Novel DNA sequences at chromosome 10q26 are amplified in human gastric carcinoma cell lines: molecular cloning by competitive DNA reassociation

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ABSTRACT

Molecular cloning of genomic sequences altered in cancer cells is believed to lead to the identification of new genes involved in the initiation and progression of the malignant phenotype. DNA amplification is a frequent molecular alteration in tumor cells, and is a mode of proto-oncogene activation. The cytologic manifestation of this phenomenon is the appearance of chromosomal homogeneously staining regions (HSRs) or double minute bodies (DMs). The gastric carcinoma cell line KATO III is characterized by a large HSR on chromosome 11. In-gel renaturation analysis confirmed the amplification of DNA sequences in this cell line, yet none of 42 proto-oncogenes that we tested is amplified in KATO III DNA. We employed the phenol-enhanced reassociation technique (PERT) to isolate 21 random DNA fragments from the amplified domain, and used 6 of them to further clone some 150 kb from that genomic region. While in situ hybridization performed with some of these sequences indicated that in KATO III they are indeed amplified within the HSR on chromosome 11, somatic cell hybrid analysis and in situ hybridization to normal lymphocyte chromosomes showed that they are derived from chromosome 10, band q26. The same sequences were found to be amplified in another gastric carcinoma cell line, SNU-16, which contains DMs, but were not amplified in other 70 cell lines representing a wide variety of human neoplasms. One of these sequences was highly expressed in both KATO III and SNU-16. Thus, the cloned sequences supply a starting point for identification of novel genes which might be involved in the pathogenesis of gastric cancers, and are located in a relatively unexplored domain of the human genome.

INTRODUCTION

The initiation and development of the neoplastic process are associated with specific alterations in a large number of cellular genes (1). Such genes can be identified by their ability to induce neoplastic transformation or via their involvement in structural alterations occurring in the genome of cancer cells. A typical such alteration is DNA amplification. To date, at least 15 proto-oncogenes have been found to be amplified at different frequencies in a variety of human tumors (2–5). Experimental and clinical observations indicate that proto-oncogene amplification is associated with the progression of certain tumors to advanced stages, making it a molecular marker for poor prognosis in specific malignant diseases (2–7).

Extensive amplification of large genomic segments often results in the formation of chromosomal homogeneously staining regions (HSRs) or double minute bodies (DMs) (8). Their frequent appearance in tumor cell lines and primary tumors indicates that DNA amplification is common in cancer cells (9–11). HSRs and DMs were recently found in tumor specimens in which none of the tested proto-oncogenes was amplified (12; O. Mor et al., unpublished). Thus, a search for novel amplified sequences might reveal new genes involved in the tumorigenic process and lead to the derivation of new molecular probes of clinical value.

We use a combined approach to identify and isolate novel DNA sequences amplified in cancer cells (13). A variety of tumor cell lines are screened by cytogenetic analysis and in-gel renaturation in search of cytologic or molecular manifestation of DNA amplification. The latter method allows the detection of DNA sequences amplified at least 20-fold, without the use of specific probes (14). At the same time, Southern blotting analysis is used to rule out the amplification of 42 known proto-oncogenes as well as the multidrug resistance gene *mdr1* in these cells. Following identification of a cell line with evidence of DNA amplification but without any alterations in these genes, we employ the phenol-enhanced reassociation technique (PERT) to isolate the

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anonymous amplified sequences. This method which was originally developed for the isolation of DNA sequences missing in deletions (15), was adapted for random cloning of amplified sequences (16).

The cell line KATO III was established from a pleural effusion of a male patient with metastatic gastric adenocarcinoma (17). The karyotype of KATO III is hypotetraploid, and a prominent HSR on chromosome 11 has been noticed since the deposition of this cell line at the American Type Culture Collection, in 1983 (18). Using our experimental approach, we have now shown that novel DNA sequences from the chromosomal region 10q26, not related to known proto-oncogenes, are amplified both in this HSR, and in another gastric carcinoma cell line, SNU-16, which has DMs.

MATERIALS AND METHODS

Cell cultures

The cell line KATO III was purchased from the American Type Culture Collection. The establishment and characterization of SNU-16 was previously described (19). The cells were maintained in RPMI 1640 medium supplemented with 15% fetal bovine serum (Beit Ha'Emek, Israel).

