Frequent alterations in the expression of tumor suppressor genes p16<sub>INK4a</sub> and pRb in esophageal squamous cell carcinoma in the Indian population

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Abstract Purpose: Alterations in the cell cycle regulatory p16<sub>INK4a</sub>/Cyclin D1/pRb pathway play a pivotal role in tumorigenesis. Knowledge of alterations in the tumor suppressor protein pRb and its negative regulator, p16<sub>CDKN2/MTS1</sub>/INK4a in esophageal squamous cell carcinoma (ESCC) from the Indian subcontinent is meager. To gain insight into the mechanisms underlying tumorigenesis and to search for diagnostic molecular markers for ESCC, we analyzed the expression of p16<sub>INK4a</sub> and pRb in ESCCs in the Indian population.

Methods: Immunohistochemical analysis of pRb and p16<sub>INK4a</sub> proteins was carried out in paraffin-embedded sections from 61 surgically resected ESCCs and matched normal tissues, and the results correlated with clinicopathological parameters using chi square and Fisher’s exact tests. Dual immunohistochemical analysis has been carried out to demonstrate the concomitant loss of expression of p16<sub>INK4a</sub> and pRb. Results: Fifty-nine of 61 (97%) cases showed aberration(s) in either or both of these proteins confirming their critical role in esophageal tumorigenesis. Loss of pRb was observed in 51 of the 61 (84%) and loss of p16<sub>INK4a</sub> was observed in 35 of 61 (57%) cases. Loss of pRb showed significant association with dedifferentiation of the tumor (P = 0.004). p16<sup>-</sup>/pRb<sup>-</sup>, and p16<sup>+</sup>/pRb<sup>-</sup> phenotypes were significantly associated with nodal metastasis (P = 0.017 and 0.027, respectively), while p16<sup>-</sup>/pRb<sup>+</sup> phenotype was associated with dedifferentiation of the tumor (P = 0.012).

Conclusion: pRb/p16<sub>INK4a</sub> pathway plays a critical role in esophageal tumorigenesis in the Indian population. The dual hits (concomitant loss) of pRb and p16<sub>INK4a</sub> expression suggest that these two components are not mutually exclusive, and can both be altered in a significant proportion of primary ESCCs serving as putative diagnostic markers for esophageal cancer. However, the impact of dual hit on tumor behavior and disease prognosis remains to be determined.

Key words p16 · pRb · INK4a · Esophageal cancer

Introduction

Esophageal cancer is one of the most common malignancies of the upper aero-digestive tract, characterized by late clinical presentation, rapid progression, and poor survival (Landis et al. 1999). It exhibits an uneven geographic distribution and a high incidence rate in developing countries like India. The complex multifactorial etiology with alarmingly poor prognosis is compounded by the synergistic effect of environmental and dietary factors and warrants an in-depth analysis of molecular aberrations in the pathogenesis of ESCC. Many dietary and habitual factors have been implicated in the pathogenesis of ESCCs (Notani et al. 1988; Yu et al. 1988; Siddiqi et al. 1991; Munoz and Day 1996; Gaur et al. 1997). 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 cell cycle regulatory pathways implicated in the pathogenesis of ESCC, which may be instrumental in understanding the molecular mechanisms underlying the process of esophageal carcinogenesis.

