Association of Ki-ras with Amplified DNA Sequences, Detected in Human Ovarian Carcinomas by a Modified In-Gel Renaturation Assay


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ABSTRACT

A modified in-gel DNA renaturation technique, which detects DNA sequences amplified >7-fold in human DNA, was used to analyze gene amplification in surgical specimens of primary and metastatic ovarian carcinomas. Amplified DNA sequences were detected in two of eight tumors. Hybridization of these samples with different oncogene probes revealed that both tumors contained an amplified Ki-ras gene, which in one case was coamplified with c-myc. In one of the tumors, Ki-ras was found to be amplified in both the primary tumor and three different metastatic nodules. No mutations at codons 12 or 61 of Ki-ras were detected in these tumors. No additional cases of Ki-ras or c-myc amplification were detected by Southern hybridization in the tumors that were found to be amplification negative by modified in-gel renaturation assays. These results indicate that gene amplification in ovarian carcinomas is likely to involve the Ki-ras oncogene.

INTRODUCTION

Gene amplification is a major mechanism for increased expression of genes involved in carcinogenesis and tumor progression. Amplification of at least a dozen different oncogenes has been observed in tumor-derived cell lines, as well as in primary human tumors (1-10). In several types of cancer, amplification of a specific oncogene has been shown to correlate with advanced stages of tumor progression, suggesting that oncogene amplification and expression may be valid prognostic markers (3, 4, 10). Examples of consistent tumor-specific oncogene amplification include the HER-2/neu gene in breast carcinoma (3), N-myc in neuroblastoma (4), c-myb, N-myc, and L-myc in small cell lung cancer (5, 6), and c-erbB in glioblastoma (7). In other types of cancer, sporadic amplification of different oncogenes has been detected at low frequency, but no consistent correlations have yet been found (1). Since most of these studies were done using a limited set of the available oncogene-specific probes, there is a possibility that some other, presently unknown genes may be amplified in some tumors at a high frequency. Identification of novel genes that are consistently amplified in such malignancies would provide important diagnostic and prognostic markers.

All carcinomas of the female genital tract arise from the same embryological precursor, the coelomic epithelium. This progenitor epithelium covers the ovary and the epithelial surfaces of the fallopian tubes, as well as the endometrium and endocervix. The range of biological behavior in ovarian carcinomas ranges from borderline malignancy to highly malignant. Of the 18,000 to 19,000 cases diagnosed each year, approximately three-fourths will have spread beyond the primary site. Ovarian carcinomas appear to have a low incidence of mutational activation of ras oncogenes, as determined by NIH 3T3 focus-forming assays (11-13). Studies of gene amplification, utilizing different oncogene probes, showed relatively infrequent amplification of Ki-ras, c-myc, and H-ras in these tumors (9, 10, 14-16). Studies of this type, however, could not determine whether the overall incidence of gene amplification in ovarian carcinomas is low, or whether genes other than the known oncogenes are amplified in ovarian tumors.

We have previously described the method of in-gel DNA renaturation, which detects amplified sequences of unknown nature in total genomic DNA (17-19). This method has been used to detect and clone the gene for multidrug resistance (20) and an oncogene, gli, which is amplified and expressed in some malignant gliomas (21). A recent modification of the in-gel DNA renaturation technique (22) permits the detection of human DNA sequences amplified as little as 7- to 8-fold. This technique was used to test for the presence of amplified DNA in 16 cell lines, derived from different types of tumors that were not usually associated with oncogene amplification. Two of these cell lines contained amplified DNA sequences, which in one case appeared only after the passage of tumor cells in nude mice. In both cases, the amplified DNA sequences were found to include known cellular oncogenes (22).