Southern and Northern blotting analyses

Genomic DNA and total RNA were extracted from cultured cells according to standard methods (20). Digestion of genomic DNA with restriction endonucleases, Southern blotting, hybridization and autoradiography were carried out as previously described (21), with the following modifications: Nytran membranes (Schleicher and Schuell) were used for blotting, and radiolabelling was performed using the random priming method (22). Northern blots were prepared using nitrocellulose membranes from 20 μ g of total RNA which was electrophoresed in formaldehyde gels. Hybridization conditions were the same as for Southern blots.

In-gel renaturation

The in-gel renaturation method allows the detection of highly amplified (>20-fold) DNA sequences without using specific probes (14). The experiments were performed essentially as described by Roninson (14). Briefly, 15 μ g of DNA was digested with HindIII (New England Biolabs); 1 μ g of the digest was end-labelled with 32 P to specific activity of 10^7 cpm/ μ g using T4 DNA polymerase (Amersham) and mixed with the rest of the digest. The mixture was electrophoresed in 1% agarose gel, which subsequently underwent two cycles of alkaline denaturation, neutralization and S_1 nuclease treatment. The gel was washed extensively to elute the S_1 nuclease degradation products, dried, and autoradiographed using Curtix RP-2 film (Agfa). Best resolution of the bands was obtained without intensifying screens. Due to their higher concentration in the gel, amplified sequences reanneal faster, consequently are less susceptible to subsequent S_1 nuclease digestion and are revealed as distinct radiocactive bands.

Construction of a fragment library enriched for amplified sequences

The phenol-enhanced reassociation technique (PERT) is based on the competitive reassociation of denatured DNA fragments in phenol emulsion and was previously used to construct genomic libraries enriched for DNA sequences amplified in neuroblastoma

cell lines (16). 1 μ g of KATO III DNA was digested to completion with MboI (Bethesda Research Laboratories) and mixed with 70 μ g of normal male DNA which had been sheared by sonication to fragments of 300–800 bp. Denaturation and PERT reaction were carried out as described previously (16). Following phenol extraction and ethanol precipitation, varying amounts of the mixture were ligated to BamHI-cut dephosphorylated pBR322 plasmid (New England Biolabs), and the ligation products were used to transform competent DH5 bacteria. The insert size was determined in individual clones following digestion with EcoRI and SalI, which together release from pBR322 a 230 bp fragment containing the BamHI site. In order to identify possible amplification of the cloned sequences in KATO III, the EcoRI-SalI fragment of each clone was separated from the rest of the plasmid by electrophoresis in low melting point agarose gel, labelled and hybridized with Southern blots made with EcoRI-digested KATO III and normal DNA.

Isolation of genomic sequences flanking the PERT fragments

A genomic library made in the phage vector lambda FIX from human lung fibroblast DNA was purchased from Stratagene. PERT probes shown to be unique in normal DNA and amplified in KATO III were used to screen 4×10^5 clones of this library by plaque hybridization. Plaques which appeared positive on duplicate filters were further purified, and the recombinant phages were analyzed by restriction mapping and Southern blotting in order to identify unique fragments and locate the sequences homologous to the original PERT probes.

Chromosomal assignment of cloned sequences using somatic cell hybrids

Unique fragments isolated from the recombinant phages were hybridized with Southern blots made from a panel of 35 human-mouse somatic cell hybrids with different human chromosome constitutions. The hybrids were characterized for human chromosome content by karyotypic analysis and mapped enzyme markers (23–25).

