	31 (72)	24 (56)	17 (40)	29 (67)	19 (44)	29 (67)
Histological grade (differential)							
Well	35	30 (86)	13 (37)	14 (40)	28 (80)	20 (57)	24 (69)
Moderate	41	27 (66)	17 (41)	14 (34)	32 (78)	16 (39)	26 (63)
Poor	16	11 (69)	7 (44)	6 (38)	8 (50)	9 (56)	11 (69)
Tumour stage							
T_1/T_2	40	31 (78)	15 (38)	16 (40)	28 (70)	14 (35)	24 (60)
T_3/T_4	33	25 (76)	9 (27)	10 (30)	26 (79)	21 (64)	21 (64)
Nodal status							
Nodes positive (N_+)	28	20 (71)	10 (36)	11 (39)	14 (50)	11 (39)	15 (54)
Nodes negative (N_0)	45	36 (80)	14 (31)	15 (33)	40 (89)	24 (53)	30 (67)
Metastasis							
M_0	62	50 (81)	17 (27)	23 (37)	46 (74)	32 (52)	40 (65)
M_X	11	6 (55)	7 (64)	3 (27)	8 (73)	3 (27)	5 (45)
Normal mucosa	100	9 (9)	5 (5)	4 (4)	6 (6)	–	4 (4)

ESCC, oesophageal squamous cell carcinomas.
TNM staging was available for 73 cases, and histopathological grading was available for 92 cases. Site was available for 98 cases.

disease-free survival by Kaplan–Meier method revealed that tumour stage (pT; $P=0.05$) and cyclin D1$^+$ ($P=0.03$) were significantly associated with disease-free survival (Fig. 3a and b).

4. Discussion

To the best of our knowledge, this is the first composite report demonstrating frequent alterations in the expression of major cell cycle regulatory proteins of the p53-MDM2-p21 and p16-pRb-cyclin D1 pathways and their association with various clinicopathological parameters of oesophageal squamous cell carcinoma and disease prognosis in the Indian subcontinent. Mutations in both of these regulatory pathways have been reported in several human tumours [18]. Interestingly, all the ESCC cases analysed herein showed alterations in at least one of the components of these pathways, underscoring their importance in oesophageal tumorigenesis. Overexpression of p53, MDM2 and cyclin D1 proteins was observed in 73, 42 and 67% of the cases, respectively, while loss of expression of p21, p16 and pRb was observed in 36, 45 and 75% cases, respectively. Earlier reports on alterations in cell cycle regulatory proteins in oesophageal cancer have shown similar expression pattern of these proteins, although the protein expression rates were different from those observed in this study [19–21]. This discrepancy in protein expression rates may be due to the differences in sample size and the ethnicity of the population studied. Alternatively, these variations may be attributed to the differences in the major dietary and habitual risk factors (smokeless tobacco) associated with the disease in the Indian population. In a preliminary report, we have observed similar pRb and p16 protein expression in an analysis of a different cohort [22]. Recent studies suggest that alterations in pRb and p53 pathways exert a synergistic effect on tumour progression and survival rates [23–25]. Our data provide evidence for cooperative effects of alterations in both p53 and pRb pathways in imparting an aggressive tumour phenotype. Cases with con-

