Amplification and Expression of the Human Cyclin D Gene in Esophageal Cancer

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Abstract

Amplification of the hst-1 and int-2 genes on chromosome 11q13 has previously been found in over 20% of human primary esophageal cancers. However, these two genes do not appear to be transcribed in appreciable amounts. Recently, the human cyclin D gene (also referred to as pradl) has been mapped to the 11q13 locus. Here, we report coamplification of the cyclin D and hst-1 genes in 5 of 20 (25%) human squamous esophageal tumors. We also detected significant levels of cyclin D transcription in two esophageal carcinoma cell lines, even though they did not express detectable amounts of hst-1 transcription. These findings provide the first evidence for the amplification of a cyclin gene in human esophageal cancer and suggest that an increase in cyclin D gene dosage could be an important factor in the pathogenesis of esophageal cancer. Additionally, because the 11q13 locus is found to be amplified in many types of human tumors, cyclin gene amplification could also play an important role in the development of other forms of human cancer.

Introduction

Esophageal cancer occurs at very high frequencies in certain areas of the world, including specific regions in China, Iran, South Africa, Uruguay, and France (1). The localized clustering of this disease has been the focus of numerous epidemiological studies. These studies have revealed several factors that are associated with the incidence of esophageal cancer. Factors implicated include nitrosamines in food sources and certain nutritional deficiencies in China (2) and high consumption of alcohol and cigarette smoking in Western countries (3).

Molecular studies of human esophageal tumors have revealed a frequent display of several genetic abnormalities. These include: (a) amplification of the cellular oncogene c-myc, the epidermal growth factor receptor gene (4), and the growth factor genes int-2 and hst-1 (5-7); (b) loss of heterozygosity at the retinoblastoma gene locus on chromosome 13 (8); and (c) loss of heterozygosity at the p53 gene locus on chromosome 17, along with point mutations in the coding region of the remaining allele (5, 9-11). Cytogenetic studies have revealed numerous chromosomal abnormalities in esophageal cancer cell lines (12). The major changes include breakpoints and deletions at 3p14, 11q11q12, and 9q11q12 and at the centromeric regions of all of the acrocentric chromosomes (12). Three esophageal cancer cell lines displayed a homogeneously staining region that occurred as an extension of 11q12 (12). Activating mutations in ras oncogenes appear to be absent or rare in human esophageal cancer (13-15).

Over 20% of human esophageal tumors display evidence of chromosome 11q13 amplification (5-7). This amplification is also found in carcinomas of the breast, bladder, and head and neck and poorly differentiated squamous cell lung carcinomas (16-18). Furthermore, chromosomal rearrangements involving the 11q13 region occur in certain B-lymphocyte malignancies and parathyroid tumors (19-20). These findings indicate that the 11q13 locus harbors targets involved in the development of several types of human cancer.

Much work has been devoted to developing a detailed genetic map of the 11q13 region with the goal of identifying genes whose overexpression might be implicated in tumorigenesis. This region has been found to encode several growth factor genes, including the fibroblast growth factor-related int-2 and hst-1 genes. The bcl-1 locus, involved in translocations found in certain B-lymphocyte malignancies, has also been mapped to this region. Paradoxically, little or no int-2 or hst-1 gene transcription is detected in either primary esophageal tumors or esophageal tumor cell lines (7).

In this context, it is of interest that Motokura et al. (21) recently reported the isolation of a potential oncogene, pradl, from a rearranged chromosome 11q13 found in parathyroid tumors. The complementary DNA sequence of pradl was found to be identical to that of the human cyclin D complementary DNA. The cyclin D gene encodes a cell cycle-regulatory protein that is found at high levels during the G1 phase of the cell cycle and was initially isolated through its ability to complement a Saccharomyces cerevisiae strain that was mutant in three known G1 cyclins (22). More recently, activation of the cyclin D gene has been implicated as a critical event in translocations involving the bcl-1 locus (23).

Based on these findings, we examined DNA from 20 primary squamous cell esophageal tumors for cyclin D gene amplification. In this paper, we report the coamplification of the hst-1 and cyclin D genes in 5 of these 20 tumors. In addition, we describe significant levels of transcripts from the cyclin D gene in two human esophageal cancer cell lines.