In situ hybridization

Confluent cell cultures were split at 1:4 ratio, and 48 hr later the mitotic cells were blocked with 10^{-6} M colchicine for 1 hr. Peripheral blood lymphocytes from a normal female were cultured for 68 hr in F-10 medium containing 20% fetal bovine serum and 3% phytohaemagglutinin, and were then blocked in the same way. In situ hybridization was performed essentially as described by Harper and Saunders (26), with slight modifications. Air-dried chromosome slides were pretreated for 30 min with $2 \times$ SSC at 70°C, and then with RNase A (100 μ g/ml in $20 \times$ SSC) for 1 hr at 37°C. Chromosome denaturation was carried out in 70% formamide, 10 mM phosphate buffer (pH 6.8), $20 \times$ SSC for 2 min at 70°C. Probes were labelled with [3 H]dCTP using the random priming method and were hybridized to the slides for 18 hr at 37°C in 50% formamide, $2 \times$ SSC, 10% dextran sulfate, 0.2 mg/ml fycoll, 0.2 mg/ml polyvinylpyrrolidone, 0.2 mg/ml bovine serum albumin, and 100 μ g/ml sonicated denatured salmon-sperm DNA. The slides were washed with 50% formamide in $2 \times$ SSC at 41°C, dried in alcohol series and coated with NTB-2 emulsion (Kodak). Exposure was carried out for 7 days at 4°C. Only silver grains situated on non-overlapping chromosomal regions were scored and their location determined on G-banded chromosomes.

RESULTS

DNA sequences not related to known proto-oncogenes are amplified in KATO III

Cytogenetic and in-gel renaturation analysis were used to screen 25 human tumor cell lines in search of amplification hallmarks. The cell line KATO III, derived from a gastric carcinoma (17), showed clear indication of DNA amplification by both criteria. Examination of 30 trypsin-banded metaphases of this cell line (not shown) reconfirmed the presence of a large HSR in chromosome 11 (18). In-gel renaturation was carried out using DNA from KATO III, normal controls, and the neuroblastoma cell line Nub 7 in which N-myc is amplified 70-fold (27). The normal controls (Fig. 1, lanes 1 and 3) revealed the expected pattern resulting from satellite and mitochondrial DNA reassociation, while Nub 7 (lane 2) showed an extensive series of additional bands, representing the N-myc amplicon. Numerous amplification bands were observed also in KATO III DNA (lane 4), their intensity being smaller than that of the amplified bands of Nub 7, indicating lower level of amplification. We tested the copy number of 42 proto-oncogenes and the multidrug resistance gene in KATO III DNA. Southern blotting analysis (not shown) indicated that all these genes were present as single copy in this cell line.

Isolation of random fragments from the amplified domain using the PERT technique

In phenol-water emulsion, the rate of DNA reassociation is highly accelerated (28). Shiloh et al. (16) applied this method to prepare PERT libraries enriched for DNA sequences amplified in two neuroblastoma cell lines. A small amount of denatured restriction fragments of tumor DNA (tracer) was reannealed with excess sheared denatured normal DNA (driver). Only double stranded fragments regenerated from reassociation of tracer-derived single-stranded molecules bear clonable ends. Shotgun cloning of the reaction products resulted in a library enriched for amplified sequences, owing to their higher chance of self reassociation.

A PERT library was constructed using KATO III DNA as tracer. Southern blotting analysis using random clones from this library as probes identified 21 unique clones which were highly amplified in KATO III DNA (Fig. 2). These clones constitute

Fig. 1. In-gel renaturation of DNA from different cell lines. Lanes 1 and 3: normal lymphoblasts. Lane 2: the neuroblastoma Nub 7. Lane 4: KATO III.

12.1% of the PERT fragments which gave a visible hybridization signal with human blots. Southern blotting analysis of serial dilutions of KATO III DNA with mouse DNA (not shown) showed that amplification of different PERT probes in KATO III DNA varied from 30-fold to 50-fold. It should be noted that 10 of these clones also detected DNA rearrangements in KATO III (Fig. 2).

Isolation of genomic sequences flanking the PERT fragments

Initial cloning of DNA sequences flanking amplified PERT fragments was carried out by screening a genomic library with six of these probes. Each PERT fragment identified between 2 and 8 partly overlapping phage clones, containing a total of 20 to 30 kb of genomic DNA. Restriction maps of these different genomic segments and cross hybridization experiments indicated that they were not overlapping. Various unique fragments from these phages were found to be amplified in KATO III to the same extent as the original PERT clones (not shown). These results indicate that the PERT clones were indeed derived from different parts of the KATO III amplicon.

