Amplification and Enhanced Expression of the c-Ki-ras2 Protooncogene in Human Embryonal Carcinomas

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ABSTRACT

Two cell lines of human embryonal carcinoma, Tera-1 and Tera-2, have been found to exhibit a 4- to 6-fold amplification of protooncogene c-Ki-ras2. The polyadenylate acid selected RNA also showed 8-fold or greater enhancement, showing marked elevation in the level of two major mRNAs, 5.7 and 4.0 kilobases, and two additional minor mRNAs, 2.3 and 1.2 kilobases, as compared with those of a normal human embryonic fibroblast cell line, MRC-5. More than one-half of the number of tumor samples obtained from metastatic human embryonal carcinomas also showed c-Ki-ras2 gene amplification and enhanced mRNA expression. However, the c-Ki-ras2 gene amplification did not always lead to enhanced mRNA expression, and some embryonal carcinomas showed mRNA overexpression without apparent c-Ki-ras2 gene amplification. These results suggest that human embryonal carcinomas may have c-Ki-ras2 amplification and/or overexpression before in vitro culture. Among various chromosomal changes observed in Tera-1 and Tera-2 cells, there were anomalies in chromosome 12 in which c-Ki-ras2 is located although these karyological changes alone could not account for the amplification observed. It is suggested that the genomic instability and active DNA replication during the early developmental period may give rise to changes involving c-Ki-ras2 which may contribute to oncogenic processes.

INTRODUCTION

Most members of cellular oncogenes have been identified experimentally by the homology of their nucleotide sequences to those of acutely transforming retroviral oncogenes. The normal cellular oncogenes (protooncogenes, c-onc), which are considered to be normal cellular progenitors of viral oncogenes (v-onc), apparently served as targets for genetic transduction by retroviruses. In recent years, major research efforts have focused on elucidating the roles of cellular oncogenes in normal as well as neoplastic cells. It has been suggested that amplification and overexpression of cellular oncogenes contribute to tumorigenesis, tumor progression, or both (1). Point mutations, promotor insertion mutagenesis, and chromosomal translocations of cellular oncogenes have also been demonstrated to play important roles in oncogenesis. Gene amplification has been associated with differentiation and development in oogenesis of lower vertebrates but has not been associated with development in mammals (2).

Amplification of a number of human cellular oncogenes has been described: c-myc in a promyelocytic leukemia cell line, HL60 (3, 4), malignant neuroendocrine cells from a colon carcinoma (5), small cell lung carcinoma cell lines (variants) (6), a stomach cancer passed in nude mice (7), and mammary carcinoma cell lines (8, 9); c-N-myc in neuroblastoma cell lines (10), c-ErbB2 in a mammary carcinoma (25, 26), and primary carcinomas of lung, colon, bladder, and rectum (1); N-ras in a mammary carcinoma cell line, MCF7 (1); c-erbB1 in an epidermoid carcinoma (23) and glioblastomas (24); and c-erbB2 in a mammary carcinoma (25) and a salivary gland adenocarcinoma (26).

In this report, we describe an amplification and overexpression of c-Ki-ras2 gene in two cell lines and several tumor tissues of human embryonal carcinoma.

MATERIALS AND METHODS

Cell Lines and Tumors. The Tera-1 and Tera-2 human embryonal carcinoma cell lines (27) were obtained from ATCC, Rockville, MD. These were cultured in McCoy's 5A medium supplemented with 10% fetal bovine serum at 37°C under a humidified atmosphere containing 5% CO₂ in air. The cells were subcultured weekly by scraping. The MRC-5 and WI-38 human fetal lung cell lines obtained from ATCC were used as normal controls. Furthermore, other human tumor cell lines were used as additional controls. These include J7, a hepatocellular carcinoma (obtained from Dr. C. S. Yang, Taipei, Taiwan); JAR and JEG-3, choriocarcinomas (obtained from ATCC); and PAI, an embryonal carcinoma (obtained from ATCC). Frozen samples of metastatic embryonal carcinomas and hydatidiform mole tissues were obtained from the Tumor Repository, Biological Carcinogenesis Branch, Division of Cancer Etiology, National Cancer Institute.

