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A POINT MUTATION OF C-KI-*RAS* GENE WAS FOUND IN HUMAN ESOPHAGEAL CARCINOMA CELL LINES BUT NOT IN PRIMARY ESOPHAGEAL CARCINOMAS

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SUMMARY: Activation of the *ras* oncogene in primary human esophageal carcinomas and cell lines established from such carcinomas was analyzed using the polymerase chain reaction (PCR)-direct sequencing method. This analysis revealed a GC→AT transition at the second base in the 12th codon of the c-Ki-*ras* gene in TE1 and TE2 esophageal carcinoma cell lines. In contrast, no point mutation was detected in the 12th, 13th, and 61st codon of the c-Ki-*ras* and c-Ha-*ras* gene in 31 primary esophageal carcinomas including those from which TE1 and TE2 cell lines were established. These results demonstrate that while activation of the c-Ki-*ras* gene by point mutation occurred in a subset of esophageal carcinoma cell lines during establishment of the cell lines, the activation events are not important in the transformation of human esophageal epithelial cells.

INTRODUCTION: Tumorigenesis is a multistep process frequently associated with the activation of several protooncogenes and/or the inactivation of one or more tumor suppressor genes. A typical example of this idea has recently been demonstrated in the study of colon cancers (1). Protooncogenes involved in the malignant transformation of cells differ from cancer to cancer; for example, activation of *ras* oncogenes has been reported to be common in carcinomas of the colon (2), the pancreas (3), and the lung (4), but it has been found to be a rare event in carcinomas of the breast (5).

Our group has focused on the characterization of esophageal carcinoma cells. In esophageal carcinoma cells, several genetic changes have been found including *hst-1* (6), *int-2* (7), or EGFR(*erbB*) (8) gene amplification, loss of heterozygosity at chromosome 17 (7) or RB locus (9), and point mutations in the p53 anti-cancer gene (10). In this regard, it is noteworthy that a carcinogen, methylbenzennitrosoamine (MBN) induces esophageal tumors in rats which accompany activation of the c-Ha-*ras* oncogene caused by the point mutation at the second base in the 12th codon of the gene (11,12). However, it remains to be investigated whether activation of the *ras* oncogene plays an important role in the malignant transformation of esophageal epithelial cells.

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Therefore, in this study, we focused on the analysis of *ras* oncogenes in primary esophageal carcinomas and cultured cell lines established from esophageal carcinoma cells. Our approach to this was to directly sequence the PCR-amplified genomic DNA fragments and determine whether the well-known *ras* activating mutations in codon 12,13, and 61 exist in c-Ha-*ras* and c-Ki-*ras* genes or not. We found two cell lines which bear a point mutation in the 12th codon of the c-Ki-*ras* gene. However, the mutation as well as the mutations in the other well-known hot spots were not observed in 31 cases of primary esophageal carcinomas, demonstrating that the observed mutation in the c-Ki-*ras* gene was acquired upon establishment of the cell lines.

MATERIALS AND METHODS:

PREPARATION OF DNA FROM TISSUES AND TUMOR CELL LINES

Primary tumor tissues were removed from esophageal cancer patients under approved protocols at Tohoku University Hospital, Sendai, Japan. After surgical removal, samples were frozen and stored at -80°C. In addition, all surgical specimens were formaldehyde fixed and paraffin embedded. Thirteen cell lines of the TE series were also used in this study. These cell lines were established from primary esophageal carcinoma cells at the Department of Surgery, Tohoku University School of Medicine. Characterization of cell lines in the TE series has been previously described (13,14).

High molecular weight genomic DNA was extracted from the frozen tissue samples using a standard method (15) and digested with *Bam*HI, since the DNA fragments amplified with PCR in this study contained no *Bam*HI sites. Extraction of DNA from paraffin-embedded tissues was carried out as described (16).