In the present study, we utilized the same general assay to analyze gene amplification directly in surgical specimens of ovarian carcinomas, obtained from the primary or metastatic sites. Gene amplification was detected in two of eight tumors, and in both cases the amplified DNA sequences were found to include the Ki-ras oncogene. In one tumor, Ki-ras was coamplified with c-myc. No mutations at codons 12 or 61 of Ki-ras were detected in these tumors. In one of the tumors, Ki-ras was amplified in both the primary tumor and different metastatic nodules. These results indicate that gene amplification in ovarian carcinomas is likely to involve known cellular oncogenes, in particular Ki-ras.

MATERIALS AND METHODS

Tumor Tissues. Fresh surgical tumor specimens obtained from the primary site (ovary) and metastatic sites were examined grossly and by frozen sections to select areas of viable tumor and high tumor cell density (50% or greater). The appropriate areas were then dissected out and snap frozen in liquid nitrogen. The samples were stored at -70°C for subsequent analysis.

Gene Amplification Assays. Frozen tumor tissues were homogenized under liquid nitrogen using a magnetic vibrator (Mikro-Dismembrator II; Braun Instruments) or with mortar and pestle. The homogenates were suspended in 0.5 ml of 10 mM Tris (pH 8.0)-1 mM EDTA, and DNA was extracted using an automatic nucleic acid extractor (Model 340A; Applied Biosystems) under the conditions recommended by the manufacturer, except that the time for lysis and proteinase K digestion was increased from 45 to 90 min.

For gene amplification assays, tumor DNA was digested with HindIII and analyzed by the modified in-gel DNA renaturation procedure, as

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Gene amplification in ovarian carcinomas was tested for gene amplification using the modified in-gel DNA renaturation technique (22). This procedure involves separation of HindIII-digested DNA in an agarose gel, followed by two rounds of in-gel denaturation, renaturation, and S1 nuclease digestion. Subsequently, short S1 nuclease-resistant duplexes formed by interspersed highly repeated sequences are electrophoretically removed from the gel, and the long repeated and amplified restriction fragments, remaining in the gel, are transferred to a nylon membrane. Amplified fragments are detected by hybridization with a cloned Alu repeated sequence. This assay detects only a small number of Alu-related fragments that are reiterated in normal human DNA, with few if any polymorphic variations among different individuals. Amplified DNA, however, gives rise to multiple restriction fragments, containing Alu sequences, which are detectable by this technique. This method was shown to detect human DNA sequences amplified as little as 7- to 8-fold (22), although variability in the hybridization background may in some cases slightly reduce the sensitivity of the technique.

In preliminary studies, this technique was used to screen DNA preparations from frozen surgical specimens of 29 malignancies of the female genital tract (ovarian, endometrial, and cervical carcinomas), 31 colon carcinomas, 10 non-small cell lung carcinomas, and one hepatoma, obtained from various other sources. No amplified bands were detectable in any of the samples (data not shown). Subsequent pathological examination of selected specimens from this set revealed that all the examined samples contained a large amount of nontumorous and necrotic tissue elements, with viable tumor cells accounting for less than 20% of each specimen. It was likely therefore that the negative results obtained with many of these specimens could be an artifact resulting from dilution of tumor cell DNA, carrying amplified sequences, with DNA from nontumorous cells lacking gene amplification. Therefore in subsequent studies, carried out with a different set of tumors, we utilized only those specimens that were excised by a pathologist (R. D. E.) from the areas of viable tumor and high tumor cell density (see "Materials and Methods").

DNA was extracted from primary and metastatic sites of six ovarian adenocarcinomas with different levels of differentiation, one malignant mixed mesodermal tumor of the ovary, and one malignant mixed mesodermal tumor of the uterus. For five of the tumors, DNA was extracted from both the primary site and one or more metastatic sites. These tumors were analyzed for gene amplification using the modified in-gel DNA renaturation procedure. By this assay, two of the tumors were found to contain amplified DNA fragments. Fig. 1, Lane 1, shows multiple amplified fragments in the DNA of one of these tumors (R.A.). In the second case (P.H.), bands corresponding to the amplified fragments were detectable by visual examination of the autoradiogram, but the intensity of these bands relative to the background was too low for photographic reproduction (data not shown).