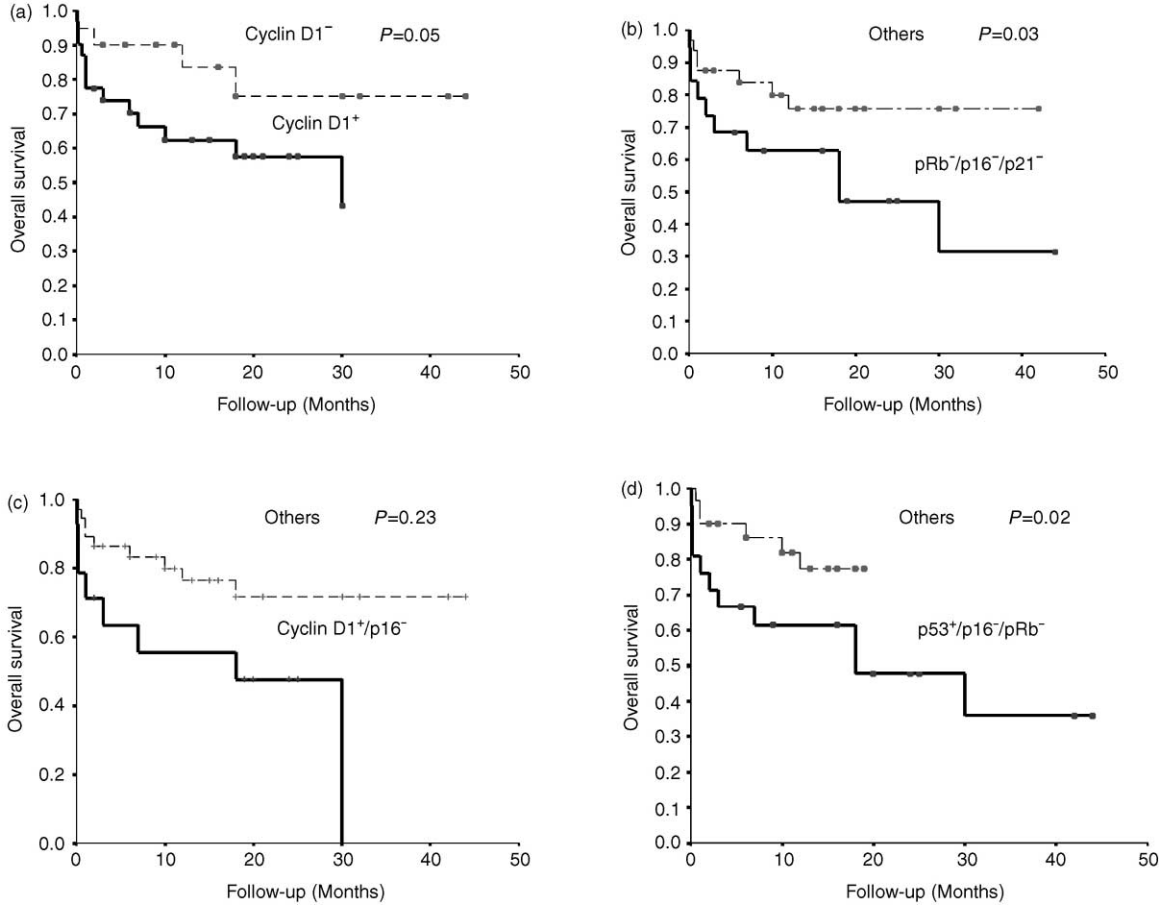

Fig. 2. Kaplan–Meier estimation of postoesophagectomy overall survival of patients with primary oesophageal squamous cell carcinoma in relation to cell cycle regulatory proteins. Patients with cancer-related death were classified as failures, whereas patients surviving without disease or dead from other causes were censored. The median time for overall survival in the different groups analysed herein was: (a) cyclin D1$^+$ group 30 months (range: 6.5–53.5 months); (b) cyclin D1$^+$/p16$^-$ group 18 months (range: 6.79–29.21 months); (c) pRb$^-$/p16$^-$/p21$^-$ group 18 months (range: 0–36.34 months); and (d) p53$^+$/p16$^-$/pRb$^-$ group 18 months (range: 0–38.95 months).

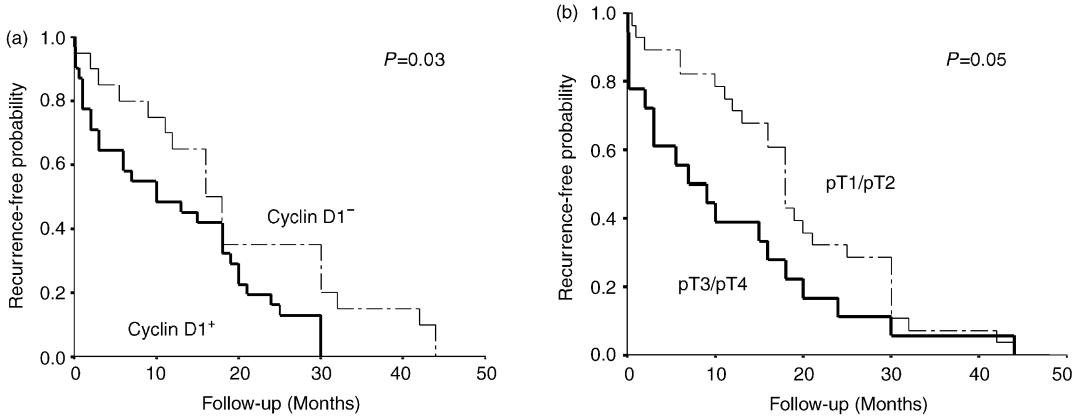

Fig. 3. Kaplan–Meier estimation of postoesophagectomy disease-free survival of patients with primary oesophageal squamous cell carcinoma in relation to tumour stage (pT1/pT2 and pT3/pT4) and expression of cyclin D1. Patients with cancer relapse were classified as failures, whereas patients without evidence of the disease or dead from other causes were censored. The median survival time for groups analysed herein was: (a) cyclin $D1^+$ group 10 months and (b) pT3/pT4 group 7 months.