Isolation of Nucleic Acids. High molecular weight DNA's were prepared from cells and tumor tissues according to the method of Gross-Bellard et al. (28). Total cellular RNA was prepared by a modification of the method of Auffrey and Rougeon (29). The washed cell pellets were homogenized in a 20x volume of lithium chloride buffer (3 M LiCl, 6 M urea, 50 mM Tris, pH 7.4, 5 mM EDTA, 0.1 M β-mercaptoethanol, and 0.1% Sarkosyl), and after overnight standing at 4°C the RNA was precipitated by centrifugation at 13,000 X g for 20–30 min. The pellet was dissolved in 10 mM Tris buffer containing 1 mM EDTA, 0.5% sodium dodecyl sulfate, and 200 μg/ml proteinase K, and extracted with phenol-chloroform twice before ethanol precipitation at −20°C. The poly(A)⁺ RNA was selected by means of an oligodeoxythymidyl acid-cellulose column (30).

Southern and Northern Blotting. DNAs were digested to completion with restriction enzymes as recommended by the supplier and electrophoresed on 0.7% agarose gels. Transfers to nitrocellulose filters were performed as described in (Ref. 30). RNAs were electrophoresed on 1% agarose gels containing formaldehyde and transferred to nitrocellulose filters as described in Ref. 30. Phage λ DNA HindIII fragments and the RNA ladder (Bethesda Research Laboratories, Gaithersburg, MD) were used as molecular weight markers and electrophoresed in each gel.

Hybridizations. The following specific probes were used: a 0.6-kilobase SstII-HindII fragment of v-Ki-ras, a 0.9-kilobase AvaI-HindIII fragment of v-mos, a 0.7-kilobase SstII-PstI fragment of v-Ha-ras, and a 1.4-kilobase PstI-AvalI fragment of v-erbB2 all supplied by Oncor, Gaithersburg, MD. A β-actin probe, which is a 2.1-kilobase insert replacing the small BamHI-Pull fragment of PBR322 was kindly provided by Dr. Gilbert Jay (National Cancer Institute, NIH). These were 3²P labeled by nick translation to a specific activity of 2–4 × 10⁸ cpm/μg. Hybridizations for Southern and Northern blots were performed according to the method described in Ref. 30 except that dextran

Received 10/16/86; revised 3/31/87; accepted 5/7/87.
c-Ki-ras2 AMPLIFICATION IN EMBRYONAL CARCINOMAS

Sulfate was omitted. Hybridizations were performed at 42°C for 24-72 h. The filters were washed 5 times with 0.1x saline sodium citrate solution containing 0.1% sodium dodecyl sulfate. The washed filters were autoradiographed at -70°C using Kodak XAR-5 and DuPont Quanta III intensifying screens. For quantitation of amplified gene, Southern blots of graded doses of DNA cleaved by EcoRI were hybridized either with a mixture of v-Ki-ras probe and v-mos probe or with each of 3 different probes, i.e., v-Ha-ras, v-myc, or v-Ki-ras, using the same blot after regeneration as described in Ref. 30. For RNA slot blot assay, the Schleicher and Schuell Minifold II Slot Blot System was used.

Chromosomal Analyses. Cells in metaphase were stained by conventional Giemsa staining and by G and C banding. For visualizing double minutes, these were stained with 1 µg of Hoechst 33258/ml of phosphate buffered saline for 30 min. At least 30 cells in metaphase were examined.