The positive DNA samples were then analyzed for amplification of known oncogenes by hybridization with c-myc, N-myc, L-myc, H-ras, v-Ki-ras, N-ras, erbA, erbB, and neu.

Fig. 1. Detection of amplified DNA sequences by modified in-gel renaturation. Each lane contains 15 μg of HindIII-digested genomic DNA, separated in a 1.2% agarose gel and analyzed by modified in-gel renaturation (26). Lane 1, DNA from an ovarian carcinoma (R.A.). Lanes 2 and 3, DNA from two colon carcinoma cell lines, showing the characteristic pattern of human DNA bands in the absence of gene amplification.
GENE AMPLIFICATION IN OVARIAN CARCINOMAS

probes. The R.A. sample, corresponding to a metastatic highly malignant mixed mesodermal tumor, was found to contain amplified c-myc and Ki-ras genes (Fig. 2). The degree of amplification, determined by densitometry of autoradiograms, was approximately 8-fold for both c-myc and Ki-ras. In the second tumor (P.H.), a poorly differentiated serous cystadenocarcinoma, only Ki-ras was found to be amplified (Fig. 3). There was no significant difference in the observed level of amplification between the primary tumor (11-fold) and the metastases from the omentum, abdominal wall, and colon (10-, 9-, and 8-fold, respectively).

The Ki-ras and c-myc probes were then used for Southern hybridization with DNA from the tumors that were negative for gene amplification by the modified in-gel renaturation assay and from the primary and metastatic sites of one additional ovarian adenocarcinoma. No amplification was detected in any of the samples (data not shown). We were also unable to detect amplification of either Ki-ras or c-myc in the previously analyzed samples of 29 carcinomas of the female genital tract, which had a low frequency of tumor cells (data not shown).

To determine if the amplified Ki-ras genes were activated by mutations at codons 12 or 61, we have used the PCR technique (30) to amplify in vitro the corresponding regions of genomic DNA. The PCR-amplified sequences were then analyzed for the presence of mutations by using specific oligonucleotide probes spanning codons 12 or 61. As shown in Fig. 4, PCR-amplified DNA from the samples showing amplification of the Ki-ras gene hybridized to oligonucleotide probes containing the wild-type sequences of codons 12 or 61, but not to the mixtures of oligonucleotide probes corresponding to different mutations at each of these codons. Control hybridizations showed that, under reduced stringency conditions, the mutant-specific oligonucleotide mixtures hybridized to PCR-amplified DNA as efficiently as the wild-type oligonucleotides (data not shown). In the case of codon 12, the absence of mutations was also confirmed by separate hybridizations with each of the individual mutant-specific probes (data not shown).

DISCUSSION

We have used a general assay for detection of amplified sequences of unknown nature to analyze gene amplification in surgical specimens of primary and metastatic ovarian carcinomas. Two of eight tumors were shown by this assay to contain amplified DNA sequences. In both cases, these sequences included the Ki-ras oncogene, which in one case was amplified together with c-myc. Although the modified in-gel renaturation procedure used in our study is unlikely to detect DNA sequences amplified less than 7-fold, no additional cases of Ki-ras or c-myc amplification were found among the samples that were negative in the original assays using a more sensitive Southern
hybridization technique. In the published large-scale studies of oncogene amplification in tumor tissues, amplification at a level lower than 7-fold was reported in fewer than 50% of the cases of HER-2/neu amplification in breast carcinomas (3) or N-myc amplification in neuroblastomas (4). It appears therefore that the modified in-gel renaturation assay can provide a reasonable approximation of the overall frequency of gene amplification in tumor DNA preparations.