Materials and Methods

Tissues and Cell Lines. A total of 20 human esophageal squamous cell carcinomas were obtained from patients who underwent surgery in the People's Republic of China. Adjacent nontumorous esophageal specimens were also available from some of these patients. A normal esophageal sample was also obtained from a postmortem autopsy specimen collected at Columbia Presbyterian Medical Center in New York City. The A431 vulval epithelial cell line and the EC 109 and EC 17 esophageal squamous carcinoma cell lines (24) were provided by Dr. J. Schlessinger and Dr. K. Xu, respectively.

DNA and RNA Analysis. High-molecular-weight DNA was isolated from frozen tissue specimens as previously described (4). Briefly, tissue was placed in liquid nitrogen and pulverized with a blender. The tissue powder was then lysed with DNA lysis buffer (10 mM Tris, pH 7.9; 10 mM NaCl; 0.1% sodium dodecyl sulfate; 200 μg/ml proteinase K). Confluent cultured cells were washed with phosphate-buffered saline and lysed with DNA lysis buffer. DNA was isolated after phenol/chloroform extraction and ethanol precipitation.

Ten μg of genomic DNA were digested to completion with the
Fig. 1. Coamplification of the cyclin D and Ast-1 genes in human esophageal squamous cell carcinomas. Approximately 10 µg of human genomic DNA from the indicated esophageal tumors and adjacent normal esophageal tissue were separately digested with EcoRI and electrophoresed through a 1% agarose gel. DNA was transferred to nylon-N membranes as described in "Materials and Methods." Separate membranes were then hybridized to the indicated probes and exposed to film. A, top, Southern blot using the cyclin D probe; bottom, Southern blot using the PSTI control probe. B, Southern blot using the Ast-1 probe. Tops of lanes, sample numbers; left ordinate, molecular weight marker sizes; right ordinate, sizes of the bands.

Restriction enzyme EcoRI (N.E. Biolabs, Beverly, MA). The digested DNA was electrophoresed through a 1% agarose gel. DNA was then transferred to Hybond-N hybridization membranes, as previously described (4).

Total RNA was prepared from cultured cell lines by lysis in guanidinium thiocyanate and centrifugation through cesium chloride, as described (25). Twenty µg of total RNA were electrophoresed through a 1% denaturing agarose gel containing 17% formaldehyde. RNA was transferred to Hybond-N hybridization membranes as described above.

Probes. The entire human cyclin D (prad1) coding region (21-22) was isolated by reverse-transcription PCR3 using the following oligo-

3 The abbreviations used are: PCR, polymerase chain reaction; PSTI, pancreatic secretory trypsin inhibitor.

nucleotide primers:

- cyclin D 5': 5'-AAG CCT CAG CCC TCC CCA GCT GCC CAG-3'

- cyclin D 3': 5'-AAC CAA CAA GGA GGA TG-3'

One µg of EC17 cell line polyadenylated RNA was reverse-transcribed using the cyclin D 3' primer and the Amersham complementary DNA synthesis kit under conditions described by the supplier. One µl of the final 250-µl volume was subject to PCR amplification using the Cetus PCR amplification kit under conditions described by the supplier. PCR amplification parameters were 94°C denaturation for 60 s, 55°C annealing for 60 s, and 72°C elongation for 90 s, for a total of 30 cycles. The amplified 1062-base pair cyclin D fragment was isolated from a
Results and Discussion

