Amplification of Epidermal Growth Factor Receptor Gene but No Evidence of ras Mutations in Primary Human Esophageal Cancers

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ABSTRACT

Primary esophageal squamous cell carcinomas from 41 patients were analyzed for the presence of proto-oncogene alterations associated with this malignancy. The occurrence of activating ras gene mutations in 25 tumors was determined using oligomer hybridization of target sequences amplified by polymerase chain reaction. We found no evidence for mutations in codons 12 and 61 of the H-ras, K-ras, and N-ras genes, nor in codon 13 of the K-ras and N-ras loci in any of these tumors. The apparent absence of activated ras oncogene in esophageal cancers represents a possible exception to the presence of these mutations found consistently in numerous other types of human malignancies, and is in striking contrast to the 40% prevalence of ras mutations in human colorectal cancers.

Southern blot hybridization experiments with DNAs from tumors demonstrated amplification of the epidermal growth factor receptor gene (c-erbB) in two of 25 carcinomas. No amplification of the structurally related c-erbB2 (neu) gene was detected. In three out of 12 carcinomas, the level of epidermal growth factor receptor RNA was significantly higher than in normal esophageal mucosal tissue. Our results suggest that enhanced transcription of the epidermal growth factor receptor gene is associated with the development of some esophageal cancers.

INTRODUCTION

Esophageal cancer is among the 10 most common cancers world-wide (1) and the large geographical variation in incidence has suggested the importance of environmental risk factors (2). Epidemiological studies have shown that this type of cancer is strongly associated with various dietary and cultural habits, such as high intake of alcohol, tobacco smoking, and certain dietary deficiencies (3,4). Some of these exposures contain carcinogenic nitrosamines (3,5). Molecular mechanisms by which such environmental carcinogens induce esophageal cancers in humans are not understood. It is feasible that cellular oncogenes represent one class of targets for these risk factors.

Proto-oncogenes that have acquired transforming activity have been identified in a variety of human malignancies (7), and of the numerous proto-oncogenes implicated in human cancers, the ras oncogene family appears to play a prominent role: activated ras genes have been detected in most types of human cancers, including cancers of the digestive system, with a frequency ranging from 5 to 40% (8,9). Mutagenic activation of the c-H-ras, c-K-ras, and N-ras genes in human tumors is due to base substitutions in codons 12, 13, and 61. Experimental studies with animals have shown that carcinogens can induce tumors with transforming ras genes in which specific point mutations equivalent to those detected in human tumor specimens have occurred (10), and that the carcinogen is critical in determining the site and nature of the mutation. Currently, attention has been given to the limitations of the transfection assay used to detect the presence of these transforming sequences that may result in an underestimation of ras gene involvement in human cancers. In a recent survey of 27 human colorectal cancer samples, particular efforts were made to remove normal tissue that would dilute a positive signal, and sensitivity was increased by amplification of target sequences and biochemical detection of c-ras mutations. With this analysis activation was observed in 40% of the tumors (11). Variations from one study to another in the percentage of tumors with c-ras mutations thus may be explained partly by the pathology of the tissue specimens and by sensitivity of the detection method.

Studies to date suggest that the prevalence and nature of proto-oncogene involvement in the development of human malignancies depend on the type of tumor. It is noteworthy that, unlike human colorectal cancer, lung cancer, and neoplasia at various other sites, neither an activated ras gene nor any other transforming gene has been demonstrated yet in human esophageal tumor DNA. A search for activated oncogenes in esophageal tumors could provide valuable insight into not only the etiology but also the natural history of this cancer. To investigate this question, we included in our samples a series of 30 primary human esophageal squamous cell tumors from the high-risk area of Normandy, France, for the study of proto-oncogene anomalies in view of the possibility that tumor incidence in some areas may be attributable in part to the presence of environmental carcinogens, many of which are capable of inducing genetic damage. In addition to activation of ras proto-oncogenes by point mutations, an event often linked to initiation or early stages of malignancy (11-13), we looked for amplification and overexpression of c-erbB and c-erbB2, two closely related proto-oncogenes coding for growth factor receptors that may play a role in later stages of tumor development.

MATERIALS AND METHODS

Tissues. The human tissue samples discussed in this report are described in Table 1. The specimens, both tumors (designated by “c”) and, in some cases adjacent normal mucosa from the same patient (designated by “n”) were obtained from 41 patients undergoing surgery for esophageal cancer. Material from 30 of these patients was obtained from the Centre Baclesse treatment center in Normandy, France (samples designated by “B”) where the incidence of esophageal cancer in some cantons is 5- to 10-fold higher than the overall national average (14). The remaining samples were obtained from Hôpital Edouard Herriot, Lyon, France (designated by “L”) and were tested only for activating mutations in the ras genes. Fresh tumor and normal tissue samples were snap frozen in liquid nitrogen, pulverised, and stored at −80°C until analysis.

