Alterations in cyclin D1 expression in esophageal squamous cell carcinoma in the Indian population

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Received: 3 May 2000 / Accepted: 10 July 2000

Abstract Purpose: The p16/cyclin D1/pRb pathway plays a critical role in tumorigenesis. We recently reported alterations in expression of tumour suppressor gene products, p16 and pRb in esophageal cancer. Knowledge of alterations in cyclin D1, a vital component of this pathway in esophageal carcinomas from the Indian subcontinent, where the etiology and pathogenesis may be confounded by various unique dietary and environmental factors, is presently scanty. In order to bridge the gap between the accentuating incidence of esophageal cancer and aberrations in the components of this vital pathway, we analysed cyclin D1 expression in esophageal squamous cell carcinoma in the Indian population. Method: Immunohistochemical analysis of cyclin D1 expression was carried out in paraflin-embedded sections of surgically resected esophageal squamous cell carcinomas (ESCC) (70 patients) and matched with histopathologically normal esophageal tissues from a distant site. The findings were correlated with clinicopathological parameters. Results: Overexpression of cyclin D1 was observed in the tumour nuclei in 41 out of 70 (59%) patients. We found concomitant alterations in 16 and cyclin D1 (p16/−/CycD1+/− phenotype) in 16 of the 70 patients (23%), while alterations of pRb and cyclin D1 (pRb+/CycD1−) were observed in 36 of the 70 (51%) patients of ESCCs. Cyclin D1 overexpression was significantly associated with the loss of p16 immunoreactivity (P = 0.005). The pRb− and p16−/pRb−/Cyc D+ phenotypes showed significant association with differentiation of the tumour (P = 0.005, 0.05, respectively). Kaplan-Meier analysis for disease recurrence showed increased disease recurrence in cyclin D1 overexpressed patients. Median time to disease recurrence in the cyclin D1+ group was 15 months as against 18 months observed in the cyclin D1− patients (P = 0.067; log-rank test). Conclusion: Alterations in at least one of the components of the p16/cyclin D1/pRb pathway in majority of the 70 patients analysed herein, and concomitant alterations in all the three proteins in 19 patients (35%) underscore the critical role of this pathway in esophageal tumorigenesis. The results of the present study taken together with our previous findings on p16 and pRb alterations in ESCCs suggest that these alterations are not mutually exclusive and may cooperatively provide greater tumour growth advantage. The prognostic significance of alterations in the expression of these components cyclin D1, p16, and pRb remains to be established in a larger cohort.

Key words cyclin D1 · pRb · p16 · Esophageal cancer · Cell cycle

Introduction

Esophageal squamous cell carcinoma (ESCC) is one of the most lethal malignancies, exhibiting wide regional variation and causal association with exogenous risk factors such as alcohol consumption and tobacco smoking in Western countries and a variety of dietary factors confounded by nutritional deficiency in several regions of India and China (Munoz and Day 1996; Gaur et al. 1997; Yu et al. 1988; El-Serag and Sonnenberg 1999). High consumption of sun-dried and pickled vegetables, red chillies and spices, bidi smoking (an Indian cigarette comprising of tobacco rolled in a dried tabunari leaf), pan chewing (pan is an Indian masticatory
consisting of betel leaf coated with lime filled with areca nut with or without tobacco) and pan-tobacco chewing are implicated as major predisposing factors to esophageal tumourgenesis in the Indian population (Notani 1988; Siddiqi et al. 1991). The differences in these causal agents and genetic background could lead to regional variations in molecular/genetic alterations observed in esophageal cancer reflected as causal heterogeneity and histological sub-types (Stemmermann et al. 1994). The Indian population, with poor nutritional status and high exposure to these dietary risk factors may therefore serve as an important in vivo model to investigate the alterations in oncogenes and tumour suppressor genes implicated in the pathogenesis of ESCC, leading to the identification of novel predisposing factors or molecular markers for early diagnosis.

The orchestration of complex process of cell cycle regulation via G1 arrest is mediated through a meticulously regulated network of events involving the tumour suppressor gene products p16 and pRb and the oncogene product cyclin D1, which is collectively referred to as the p16/cyclin D1/pRb pathway. The proteins involved in this regulatory pathway include the cyclin-dependent kinases (CDKs) which, when activated by binding to cyclins, phosphorylate the retinoblastoma protein (pRb), resulting in the release of transcription factors of the E2F family, required for activating the S phase genes. Therefore, a perturbation in any individual component is likely to have a profound oncogenic effect, as in the case of amplification and/or overexpression of cyclin D1 (Lammic et al. 1991; Nakagawa et al. 1995; Montesano et al. 1996; Shimada et al. 1999), mutation of CDK 4 (He et al. 1995), and loss of p16 (Reed et al. 1995; Sonoda et al. 1995) or pRb (Harbour et al. 1988).