High-molecular-weight DNA was isolated from 20 human primary esophageal tumors and adjacent nontumorous esophageal tissue when available, as well as one autopsy specimen from a patient who died of other causes. Ten μg of each DNA sample were digested to completion with the restriction endonuclease EcoRI, electrophoresed through an agarose gel, and subjected to Southern blot analysis using 32P-labeled cyclin D and hst-1 probes. The cyclin D probe detected three EcoRI fragments of 4.0, 2.2, and 2.0 kilobases (Fig. 1A), and the hst-1 probe detected three EcoRI fragments of 5.8, 2.8, and 0.8 kilobases (Fig. 1B). As a control, these Southern blots were also hybridized to a PSTI gene probe (Fig. 1A) (26). The cyclin D and hst-1 genes were coamplified in tumors 10 and 13 when compared to samples from nontumorous adjacent tissue specimens (Fig. 1). Using the PSTI bands as controls in densitometric scans of these blots, a 5-fold amplification of both the cyclin D and hst-1 genes was seen in tumor 10, and a 2-fold amplification was seen in tumor 13. Another three esophageal tumor samples showed a coamplification of both genes of 2- to 3-fold (data not shown). The same Southern blots were hybridized to a human H-ras probe to test for the possibility of chromosome 11 aneuploidy. Band intensities similar to those of the PSTI probe were seen, implying that cyclin D and hst-1 were truly amplified in the five tumors referred to above (data not shown). Fifteen additional esophageal tumor samples were analyzed and did not show evidence of cyclin D or hst-1 gene amplification when compared to the normal esophagus autopsy specimen (data not shown). No gross DNA rearrangements in the cyclin D gene or in the hst-1 gene were seen in any of the esophageal tumor samples. Although our frequency of amplification is toward the lower end of previous reports (25% versus 28–50% (5–7)), we can attribute these differences to the origin of the samples and statistical variability.

We next wanted to determine whether the cyclin D gene is transcribed in esophageal tumor cells. We were unable to obtain adequate amounts of RNA of sufficient quality to perform an analysis of cyclin D expression on the 20 primary esophageal tumor specimens. Therefore, we analyzed RNA from the human EC17 and EC109 esophageal carcinoma cell lines as well as from the A431 vulval epithelial cell line (which had previously been shown to have an amplified hst-1 gene) (28). Although the...
cyclin D and hst-1 genes did not appear to be amplified in either of the two esophageal carcinoma cell lines (Fig. 2A), significant expression of the cyclin D gene was found in the EC109 cell line, while a low level of expression was found in the EC17 cell line. The A431 vulval epithelial cell line showed both amplification of the cyclin D gene (Fig. 2A) and very high levels of 4.7- and 1.8-kilobase cyclin D transcripts (Fig. 2B). High levels of cyclin D expression were also found in the human GM6167 fibroblast cell line, the human T24 bladder carcinoma cell line, and the human HCT116 colon carcinoma cell line. Lower levels could also be detected in the human Caki-1 and Caki-2 kidney carcinoma cell lines and the human HeLa and Caski cervical carcinoma cell lines (data not shown). Expression of the hst-1 gene could not be detected in the EC109, EC17, or A431 lines or in any of the cell lines examined (data not shown).

The above results provide the first evidence that a cyclin gene, namely cyclin D, is amplified and expressed in human esophageal cancer. During the preparation of this paper, we learned that Dr. Masaaki Terada had also detected amplification of the cyclin D gene in human esophageal tumors. The chromosome 11q13 region, on which the cyclin D gene resides, is amplified in a number of different epithelial tumor types (see "Introduction"). Even though our findings are confined to squamous cell carcinomas of the esophagus, it seems likely that they might be relevant to other types of human cancer. In this vein, a recent study showed that the cyclin D gene is amplified in about 20% of human breast tumors (29). Therefore, amplification of the cyclin D gene may play an important role in the development of a variety of human cancers.

Cyclin D has been classified as a G, cyclin, as opposed to a M cyclin, based on its timing of RNA expression, its protein turnover pattern, its binding to the cdc2-related p34 protein, and its ability to complement GÌ cyclin mutants in budding yeast (22, 23, 30-31). Therefore, amplification and increased expression of cyclin D could allow cancer cells to traverse the G0 to G1 and/or the G1 to S transition under conditions that limit the growth of normal cells. Thus, amplification or rearrangement of the cyclin D gene (and possibly other cyclins), by providing an increased gene dosage effect, could play a critical role in multistage carcinogenesis. It remains to be determined whether the increased expression of cyclin D can be by itself, or in combination with other oncogenes, lead to cell transformation. These experiments are currently in progress.

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