PRIMERS AND POLYMERASE CHAIN REACTION (PCR)

Oligodeoxynucleotides used for PCR were synthesized on an Applied Biosystem Model 380A DNA synthesizer. Primers used in PCR were as follows: K12A, 5'-GGCCTGCTG AAAATGACTGA-3'; K12B, 5'-GTCCTGCACCAGTAATATGC-3'; K61A, 5'-TTCCTACA GGAAGCAAGTAG-3'; K61B, 5'-CACAAAGAAAGCCCTCCCCA-3'; H12A, 5'-CAGGCC CCTGAGGAGCGATG-3'; H12B, 5'-TTCGTCCACAAAATGGTTCT-3'; H61A, 5'-TCCT GCAGGATTCCTACCGG-3'; H61B, 5'-GGTTCACCTGTACTGGTGGA-3'. Genomic DNA (0.5-1.0μg) was amplified in a 50-100 μl reaction mixture containing 1 μM of a set of primers, 10 mM Tris-HCl (pH8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM dNTPs (Boehringer Manheim) and 2.5 units/100 μl of *Taq* polymerase (Perkin Elmer/Cetus). Thirty cycles of polymerase chain reactions were performed: denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min and primer extension at 72°C for 1 min in an automated thermal cycler (Perkin Elmer/Cetus). The PCR product was precipitated once with ethanol and resuspended in sterile water. After electrophoresis on 2% agarose gel (Seakem GTG) containing 0.5 μg/ml ethidium bromide, the amplified DNA fragments were extracted from the gel as follows. The gel slices were centrifuged and filtered through a 0.22 μm pore filter in SUPREC-01 (Takara Shuzo, Kyoto, Japan). The eluate was ethanol precipitated prior to subsequent sequencing.

DIRECT SEQUENCING OF AMPLIFIED DNA

The PCR amplified DNA was sequenced without any cloning steps. The alkali denatured DNA templates and sequencing primers were heated at 65° for 2 min and cooled at room temperature for 15-30 min for annealing. The primers used were the same as those used for PCR amplification. Dideoxy chain termination reactions were performed by the internal labeling method according to the protocol of Sequenase II (USB, Cleveland, OH) using [α-^{32}P]dCTP (3000 Ci/mmol; Amersham Japan, Tokyo).

RESULTS: Mutations in the *ras* oncogenes were analyzed using the direct-sequencing method of the PCR-amplified genomic DNA fragments. As an initial step for this study, we tried several published protocols (17,18), but our attempts were repeatedly hampered. Clear sequence ladders were not reproducibly obtained. Therefore, we designed a modified conventional direct-sequencing method of PCR-amplified products as described in MATERIALS AND METHODS. In our protocol, PCR-amplified genomic DNA fragments were first fractionated with an agarose gel electrophoresis, and then eluted from the gel with centrifugation in a tube with a 0.22 μm filter. The sequencing reaction was performed in the same manner as described for the sequencing of double strand plasmid templates. The method is simple and rapid, and even applicable to the case in which only the DNAs extracted from formaldehyde-fixed and paraffin-embedded samples are available as templates (see below).

To study the characteristic features of the transformed esophageal epithelial cells, 16 cell lines have been established in this labolatory from surgical removed esophageal tumors. Pathological studies revealed that all the original tumors from which these cell lines were established were squamous cell carcinomas, except the TE7 cell line which had been derived from adenocarcinoma of the esophagus (Table 1). Use of these cell lines has an advantage; we can obtain large quantities of fresh materials easily. Therefore, we first analyzed genomic DNAs in these cell lines by the above-described method. The results of this analysis are summarized in Table 1. Of the 13 cell lines analyzed, two cell lines have a guanine (G) to adenine (A) transition at the second base in codon 12 of the c-Ki-*ras* gene (Fig.1). The

Table 1. *ras* gene mutations in human esophageal carcinoma cell lines and classification of patients from whose tumors these cell lines were derived