The relatively low frequency of gene amplification in ovarian tumors observed in this study (two of eight by modified in-gel renaturation, two of nine by Southern hybridization) is similar to the values reported by other investigators, using Southern hybridization with oncogene-specific probes (see below). Underestimation of the amplification frequency, however, may be an intrinsic problem for any assay carried out on total tumor DNA preparations. The failure of our preliminary studies to detect gene amplification in any tumor samples obtained without direct participation of a pathologist underscores the need for careful pathological evaluation in the specimen preparation. The possibility of false negative results in gene amplification assays, resulting from the presence of nontumorous cells in the sample, has been recently emphasized (31). Furthermore, gene amplification may be missed even in a representative tumor specimen in the case where the gene of interest is amplified only in a genetically distinct subpopulation of tumor cells. This may be the reason why cytogenetic studies have suggested a higher frequency of gene amplification in ovarian carcinomas than that observed in the present work. Double minutes, cytogenetic markers of gene amplification, were found in malignant effusions from 65% to 79% of ovarian carcinoma patients, but in most cases double minutes were detectable only in a minority of tumor cells (32–34).

The finding of amplified oncogenes in both cases where gene amplification was first detected by the general assay suggests that known cellular oncogenes, rather than some presently unknown genes, are likely to be associated with most cases of gene amplification in ovarian carcinomas. Assuming that the size of amplified DNA is on the order of 10^6 kilobases, or less than 0.01% of the genome (35), the finding that amplified DNA in both amplification-positive tumors includes the Ki-ras gene appears to be highly significant. Ki-ras amplification was previously reported in one of seven (14) and one of five (9) cases of ovarian carcinomas. While this work was in progress, two other groups, using various oncogene probes to study gene amplification, reported an association between Ki-ras amplification and ovarian carcinomas. Masuda et al. (10) found Ki-ras amplification in 2 of 5 ovarian carcinoma samples, whereas Van't Veer et al. (16) reported that Ki-ras was amplified in 3 of 37 samples of ovarian tumors. In the latter study, Ki-ras was the only oncogene found to be amplified in ovarian carcinomas, and amplification of this gene was shown to be associated with increased mRNA and protein expression. Although there are sporadic reports of Ki-ras amplification in other types of human cancer (12), to the best of our knowledge there is no other tumor where Ki-ras amplification would have been detected in a significant minority of cases. For example, in the study of Masuda et al. (10), Ki-ras was amplified in 2 of 5 ovarian carcinomas, but only in 1 of 107 other tumors. The common findings of Ki-ras amplification can be contrasted with the very low incidence of mutational activation of Ki-ras in ovarian carcinomas (11–13, 16). The amplified Ki-ras genes in both tumors analyzed in our studies or in the three ovarian tumors studied by Van't Veer et al. (16) did not contain mutations in codons 12 or 61. Assuming that Ki-ras amplification in all cases played a functional role in tumor growth and was not a result of incidental coamplification with some unknown linked oncogene, it appears that amplification rather than point mutation may be the main mechanism for Ki-ras activation in ovarian carcinomas.

Aside from Ki-ras, only c-myc (9, 10, 15) and H-ras (10) amplification has been reported in ovarian carcinomas. In one of our amplification-positive samples, both Ki-ras and c-myc were amplified. Coamplification of these two genes was previously described in carcinomas of the lung (36), pancreas (37), and ovary (10). c-myc amplification has been observed in a wide variety of solid tumors (1, 10), and it appears to correlate with clinical stage in adenocarcinomas of other organs (10).

There was no apparent association between the presence of gene amplification and histological classification of the tumors used in this study. Both amplification-positive tumors were highly malignant and metastatic. In one of the tumors, Ki-ras amplification was found in both primary and all the metastatic sites, indicating that amplification occurred relatively early in the course of tumor progression. This result is in agreement with the studies of Wong et al. (38), who found that amplification of c-myc and N-myc in small cell lung cancer always occurred in parallel in the primary and metastatic sites. It appears therefore that generation of metastases does not generally involve de novo amplification of known oncogenes.

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GENE AMPLIFICATION IN OVARIAN CARCINOMAS


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