Amplification in other tumor cell lines

The copy number of the PERT clones and the genomic phages was tested in the DNA of 71 different cell lines representing a wide variety of human solid neoplasms (tumors of the gastrointestinal system, breast, ovary, lung, bone, nervous system, prostate and skin). While being present in single copy in 70 of these cell lines, all of our probes were amplified in one of the 12 gastric carcinoma cell lines tested, SNU-16 (19). The

Fig. 2. Hybridization of various PERT clones to Southern blots containing EcoRI-digested DNA from normal cells (left lane) and KATO III (right lane). Probe no. 1 is present in single copy in KATO III, while the others are amplified in this cell line. Probes no. 6 and 7 detect in KATO III rearrangements representing novel joints formed during the amplification process.

Fig. 3. Southern blots of normal DNA (1), KATO III (2) and SNU-16 (3), probed by 2 different PERT clones (A and C) and the phage clone 24-1 (B). Note the different organization in the two cell lines of the sequences corresponding to two of the probes.

Fig. 4. In situ hybridization of the probe 69-8B-1.1 to KATO III chromosomes. Note the clustering of silver grains on the HSR on chromosome 11.

degree of amplification was similar in KATO III and SNU-16, however occasional variations in the hybridization patterns indicated different organization of these sequences in the two cell lines (Fig. 3).

Localization of the sites of amplification in KATO III and SNU-16

Two unique fragments, 69-8B-1.1 and 12-10-2.8, derived from different genomic phages were hybridized in situ to KATO III and SNU-16 metaphases. While heavy clustering of silver grains on the HSR of chromosome 11 was noticed in KATO III with each of the two probes (Fig. 4), no such clustering was observed on any specific chromosome in SNU-16 (not shown). It should

Fig. 5. Distribution of silver grains scored on chromosome 10 in normal lymphocytes following in situ hybridization of probes 69-8B-1.1 and 12-10-2.8

Fig. 6. Hybridization of the probe 24-1 to a Northern blot of KATO III (1) and SNU-16 (2). The smear in the upper part of the lanes was noticed consistently and probably represents hybridization to intermediates of RNA processing.

be noted that in situ hybridization to DMs is known to present difficulties, probably due to DNA organization within these structures (27a,b). Therefore, while the site of amplification of these sequences in KATO III is definitely the HSR in chromosome 11, in SNU-16 it is probably extrachromosomal.

Localization of the amplified sequences in the normal genome

Somatic cell hybrid analysis and in situ hybridization were used in parallel to map in normal cells the genomic domain amplified in KATO III. The same two probes mentioned in the previous section and a fragment from an additional genomic segment served for both purposes. Under normal stringency, these probes did not hybridize with mouse DNA. The segregation of human bands detected by the three probes in 35 human-mouse hybrids was similar, and was concordant only with presence of chromosome # 10 in the hybrids (Table 1). Lack of hybridization of the probes with DNA from the hybrid XTR-3BSAgB, containing a derivative of chromosome 10 (10pter-10q23:) and no intact chromosome 10, indicated that these sequences localized to the q23-qter region of this chromosome. In situ hybridization of these probes to normal lymphocyte chromosomes resulted in a significant peak over the most distal portion of 10q, band q26 (Fig. 5).

Table 1. Segregation of DNA sequences corresponding to probes 69-8B-1.1, 25-1.8 and 12-10-2.8 with human chromosomes in EcoRI-digested human-mouse cell hybrid DNA.

Chromosome	Concordant # of Hybrids		Discordant # of Hybrids		% Discordancy
	(+/+)	(-/-)	(+/-)	(-/+)	
1	6	12	12	0	40
2	11	8	10	5	44
3	15	8	6	4	30
4	9	8	13	5	51
5	13	9	9	4	37
6	10	11	12	2	40
7	12	8	8	4	37
8	12	11	10	2	34
9	4	11	18	2	57
10	22	12	0	0	0
11	15	9	7	4	31
12	15	7	7	6	37
13	10	8	12	5	49
14	17	6	5	7	34
15	12	11	9	2	32
16	8	12	14	1	43
17	18	5	3	8	32
18	14	8	8	5	37
19	6	9	16	4	57
20	14	7	8	6	40
21	14	4	8	9	49
22	6	8	15	5	59
X	11	5	7	7	47