Cell line	Age (year)	Sex	Origin	Histology	Stage[§]	*ras* mutation[†]	Sequence at mutation codon
TE1	58	M	Primary tumor	Sq.C.C.	II	K12	GGT → GAT
TE2	56	M	Primary tumor	Sq.C.C.	IV	K12	GGT → GAT
TE3	48	M	Skin metastasis	Sq.C.C.	IV	W.T.	
TE4	48	M	Primary tumor	Sq.C.C.	III	W.T.	
TE5	73	F	Primary tumor	Sq.C.C.	IV	W.T.	
TE6	71	M	Primary tumor	Sq.C.C.	IV	W.T.	
TE7	72	M	Primary tumor	Adenoca.		W.T.	
TE8	63	M	Primary tumor	Sq.C.C.	III	W.T.	
TE9	48	M	Pleural effusion	Sq.C.C.	IV	W.T.	
TE10	50	M	Primary tumor	Sq.C.C.	IV	W.T.	
TE11	58	M	Primary tumor	Sq.C.C.	IV	W.T.	
TE12	54	M	Primary tumor	Sq.C.C.	III	W.T.	
TE13	65	F	Primary tumor	Sq.C.C.	IV	W.T.	

[§]Carcinoma in patients was classified into five stages (0,I,II,III,and IV) according to the depth of invasion, degree of lymph node metastasis, presence or absence of organ metastasis, and presence or absence of pleural dissemination.

[†]K12, mutation at codon 12 of c-Ki-*ras* gene; W.T., wild type of *ras* genes,i.e., no mutation identified.

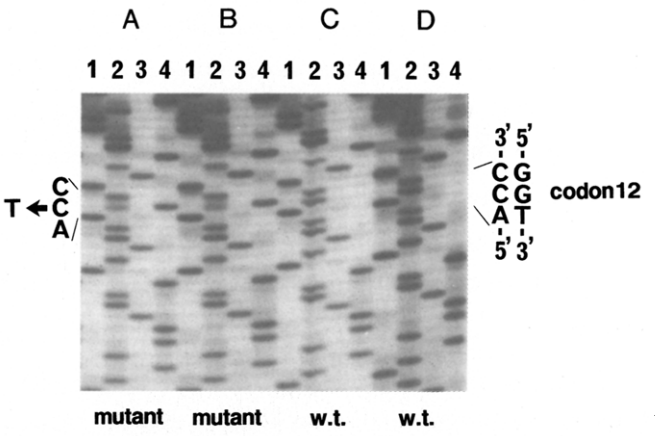

Figure 1. Identification of point mutations in c-Ki-*ras* gene by PCR-direct sequencing method. High molecular weight DNA was isolated from human esophageal carcinoma cell lines TE1(A), TE2(B), TE3(C), and TE10(D) and subjected to amplification of exon 1 of the c-Ki-*ras* gene using oligonucleotide K12A and K12B as primers. PCR products were gel-purified and sequenced directly with K12B as a primer (see MATERIALS AND METHODS). The sequencing reaction was analyzed by electrophoresis in 6% polyacrylamide/urea gel. Lanes 1, 2, 3, and 4 in each panel from left to right are A,C,G, and T, respectively. W.T., wild type c-Ki-*ras* gene; mutant, mutant c-Ki-*ras* gene. Both wild type (5'-GGT-3') and mutant (5'-GAT-3') alleles are present in these tumor samples (A and B).

mutation occurred in one allele in both cases, since both A and G bands were detected on the mutant DNA lanes. Other mutations were not observed in any other portion of the sequences examined (Table 1).

There is a possibility that the point mutation in the c-Ki-*ras* gene significantly contributes to the maintenance of the transformed phenotype of the esophageal carcinoma cells. Another

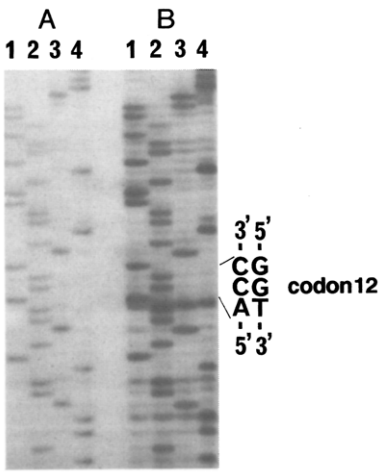

Figure 2. No point mutation was revealed in c-Ki-*ras* gene in paraffin-embedded tumor DNAs. Sections cut from the paraffin-embedded human esophageal tumors which were used to establish TE1(A) and TE2(B) cell lines were deparaffinized in xylene, cleared with ethanol and completely dried before DNA extraction. PCR-direct sequencing was carried out as described in the legend of Figure 1.