RESULTS

Amplification of c-Ki-ras2 Gene in Human Embryonal Carcinoma Cell Lines and Tumor Tissues. EcoRI, BglII, PvuII, HindIII, and PstI were used for cleavage of high molecular weight DNA derived from 2 human embryonal cell lines, Tera-1 and Tera-2, and a normal human fetal lung cell line, MRC-5. Both Tera-1 and Tera-2 DNAs showed similar patterns of hybridization bands on the Southern blot as did MRC-5 DNA except that the signals were generally more intense for most of the bands in Tera-1 and Tera-2 than in MCR-5 DNAs (Figs. 1 and 2). Further analyses by referring these bands to restriction maps (31) led to the following conclusions. It is evident that the characteristic BglII 4.2-kilobase fragment representing the pseudogene Ki-rasl is not amplified. Both the EcoRI 6.7-kilobase fragment which includes Ki-ras2 exon 1 and the 3.0-3.2-kilobase fragment which contains exons 2, 3, and 4A are amplified. Both the BglII 25-kilobase fragment which represents exons 0, 1, and 2, and the 12-14-kilobase fragment which contains exon 4A are also amplified. Amplification of both the HindIII 23-kilobase fragment which contains exons 0 and 1 and the 11-12-kilobase fragment which includes exon 4A are in agreement with the above observations. No conclusion can be drawn on exon 4B, since the BglII 5.0-kilobase fragment containing this exon is not detected in this Southern blot, although the EcoRI 2.4-kilobase fragment containing this exon is visible in Tera-1 but not in Tera-2 or MRC-5 cell DNAs.

In addition to MRC-5, another normal cell line, WI-38, and other human tumor cell lines such as J7 (hepatoma), JAR and JEG-3 (choriocarcinomas), and PA1 (embryonal carcinoma) were tested along with Tera-1 and Tera-2 for the presence of amplification and/or rearrangement of c-fos, c-mos, c-N-ras, c-abl, c-Ha-ras, c-myc, c-fes, and c-sis. None of these protooncogenes showed alterations in these cells (data not shown).

In order to control for the quantity of DNA that had been loaded in each lane of agarose gel we used v-mos and v-Ki-ras as probes simultaneously for hybridization on the Southern blots. The 2.8-kilobase EcoRI band of c-mos (32) was not amplified in either Tera-1 or Tera-2 DNAs. By contrast, the results of quantitation of the Tera-1 and Tera-2 DNAs for densities of the 6.7 and 3.2-3.0-kilobase EcoRI fragments indicated a 4- to 8-fold amplification (Fig. 3). Results of further quantitation experiments using 3 different (v-Ha-ras, v-myc, and v-Ki-ras) probes for the same Southern blot after repeated regeneration procedures are presented in Fig. 4. The hybridi-
c-Ki-ras2 AMPLIFICATION IN EMBRYONAL CARCINOMAS

![Quantitation of the amplified c-Ki-ras2 in Tera-1 and Tera-2 cells with the unamplified c-Ha-ras1 and c-myc as controls. Graded doses (10, 5, 2.5, and 1.25 µg) of DNA from Tera-1 and Tera-2 and a single dose (10 µg) of DNA from MRC-5, J7 (hepatoma), and JAR (choriocarcinoma) cell DNAs were cleaved with EcoRI and blot hybridized with v-Ki-ras probe. The same blot was rehybridized with v-Ha-ras probe or v-myc probe after repeated regenerations (30). The single 21.0-kilobase (kb) c-Ha-ras1 band and the single 12.5-kilobase c-myc band served as internal controls.](image1)

**Fig. 4.** Quantitation of the amplified c-Ki-ras2 in Tera-1 and Tera-2 cells with the unamplified c-Ha-ras1 and c-myc as controls. Graded doses (10, 5, 2.5, and 1.25 µg) of DNA from Tera-1 and Tera-2 and a single dose (10 µg) of DNA from MRC-5, J7 (hepatoma), and JAR (choriocarcinoma) cell DNAs were cleaved with EcoRI and blot hybridized with v-Ki-ras probe. The same blot was rehybridized with v-Ha-ras probe or v-myc probe after repeated regenerations (30). The single 21.0-kilobase (kb) c-Ha-ras1 band and the single 12.5-kilobase c-myc band served as internal controls.