Blot Hybridization Analysis of DNA and RNA. To obtain high molecular weight DNA for gene amplification analysis, 0.2 g of tissue were incubated at 55°C for 30 min in Tris lysis buffer containing 0.5% n-lauroylsarcosine, 4 M urea, and 20 units protease K. Following extractions with phenol and chloroform, and multiple washes by ethanol precipitation, DNA was digested with a 20-fold excess of restriction endonuclease EcoRI. Five μg of each sample were resolved by electrophoresis on 0.7% agarose gels, blotted onto nylon membranes (Gene Screen Plus, Dupont) in alkaline solution, and hybridized under stringent conditions (42°C, in 50% formamide) with probe radioactively labeled to 1–3 × 10⁸ cpm/μg, by 32P incorporation using random primers. Blots were washed for 1 h at 60–65°C in 2x SSC (0.3 M NaCl, 0.03 M sodium citrate) and 1.0% sodium dodecyl sulfate with constant...
agitation, followed by incubation for one hour at ambient temperature in 0.1× SSC, and then exposed to Amersham MP X-ray film at ~80°C for 12 h to 4 days. The human c-erbB-specific probe pE7 (15) and the human c-mos probe (16) were obtained from the American Type Tissue Collection. The human c-erbB2 probe was purchased from Amersham, Ltd., and the actin probe from Oncor, Inc.

Northern and RNA slot blot analyses were performed to determine the correlation between amplification of the EGFR gene and abundance of the gene transcript. When available, up to 0.2 g of tissue were used to prepare total RNA by the guanidium isothiocyanate-cesium chloride ultracentrifugation method (17). RNA was denatured by heating in 2.5 M sodium citrate, pH 7.0; EGFR, epidermal growth factor receptor; RFLP, restriction fragment length polymorphism.

Table I Human tissue specimens analyzed for proto-oncogene modifications

<table>
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<tr>
<th>Patient</th>
<th>Tissue specimen and designation</th>
<th>Gene amplification</th>
<th>Overexpression&lt;sup&gt;a&lt;/sup&gt;</th>
<th>ras&lt;sup&gt;b&lt;/sup&gt; mutation</th>
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<sup>a</sup> Prefix B, patient was hospitalized at Centre Baclesse, Caen, France; prefix L, patient underwent surgery at Hôpital Herriot, Lyon, France.
<sup>b</sup> Specimen is either from a carcinoma (c) or normal mucosa (n).
<sup>c</sup> Levels of EGFR mRNA were considered overabundant (positive) if amounts were at least 4-fold higher than levels found in RNA samples from normal mucosa specimens B9-n or B23-n. -, negative results; +, positive result (see text); no entry, not tested.
<sup>d</sup> All samples tested were screened for mutations in codons 12 or 61 of the c-H-ras, c-K-ras, and N-ros genes, and codon 13 of the K-ras and N-ros genes.

RESULTS

Analysis of Tumor DNA for Presence of ras-Activating Mutations. To ascertain the involvement of ras oncogene mutations in human esophageal squamous cell carcinomas, DNAs of 25 tumors were screened biochemically for mutations in codons 12 and 61 of the H-ras, K-ras, and N-ras genes, and codon 13 of the K-ras and N-ras genes, following polymerase chain reaction amplification of ras sequences. All activating ras mutations found thus far in human tumors have been localized exclusively to these sites (9). Our approach to the detection of activated ras genes in human tissue specimens has a number of distinct advantages over the DNA transfection assay. The sensitivity of the analysis is enhanced considerably by increasing the ratio of specific target ras sequences to all other genomic sequences. Also, partial degradation of high molecular weight DNA was spotted onto nylon filters and hybridized to each of a series of synthetic oligodeoxynucleotides corresponding to activating mutations. The series of specific oligodeoxynucleotide probes radioactively labeled with 32P and hybridized to amplified genomic ras sequences on filters are listed in Table 3 of Verlaan-de Vries et al. (18). The oligomer panel included probes specific for all ras mutations known to occur in human or animal tumors, and has been used successfully in previous studies to show the presence of point mutations in various human tumors at these loci. After hybridization, blots were washed as described previously (11).