comitant alterations in tumour suppressor proteins, p16, pRb and p53 ($p53^+/p16^-/pRb^-$) analysed in our study, showed significant association with advanced tumour stage (pT) and poor prognosis. Furthermore, tumours with alterations in both pathways such as, $p53^+/pRb^-$, $MDM2^+/pRb^-$ and $p53^+$/cyclin $D1^+$ phenotypes showed significant association with nodal metastasis and distant organ metastasis, respectively, in the cohort of patients analysed. Thus, our data suggest that poor prognosis in this cohort of patients may be attributable to the combination of alterations of p53 and pRb pathways. Different combinations of genetic/epigenetic alterations have been reported to inactivate p53 and pRb pathways in other human cancers such as bladder cancer [26] and lung cancer [27]. Furthermore, mice mutants for pRb and p53 have reduced viability and increased tumour burden and metastasis [28]. More substantial evidence of the interaction between these two pathways is demonstrated by the recently proposed trimeric complex model which suggests that regulation of p53 by MDM2 is modulated by pRb [23]. According to this model, pRb regulates the apoptotic function of p53 through binding to MDM2, thus preventing MDM2 from targeting p53 for degradation [29]. The ability of pRb to counteract the negative regulation of MDM2 on p53-induced apoptosis would predict that loss of pRb will result in the impaired tumour suppression function of p53. Our observation that $MDM2^+/pRb^-$ phenotypes significantly correlate with distant organ metastasis further strengthens this paradigm. It may therefore be hypothesised that the loss of pRb in these tumours imparts a selective growth advantage by releasing MDM2, facilitating p53 degradation. Furthermore, loss of pRb is also identified as an independent risk factor for lymph node metastasis in our study suggesting its involvement in tumour growth.

Downregulation of the pRb pathway may also occur due to loss of p16 or overexpression of cyclin D1 despite harbouring a functional pRb protein. Alterations in the p16 protein are shown to be closely associated with the pathogenesis of oesophageal cancer [19]. Consistent with this, we find a significant association of the cyclin $D1^+/p16^-$ phenotype with lymph node metastasis, distant organ metastasis and overall survival. Furthermore, loss of p16 ($p16^-$) and overexpression of cyclin D1 (cyclin $D1^+$) were identified as independent prognosticators for tumour stage (pT) and disease-free survival, respectively. The loss of expression of $p16^{INK4a}$ may be due to the homozygous deletion of the *INK4a* locus, which in turn might result in a concomitant loss of $p19^{ARF}$ and taken together with the fact that *Rb* is a common target for both cascades, we propose that $p16^-/pRb^-$ may represent an aggressive phenotype. Analysis of the significance of this phenotype in presenting an aggressive phenotype revealed significant correlation of this double hit phenotype ($p16^-/pRb^-$), with nodal metastasis ($P=0.03$) corroborating our previous preliminary findings in another cohort of ESCCs [30].

Another important observation of our study was the significant association of MDM2 immunoreactivity with distant organ metastasis in multivariate analysis by stepwise logistic regression ($P=0.05$; OR = 4.6). ESCC is one of the most malignant neoplasms with a high propensity for invasion and metastasis. Significant association of MDM2 overexpression with aggressive tumour characteristics such as advanced tumour stage (pT) and metastasis have been demonstrated in intrahepatic cholangiocarcinoma [31], testicular germ cell tumours [32] and breast carcinoma [33]. In this context, the association of MDM2 accumulation with distant organ metastasis in the logistic regression analysis observed in our study suggests that MDM2 may serve as a putative marker for identifying aggressive ESCCs. Moreover, cyclin D1 overexpression showed significant association with disease-related mortality in univariate analysis by Chi square test ($P=0.05$). Survival analysis

by the Kaplan–Meier method showed a significant inverse association between cyclin D1 overexpression and disease-free survival (OR; 4.34; $P = 0.03$). Cyclin D1 and MDM2 have been shown to be associated with a shorter survival in oesophageal cancer patients [34]. The relationship between cyclin D1 expression and haematological recurrence has been proposed as a prognostic marker in ESCCs [4,35]. Our studies further support the role of cyclin D1 overexpression as a predictor for disease recurrence in ESCCs.

In conclusion, we report the biological impact of inactivation of different components in the pRb and p53 pathways in ESCCs highlighting the significance of disruption of pRb and p53 cell cycle checkpoints in oesophageal tumorigenesis and disease prognosis. It may be emphasised that many of the respective proteins interact with each other in complex networks, that display negative or positive feedback loops. Therefore, the accumulated effects of multiple alterations in these tumour cells can lead to aggressive tumour behaviour as observed in our study. These findings may have a potential impact on the clinical prognosis of oesophageal cancer.

Acknowledgements

The contract grant sponsor was the Council of Scientific and Industrial Research, India.

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