![Amplification of c-Ki-ras2 in human embryonal carcinomas. DNAs from MRC-5 and tumor tissues of embryonal carcinoma patients were cleaved with EcoRI and blot hybridized with v-Ki-ras probe. All samples contained 10 µg DNA except that EC9 contained 3 µg DNA. After regeneration (30), the same blot was rehybridized with the v-myc probe, and the 12.5-kilobase c-myc fragment showed no significant difference in signal intensities among these samples (EC9 DNA showed an intensity corresponding to 3 µg DNA). These results suggest that c-Ki-ras2 amplification occurs frequently even before in vitro cultivation of embryonal carcinomas. Since all of these tumors were taken from metastatic tissues, they are considered to be malignant. No correlation was found between the presence of c-Ki-ras2 gene amplification and the patient’s age, history of previous chemotherapy, metastatic organ site, etc.](image2)

**Fig. 5.** Amplification of c-Ki-ras2 in human embryonal carcinomas. DNAs from MRC-5 and tumor tissues of embryonal carcinoma patients were cleaved with EcoRI and blot hybridized with v-Ki-ras probe. All samples contained 10 µg DNA except that EC9 contained 3 µg DNA. After regeneration (30), the same blot was rehybridized with the v-myc probe, and the 12.5-kilobase c-myc probe in order to provide a control for the amount of DNA tested. kb, kilobase.

![Enhanced Expression of c-Ki-ras2 Gene in Human Embryonal Carcinoma Cell Lines and Tumor Tissues. Using a slot-blot assay with v-Ki-ras as a probe, the poly(A)+ RNAs of Tera-1 and Tera-2 cells were compared quantitatively with that of MRC-5. The results indicated that the amount of c-Ki-ras specific mRNA in both Tera-1 and Tera-2 DNAs was 8-fold greater than that of MRC-5 (Fig. 5).](image3)

**Fig. 6.** Slot blot assay of poly(A)+ RNA derived from MRC-5, Tera-1, and Tera-2 cells. Graded doses (12.5, 6.25, 3.2, 1.6, 0.8, 0.4, and 0.2 µg) of poly(A)+ RNA were blotted and hybridized with v-Ki-ras probe.

4194

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To investigate the Ki-ras2 mRNA expression of the EC tumor tissues, the poly(A)+ RNA from each tumor tissue was quantitatively compared with that of MRC-5. The results indicated that the amount of c-Ki-ras specific mRNA in both Tera-1 and Tera-2 DNAs was 8-fold greater than that of MRC-5 (Fig. 6).

Since Tera-1 and Tera-2 cells are in vitro established cell culture lines, the c-Ki-ras2 amplification observed may have been acquired during in vitro passages and does not represent the original tumor. The question whether similar amplification could be detected with the tumor tissues of human embryonal carcinoma was examined by similar Southern blot analysis of tumor DNAs cleaved with EcoRI. Ten µg DNA were used for each sample except that only 3 µg DNA were used for EC9. It was found that 5 (EC5, EC6, EC9, EC14, and EC16) of 9 embryonal carcinomas tested showed a relative increase in densities of both 6.7- and 3.2-kilobase fragments hybridizing with v-Ki-ras probe, as compared with MRC-5 cells, although the degree of amplification varied with each tumor examined (Fig. 5). The same Southern blot after regeneration was hybridized with the v-myc probe, and the 12.5-kilobase c-myc fragment (34) showed no significant difference in signal intensities among these samples (EC9 DNA showed an intensity corresponding to 3 µg DNA). These results suggest that c-Ki-ras2 amplification occurs frequently even before in vitro cultivation of embryonal carcinomas. Since all of these tumors were taken from metastatic tissues, they are considered to be malignant. No correlation was found between the presence of c-Ki-ras2 gene amplification and the patient’s age, history of previous chemotherapy, metastatic organ site, etc.

Enhanced Expression of c-Ki-ras2 Gene in Human Embryonal Carcinoma Cell Lines and Tumor Tissues. Using a slot-blot assay with v-Ki-ras as a probe, the poly(A)+ RNAs of Tera-1 and Tera-2 cells were compared quantitatively with that of MRC-5. The results indicated that the amount of c-Ki-ras specific mRNA in both Tera-1 and Tera-2 DNAs was 8-fold greater than that of MRC-5 (Fig. 6).