possibility is that this is an acquired characteristic of the carcinoma cells, which may help the cells to survive *in vitro* in culture. In order to examine these possibilities, we next analyzed the DNAs extracted from the primary tumors from which the TE1 and TE2 cell lines had been established. The results are shown in Figure 2. Both DNA sequences show the wild type sequence, supporting the latter hypothesis unequivocally. The results further suggest that the activation of *ras* oncogenes is probably not involved, at least not exclusively, in the establishment of the transformed phenotype of esophageal carcinoma cells.

To test this hypothesis, DNA extracted from 29 primary esophageal carcinomas were also PCR-amplified and sequenced. All the cancer cells were judged to be squamous cell carcinomas through pathological analysis. This analysis showed all 29 samples to have wild type sequences in all the regions examined (data not shown). The activation of *ras* oncogenes is therefore concluded to be a rare event in esophageal carcinoma cells.

DISCUSSION: This is the first reported study in which the activation of c-Ki-*ras* gene, *i.e.*, the point mutation at the 12th codon of this gene, was detected in two esophageal carcinoma cell lines but in which there was no mutation in primary tumors.

Several kinds of oncogenes are activated in human tumors and contribute to carcinogenesis of the tumors, frequently in concert with the loss or inactivation of tumor suppressor genes. Among activated oncogenes, *ras* genes are the ones most frequently detected in a wide variety of human cancers and cell lines (19). In this paper we analyzed *ras* gene mutations in human primary esophageal carcinomas and cell lines by the PCR-direct sequencing method. Thanks to this method, we clearly marked not only the actual site of the point mutations but also the molar ratio between the heterogeneous bands. Figure 1 shows that two out of 13 cell lines had a point mutation in codon 12 of the c-Ki-*ras* gene at one allele. However, we did not detect the existence of the same mutation in the carcinoma specimens from which cells these cell lines were derived (Fig. 2). These results indicated that the mutation of the c-Ki-*ras* gene in TE1 and TE2 cells could have occurred during culture *in vitro*. In fact, several investigators have reported that activation of c-Ha-*ras*, c-Ki-*ras* and c-N-*ras* genes occurred during culture *in vitro* (20-22). Although the role and mechanism of *ras* activation are not clear in TE1 and TE2 cell lines, the activation of c-Ki-*ras* gene could be needed for these cell lines to be immortalized and maintain the cancerous phenotypes, but apparently were not needed for the transformation of the epithelial cells into carcinomas from which these cell lines were derived. In support of this hypothesis, no point mutation in c-Ki-*ras* and c-Ha-*ras* genes was detected in 29 other esophageal carcinomas (data not shown). On the other hand, some carcinogens induce *ras* gene mutations. Among these carcinogens, especiall methylbenzennitrosamine (MBN) induced esophageal tumors in rats and activated the c-Ha-*ras* gene by point mutation at the second base in codon 12 (11,12). Since no *ras* gene mutations were observed *in vivo*, these animal models do not parallel the human esophageal carcinomas. Perhaps this animal model of esophageal

tumors may have a mechanism of transformation of epithelial cells different from that of human esophageal epithelial cells. Therefore, according to our results and a previous report (8), we concluded that the point mutation of c-Ha-*ras* and c-Ki-*ras* genes was a rare event in human esophageal carcinomas and that the activation of *ras* genes could not contribute to the transformation of human esophageal epithelial cells. The biological characteristic features that differentiate these two esophageal carcinoma cell lines from others remain to be clarified. To determine whether the mutant protein product of the c-Ki-*ras* gene plays a key role in the function of these cells in the maintenance of the cancerous phenotype or their immortalization, loss of function experiments are needed.

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