Overexpression of cyclin D1 is observed in various human carcinomas including that of the esophagus (Jiang et al. 1992; Sheyn et al. 1997). Altered expression of cyclin D1 results from rearrangement (isolated as PRAD-1 in parathyroid adenomas) (Arnold et al. 1989), translocation (isolated as bcl-1 in B-lymphocytic malignancies) (Withers et al. 1991), amplification and/or overexpression in head and neck, breast, esophageal squamous cell carcinoma, non-small cell lung cancer, colon, urinary bladder, and ovarian cancer (Hall et al. 1996; Worsley et al. 1997; Shigemasa et al. 1999). Loss of pRb function either by mutation or viral inactivation correlates with a decrease in cyclin D1 expression, suggesting an interplay between pRb and cyclin D1 in tumour cells (Bajersbergen et al. 1996). The significant role played by cyclin D1 is evident from the fact that microinjection of cyclin D1 antibodies into mid-G1 phase causes late G1 arrest in tumour as well as normal cells (Lukas et al. 1994). Differential expression of cyclin D1 and pRb proteins has been reported, suggesting additional mechanisms affecting G1/S phase control in esophageal carcinogenesis (Montesano et al. 1996). Correlations between the abnormalities in p16 and pRb as well as between cyclin D1 and pRb have been observed in ESCCs (Enders et al. 1996). Furthermore, interest has been focussed on these two genes following the finding that phosphorylation of pRb in the early G1 phase is regulated positively by cyclin D1/CDK4 and negatively by p16. Alterations in p16INK4a and cyclin D1 genes disrupt their expression leading to p16 loss and cyclin D1 overexpression (Kratzke et al. 1995; Narito et al. 1995; Garadts et al. 1996; Fueyo et al. 1996; Maesawa et al. 1996; Reed et al. 1996).

It has been proposed that environmental and genetic factors cooperate in the molecular pathogenesis of ESCCs and that abnormalities in cyclin D1 expression play a key role. Nevertheless, very little is known about the molecular events in the development of ESCCs in the Indian population. The association between p16 and cyclin D1 alterations in ESCCs has been investigated (Takeuchi et al. 1997), however, to our knowledge there are no reports on concomitant alterations in these proteins in the Indian population. The aim of the present study was to determine the alterations in cyclin D1 protein expression in ESCCs of Indian origin and determine their association with clinicopathological parameters. Furthermore, alterations in cyclin D1 expression were correlated with aberrations in p16 and pRb expression in the same cohort of tumours with the goal of gaining insight into the role of the p16/cyclin D1/pRb pathway in esophageal tumourgenesis in the Indian population.

Methods

Tissue specimens

Seventy esophageal cancer patients enrolled in the Surgical Oncology Unit, Institute Rotary Cancer Hospital, All India Institute of Medical Sciences, India, were inducted into the study with prior consent. A structured questionnaire was used to collect information on various demographic, socio-economic, occupational, nutritional, and life-style variables. Surgical specimens from untreated primary esophageal tumours (70 patients) and matched histopathologically normal esophageal tissue specimens taken from a site distant from the cancerous lesion were formalin-fixed and paraffin-embedded. The histopathologically confirmed ESCCs and matched normal esophagal tissue sections having no contaminating tumour cells as determined by hematoxylin and eosin staining were used for immunohistochemical analysis. Each patient’s clinical status was classified according to the clinical tumour, node, and metastasis (pTNM) classification system (Sobin and Wittekind 1997). The patients were grouped based on the tumour stage (pT1/ pT2 patients; pT3/pT4 patients), nodal metastasis (pN0 patients; pN1 patients), and distant organ metastasis (pM0 patients; pM1 patients).

Immunohistochemistry

Paraffin-embedded (5-μ thickness) human ESCC and matched normal tissues were used for histopathological analysis after antigen retrieval. Briefly, tissues were deparaffinized in xylene, hydrated, and incubated with 0.5% H2O2 in methanol for 20 min to inactivate endogenous peroxidase. Slides were then washed with Tri-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