To investigate the Ki-ras2 mRNA expression of the EC tumor tissues, the poly(A)+ RNA from each tumor tissue was quantitatively compared with that of MRC-5 and other tumor cell lines including J7 (hepatoma), JEG-3 (choriocarcinoma), PA1 (embryonal carcinoma), and tumor tissues of HYD-4 (hydatidiform mole). As illustrated in Fig. 7A, out of 10 embryonal carcinomas tested 7 (EC6, EC8, EC13, EC14, EC15, EC10 and EC17) exhibited 4-fold or greater enhancement in their mRNA expression, whereas other tumors did not show mRNA overexpression relative to MRC-5 cells. These results indicated that c-Ki-ras2 gene amplification did not necessarily lead to enhanced mRNA expression (e.g., EC5) and some ECs (e.g., EC8, EC13 and EC15) which did not show amplification nevertheless manifest mRNA overexpression. As a control for the amount of RNA applied to the slot-blot assay, a duplicate blot was hybridized with v-myc probe. As shown in Fig. 7B, there
was no significant variation in the intensity of hybridization signal among these samples.

Both Tera-1 and Tera-2 cells showed 4 different sizes of poly(A)* selected mRNA which hybridized with v-Ki-ras probe on the Northern blot. Their 2 major mRNAs, 5.7 and 4.0 kilobases, were markedly more prominent than those of MRC-5, and the 2 minor mRNAs, 2.3 and 1.2 kilobases, were not detectable with MRC-5 cells under the same experimental conditions (Fig. 8A). A 32P-labeled β-actin probe was also used for hybridization with duplicate poly(A)* RNA blots prepared the same way as the above experiments. The results indicated that equivalent amounts of poly(A)* RNA were loaded for MRC-5, Tera-1, Tera-2, or EC groups, since the actin hybridization bands showed similar intensities at comparable doses (Fig. 8B).

Chromosomal Analysis. As illustrated in Figs. 9 and 10, the majority of Tera-1 cells showed 61 or 62 chromosomes, while the chromosome number of Tera-2 cells varied between 60 and 64. Both were hypotriploid. Double minute chromosomes were not present in these preparations. Tera-1 cells exhibited the following marker chromosomes: 1p−; i(2q); 3q+; inv(4); 5q+; i(6p); 7q+; 7p−; 8q−; 9q+; 10p+; i(11p); 12p−; 12q−; i(12p); 13q+; 15p+; and 21q+ (Fig. 9). Tera-2 cells displayed the following marker chromosomes: 1p−; 10q−; 11p−; i(12p); and Xp+ (Fig. 10). Chromosomes with homogeneously staining regions have not been detected.

DISCUSSION

The human genome contains a pseudogene, c-Ki-ras1, which is located in chromosome 6, and a complete gene, c-Ki-ras2, which is located in chromosome 12 (35). The present study showed that amplified c-Ki-ras exons detected in Tera-1 and Tera-2 DNAs are derived from the c-Ki-ras2 gene because (a) the amplification has not grossly changed the size of typical restriction fragments of c-Ki-ras2, (b) the amplified fragments do not entirely represent the pseudogene, and (c) the sizes of major K-ras mRNAs from Tera-1 and Tera-2 cells are 5.7 and 4.0 kilobases as described for the normal c-Ki-ras2 gene (22). In addition to the amplification of otherwise apparently normal c-Ki-ras2 loci, Tera-1 DNA showed a v-Ki-ras-related 5.0-kilobase EcoRI fragment, the origin of which is unknown.