<sup>2</sup> The abbreviations used are: SSC, standard saline citrate (0.015 M NaCl-0.015 M sodium citrate, pH 7.0); EGFR, epidermal growth factor receptor; RFLP, restriction fragment length polymorphism.
DNA from primary tissue, which hampers the ability of the transfection assay to reveal transforming sequences, does not interfere with the effectiveness of the assay. Furthermore, DNA of primary tumors was analyzed directly, avoiding possible artifacts from cell culture. No mutations were found in any of the esophageal tumor samples, 14 of which (Table 1) were from patients living in the high incidence area of Normandy; the remaining 11 samples were obtained from patients undergoing surgery at Hôpital E. Herriot, Lyon. A representative example of these hybridization assays is shown in Fig. 1. The apparent absence of ras gene activation in esophageal cancers contrasts with the 40–50% prevalence of ras mutations in human colorectal cancers (11).

EGFR Gene Amplification and Expression. Southern blot analysis of tumor tissue DNA from 25 samples obtained from Centre François Baclesse was performed to determine the possible involvement of EGFR oncogene amplification and/or overexpression in these cancers. Two of the tumors, B17-c, and B24-c, showed amplification (Fig. 2) of EGFR-specific sequences hybridizing with the probe pE7, which contains cloned human EGFR cDNA sequences. We define amplification as an at least threefold increase in band intensity relative to controls. Controls are normal tissue from the same individual (when available), or tumor tissue with the same oncogene band intensity as in normal tissues. DNA from B17-c, for which there was preliminary evidence for amplification, was re-electrophoresed adjacent to an equivalent amount of DNA from B22-c, in which no indication of amplification was obtained (Fig. 3A). The figure and subsequent slot blot analysis (not shown) shows that EGFR-specific fragments from B17-c are at least 10-fold as intense as from sample B22-c. The probe hybridizing to the membrane autoradiographed in Fig. 3A was stripped from the blot, and the membrane rehybridized (Fig. 3B) with a 2.75-kilobase human c-mos fragment to show that equivalent amounts of DNA had been loaded into each of the two lanes, and thus that the amplification in sample B17-c is oncogene-specific. As normal tissue from patient B17 was available, the EGFR-specific DNA from this tissue was compared with tumor samples in which there is no EGFR gene amplification. As Fig. 3C indicates, there is no apparent increase in EGFR gene copy number in DNA from histologically normal esophageal tissue B17-n, indicating that overall there is no change in copy number of the EGFR gene in normal cells surrounding the neoplasm in which amplification was detected.

Blots were treated to remove hybridized probe and were rehybridized with the human c-erbB2 probe. No evidence for
amplification of this structurally related oncogene in any of the samples was obtained. These results are consistent with previous observations by others that c-erbB2 (new) amplification occurs preferentially in adenocarcinomas whereas c-erbB (EGFR) amplification or overexpression is associated with squamous cell carcinomas (19); all of the samples described in this report are squamous cell carcinomas.

Amplification may be preceded by, and/or may be biologically equivalent to overexpression; therefore, as a further inquiry into the role of the EGFR proto-oncogene in the development of esophageal squamous carcinomas we analyzed the EGFR mRNA levels in 12 tumor samples by Northern and RNA slot blot determinations. Samples were analyzed as having elevated levels of transcript if the amount of EGFR mRNA was at least 4-fold higher than amounts detected in RNA from histologically normal esophageal tissue. We found elevated levels of the EGFR gene transcript in three tumors (Table 1 and Fig. 4). The highest mRNA level detected was in tumor sample B24-c, in which we had estimated that the EGFR gene was amplified 10-fold. Control hybridizations of RNA slot blots with 32P-labeled actin-specific sequences revealed signals of comparable intensities for tumor samples and normal mucosa (Fig. 4).

DNA from tumor sample B17-c in which multiple copies of the EGFR gene had been detected was used to investigate whether a RFLP potentially indicative of gene rearrangement was associated with the amplification. Southern analyses of tumor and histologically normal esophageal tissue DNA were identical in the two samples upon digestion with restriction enzymes EcoRI, HindIII, BamHI, Mspl, TaqI and Xhol (data not shown). An additional DNA fragment, however, was present in the B17-c digest but absent in normal tissue from this patient (B17-n) when samples were digested with XbaI or PstI, suggesting mechanisms by which the RFLP, in conjunction with EGFR gene amplification may have contributed to the development of this tumor.