p16<sub>INK4a</sub> protein, encoded by the INK4a locus mapped to the chromosomal location 9p21, is a frequent
site of allelic loss (LOH) in many human malignancies. This locus has two unique first exons splicing into common exons 2 and 3 and codes for a second protein p19ARF through an alternative reading frame. The functional consequence of ARF regulated p53 levels via MDM 2 mediated proteolysis is evidenced by the ability of ectopically expressed ARF to restore a p53 imposed G1 cell arrest that is otherwise abrogated by MDM 2. The subcellular events leading to the deletion of this locus can, therefore, result in the loss of expression of both the proteins and simultaneously impair both the INK4a-CyclinD1-cdk4 and ARF-MDM 2-p53 pathways. In human cancers the estimated frequency of genetic alterations involving the p16INK4a locus is believed to be second only to the alterations in p53. In addition, alterations in other tumor suppressor genes such as pRb may also be involved in promoting malignant transformation by accelerating proliferation in a synergistic mode of action. Functional loss of Rb, due to loss of one allele by germ line or early somatic mutation and subsequent alteration of the other allele has been reported in many types of cancers. LOH at the Rb locus is therefore an important event reflecting potential functional loss in the Rb gene and has been shown to be the main target during the development of ESCC (Xing 1999). Loss of expression of pRb as detected by immunohistochemistry has been correlated with the loss of heterozygosity at the pRb locus in ESCC (Xing 1999). An imbalance in the cell cycle regulatory pathway involving p16INK4a/pRb may interfere with the terminal differentiation eventually leading to unrestricted proliferation and metastasis. Abnormalities in p16INK4a/Cyclin D1/pRb pathway are common in human neoplasia (Geradts et al. 1995). These and other data led to the concept of cyclin D, p16INK4a, and pRb as alternative targets within a common route to tumorigenesis. The emerging critical role of the p16INK4a-cyclin D/cdk4-pRb-E2F pathway in cell cycle regulation is further supported by frequent aberrations of the individual components of this pathway (Kamb et al. 1994; Sherr and Roberts 1995; Weinberg et al. 1995). A novel role for pRb has been recently reported in the inhibition of S-phase progression that is distinct from the inhibition of the G1/S transition, which suggests that continued phosphorylation of pRb beyond G1/S is required for the completion of DNA replication (Knudsen 1998). G1 arrest in response to the cyclin-dependent kinase inhibitors p21Cip1/Waf1 expression also correlates with the presence of functional pRb, suggesting the role of pRb in the prevention of DNA replication in p21 mediated G2-arrested cells. These data provide a cogent support for the view that the primary target of the Cip/Kip family of tumor suppressor genes leading to efficient G1 arrest as well as to blockade of DNA replication from either G1 or G2 phase is indeed the pRb pathway (Niculescu et al. 1998), and are therefore important targets for neoplastic alterations.

We have previously reported high prevalence of p53 protein accumulation and the humoral immune response to intratumoral p53 protein accumulation in esophageal squamous cell carcinomas (ESCCs) (Gaur et al. 1997; Ralhan et al. 2000) which have been considered to be early events in the development of esophageal cancer (Montesano et al. 1996). However, very little is known about the clinical and biological implications of the inactivation of positive/negative regulators involved in the Rb pathway of the cell cycle regulation such as p16INK4a and pRb in the esophageal oncogenesis in the Indian population. The information on the interrelationships between underlying targets implicated in esophageal tumorigenesis will immensely help in identifying the individuals at high risk for esophageal cancer in the Indian population, wherein the poor prognosis is known to be associated with the late clinical presentation and diagnosis of the disease. The paucity of information regarding expression status of these proteins and their biological consequences in pathogenesis of Indian ESCC prompted us to undertake the present study so as to characterize the expression of p16INK4a and pRb in a series of ESCC and ascertain their putative role in esophageal tumorigenesis, interrelationships, and clinical implications.

Materials and methods

Tissue specimens

Sixty-one esophageal cancer cases 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 including the consumption of red chillies, spices, polyunsaturated fats (oil), hot tea, alcohol, and tobacco (chewing and/or smoking). Surgical specimens from untreated primary esophageal tumors, and paired normal esophageal tissue specimens (61 cases) taken from a site distant from the cancerous lesion were snap frozen and stored at ~70 °C till further use. The histopathologically confirmed ESCCs and paired normal esophageal tissue sections having no contaminating tumor cells as determined by hematoxylin and eosin staining, were used for analysis. Each patient’s clinical status was classified according to the pathological grade of the tumor, node, metastasis (pTNM) classification system (Sobin and Wittekind 1997). The patients were grouped based on the tumor stage (pT1/pT2 or pT3/pT4), nodal metastasis (pN0 or pN1), and distant organ metastasis (pM0 or pM1).

Immunohistochemistry

Paraffin embedded sections/cryosections (5 μm) thickness of human ECs were used for the analysis. Paraffin-embedded sections were collected on gelatin coated slides. Matched tissue sections were stained with hematoxylin and eosin. Antigen retrieval was carried out by microwaving in citrate buffer (pH 6.0) as described (Shimada et al. 1999). The sections were incubated in methanol containing hydrogen peroxide (0.3% v/v) for 20 min to quench the endogenous peroxidase activity, followed by incubation with 1% BSA for 1 h to block the non-specific binding. The sections were subsequently incubated with primary mouse monoclonal anti pRb antibody (IF8) or rabbit polyclonal p16INK4a antibody (C-20) (Santa Cruz Biotechnology, Santa Cruz, Calif., USA) for 16 h at 4 °C and washed with PBS. The primary antibody was detected using biotinylated secondary antibody and avidin-biotin complex by ABC method as described previously (Gaur et al. 1997). Incubations were performed at room temperature in a moist