The role of the ras gene family in either initiation or progression of various tumors has been reported. While the ras gene family can act early as an initiator of two-stage mouse skin carcinogenesis (36), other evidence suggests that it may be activated relatively late in the course of tumor progression (37, 38). We do not know if the original tumor of Tera-1 and Tera-2 had the c-Ki-ras2 amplification. However, the fact that tumor tissues derived from at least about one-half of the patients with embryonal carcinoma showed amplification of c-Ki-ras2 gene in their DNAs suggests that the original tumor tissues from which Tera-1 and Tera-2 cell lines were derived may have
already acquired the amplified c-Ki-ras2 gene in their DNAs. It is also possible that c-Ki-ras2 overexpression gave the original tumor cells the advantage to adapt to in vitro growth and become established lines. Although all ECs tested did exhibit amplification and/or overexpression of c-Ki-ras2, EC5 showed an increase in gene dosage without overexpression. This increase may be due to an amplification accompanied by strict cellular regulation of its transcription. It is possible that another...
mechanism such as point mutations in this or other protooncogenes may play a more important role in carcinogenesis of EC5. It is known that strong selective pressure such as that from methotrexate and other drugs is required to bring the spontaneously occurring gene amplification into prominent view (2). If these changes took place during in vitro culture, the putative selection factor, if any, that accounts for the amplification of c-Ki-ras2 in Tera-1 and Tera-2 cells is not clear. It is of interest to note that these cells spontaneously produce type-C retrovirus particles, the identity of which has not been established (39). The possible role of these viruses in oncogenesis of EC remains to be investigated.

Karyological analysis indicated that both Tera-1 and Tera-2 cells were hypotriploid with many marker chromosomes suggesting the possibility that translocations affect various chromosomes. Examination of chromosome 12, in which c-Ki-ras2 is located at p11.1-12.1 (40), revealed the following changes, i.e., 12p+, 12q+, 12p−, 12q−, and i(12p) for Tera-1, and tetrasomy of chromosome 12 with an additional isochromosome i(12p) for Tera-2 cells. No marked change was observed with chromosome 6, in which c-Ki-ras1 pseudogene is located at p11−12 (40), except that an isochromosome i(6p) has replaced one of the 2 Tera-1 chromosomes 6. However, these changes alone cannot account for the 4- to 6-fold amplification of c-Ki-ras2 gene and the 8-fold or greater overexpression of its mRNA as observed in the present study. Nevertheless, it is possible that translocations involving the short arm of chromosome 12 may bring the c-Ki-ras2 gene into proximity with and under the influence of a more effective enhancer/promoter in another chromosome, resulting in its overexpression without amplification. Other chromosomal anomalies such as double minutes or homogeneously staining regions have not been detected in these cells despite a careful search for them.

An activated c-Ki-ras2 gene with a 3T3-cell transforming ability has been found in various human tumor cell lines derived from lung (21, 41-47), colon (42, 45, 48, 49), pancreas (42, 50), bladder (42, 43), gallbladder (42), rhabdomyosarcoma (42), and ovarian carcinomas (51), but to our knowledge, none from embryonal carcinoma has been reported. A transforming N-ras gene containing a point mutation has been found in the late but not the early passage line of a human embryonal carcinoma cell line, PA1 (52). It remains to be elucidated whether the amplified c-Ki-ras2 gene in Tera-1 and Tera-2 cells has point mutations. Although the cause of embryonal carcinoma is unknown, it is conceivable that during early embryogenesis when vigorous DNA replication and gene expression are taking place, some environmental or endogenous factors may cause c-Ki-ras2 mRNA overexpression through chromosomal translocation and/or amplification. These instabilities may give more chance of point mutations, which could contribute effectively to the carcinogenesis. Some embryonal carcinomas are known to have the potentiality to be induced by retinoic acid and other agents to differentiate into other types of cells (53). Further investigations of the causes and consequences of c-Ki-ras2 gene amplification and enhanced expression under the undifferentiated and differentiated conditions of human embryonal carcinomas should provide a better understanding of the role of this gene in normal and neoplastic cells.

ACKNOWLEDGMENTS

The authors wish to acknowledge the technical assistance of Dr. Shien Tsai (Biotech Research Laboratories, Rockville, MD) for karyological analyses. We are grateful to Dr. Douglas Lowy for helpful discussions and critical reading of the manuscript. Thanks are due to Virginia Zimmerman and Barbara Hosier for typing the manuscript.


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