DISCUSSION

We obtained no evidence of ras mutations in codons 12, 13, or 61 in 25 primary esophageal cancers. While the overall prevalence of transforming c-ras sequences in human cancer is estimated at 10–20%, the frequency varies markedly with the tumor type. For example, approximately 40% of colon tumor biopsies tested thus far have a mutated ras allele, whereas such alterations are rare in stomach or breast cancers (9). Our survey has shown that squamous cell carcinomas of the esophagus may fall into this low incidence category. In the present study we used methods as specific and sensitive as those used to show the high prevalence of this oncogene in colon cancers (11). The difference between colon and esophageal carcinomas with regard to c-ras involvement could indicate that distinct oncogenes are involved in the induction or early stages of these cancers, and this may offer interesting clues to the etiologies of these malignancies. The fact that cellular c-ras oncogenes in human cancers are activated almost exclusively by specific point mutations suggests a possible link between exposure to DNA-damaging agents and the human colorectal cancers screened by Bos et al. (11) and Forrester et al. (20). As exposure to environmental genotoxic factors are likely to be important in the etiology of geographical pockets of high tumor incidence, it is noteworthy that the esophageal cancer samples analyzed here, and found negative, originated from Normandy, France, where the incidence of this cancer is unusually high in comparison to the overall national average. Similar negative findings for ras activation were observed in 11 esophageal cancer tissue specimens collected in Lyon. We emphasize, however, that information on the geographical origins of the patients in this study was not pursued. It would be of interest to analyze esophageal cancers from a different high-incidence area such as Linxian County, People's Republic of China.

DNA adducts (O6-methyldeoxyguanosine) attributable to nitrosamine exposure are known to induce G to A transition mutations that can activate c-ras genes (5). Elevated levels of these adducts have been detected in a significant number of esophageal tissue specimens from Linxian County (21) and in a few samples from Normandy. The data we have collected so far on c-ras activation, however, do not seem to support a role for this oncogene in the natural history of esophageal cancer. Moreover, our preliminary analysis of rat esophageal tumors induced by methylbenzyl-nitrosamine showed no mutations at codons 12/13 of H-ras. One can speculate that mutations activating other unidentified oncogenes may be involved in early stages of these neoplasms.

Oncogenes coding for growth factors or their receptors represent a second class of oncogenes consistently implicated in the development of a variety of human malignancies. Structural alterations of these genes, such as amplification leading to overabundance of the factor or receptor, can amplify the mitogenic stimulus. An autocrine loop hypothesis has been proposed explaining how oncogenes may render cancer cells growth factor autonomous (22). This may be important in postsinitiation stages of the disease as a mechanism whereby an aberrant cell acquires independent growth.

In our study, approximately 8% of esophageal squamous cell carcinomas from Normandy appear to contain an amplified EGFR gene. In a brief communication others have reported amplification of the EGFR gene in one of four esophageal squamous cell carcinomas (23) and experiments on established cell lines derived from five human esophageal tumors revealed the presence of multiple copies of the gene in three cases (24). Our positive results with two out of 25 tissue samples suggest that EGFR amplification occurs infrequently in vivo.
binding have been found in tissue specimens from many types of human cancers (23, 25-28) as well as in primary biopsy samples from gliomas (29) and breast tumors (30), and in squamous carcinomas of various organs (31); analysis of EGFR mRNA in a wide variety of human tumor cell lines and tumor specimens showed elevated levels in a number of different samples (32). While the viral erbB oncogene derived from the cellular EGFR gene has acquired transforming properties by structural modifications including absence of sequences coding for the NH2-terminal extracellular domain (33), the evidence to date has suggested that in human cancers the c-erbB gene may contribute to malignancy by overexpression of the normal receptor protein though structural alteration of the gene product is not excluded. The presence of an RFLP in one of the tumors gives a preliminary suggestion that EGFR re-arrangement does occur in human esophageal cancers. Gene rearrangement also can lead to transcription deregulation, but as yet there is no evidence to show that this occurs in esophageal neoplasia, and it is still unclear what physiological or environmental stimuli may cause up-regulation of the EGFR gene. In vitro, 3T3 cells can be transformed in the presence of ligand by overexpression of a structurally normal human c-erbB proto-oncogene product (34). Interestingly, in malignant gliomas, EGFR overexpression occurs only if there are multiple gene copies (35). In esophageal cancer, amplification may be preceded by overexpression of the EGFR gene which perhaps predisposes to gene duplication, and overexpression may confer a selective growth advantage to an initiated (aberrant) preneoplastic cell. If enhanced transcription is the principal mechanism by which this proto-oncogene is associated with the malignant process, then elevated EGFR mRNA transcripts found in 25% of the samples we tested may be the best index of the prevalence of EGFR gene involvement in development of human esophageal cancers.

REFERENCES


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