This table is compiled from 35 cell hybrids involving 15 unrelated human cell lines and 4 mouse cell lines (21–23). The DNA probes were hybridized to Southern blots containing EcoRI digested DNA from the human-mouse hybrids. The scoring for the probes was determined by the presence (+) or absence (–) of human bands in the hybrids on the blots. Concordant hybrids have either retained or lost the human bands together with a specific human chromosome. Discordant hybrids have the human bands, but not a specific chromosome or the reverse. Percent discordancy indicates the degree of discordant segregation for a marker and a chromosome. A 0% discordancy is the basis for chromosome assignment. Only chromosome 10 showed 0% discordancy with the two probes.

Identification of an expressed sequence in the amplified domain

In search for functional genes, unique fragments from the six genomic segments isolated from the amplified domain were used as probes against Northern blots of KATO III and SNU-16. Two such fragments, located 10.6 kb apart within one of these segments identified a major 4.5 kb and a minor 1.8 kb transcripts in RNA from KATO III and SNU-16. In SNU-16 a second major transcript appeared at 5.7 kb (Fig. 6). No hybridization was detected on Northern blots from nine normal tissues. These results indicate that both probes probably contain exons of a gene whose expression is highly elevated in the two cell lines.

DISCUSSION

There is growing evidence that genomic aberrations occurring in cancer cells signpost the location of genes involved in the tumorigenic process (1,29–31). Since DNA amplification is believed to occur mainly in advanced stage tumors, our working hypothesis is that identification of novel DNA sequences amplified in tumor cells might reveal new genes that contribute to tumor progression. Evidence for amplification in cell lines or tumor material can be achieved using combined karyotypic and in-gel renaturation analysis. Various methods devised for random cloning of anonymous amplified sequences are based on differential DNA hybridization (32,33), molecular cloning from

renaturation gels (34), physical isolation of DMs or HSR-bearing chromosomes (35–37), and microdissection of HSRs (38). The PERT method (16) is relatively simple and can be applied even to partly degraded DNA.

The different restriction maps around the various PERT fragments, their different hybridization patterns to Southern blots and their variable copy numbers in KATO III (ranging between $\times 30$ and $\times 50$) indicate that the PERT experiment picked fragments scattered throughout the KATO III amplicon. Using a human genomic library, we have isolated to date some 150 kb of genomic DNA from this amplicon, which might be much larger, as indicated by the large size of the HSR but moderate level of amplification. Previous studies have indeed shown that amplicon size in mammalian cells is usually in the range of hundreds and even thousands kb (8,37,39,40). Isolation of additional PERT clones and extensive chromosome walking should lead to molecular cloning of increasing portions of this amplicon.

The sequences cloned from the KATO III amplicon are not frequently amplified in human cancer cells, as shown in our screening of a large panel of tumor cell lines. On the other hand, their amplification in another, independently derived gastric carcinoma cell line indicates that this phenomenon is not peculiar to KATO III only, but may rather characterize a subset of gastric carcinomas. A search for functional genes within the cloned genomic domain should eventually reveal one or more 'target

genes', whose amplification supplies the driving force underlying the amplification of the whole domain. The amplification of such genes might play a role in the progression of gastric carcinomas. A recent example of a novel gene identified within anonymous amplified sequences is the gli gene, which was cloned from an amplicon found in glioblastomas (34) and later found to code for one of the Kruppel zinc finger proteins (41). Our finding of a gene which is located within the genomic domain amplified in KATO III and SNU-16 and is highly expressed in both cell lines demonstrates once again the feasibility of this approach. The expression of this gene is probably very limited in normal tissues. An attempt to clone the cDNA of this gene from KATO III is now in progress.

The cloned sequences map to chromosome 10q26. It is noteworthy that to date only one gene, ornithine aminotransferase (OAT), one fragile site and a few random DNA fragments have been mapped to 10q26 (Human Gene Mapping 10, 1989). The sequences cloned in this study should furnish new starting points for the investigation of this relatively unexplored region.

The amplification of DNA sequences from 10q26 also involves their transposition to an HSR on chromosome 11 in KATO III and most probably to DMs in SNU-16. This phenomenon is characteristic of many amplicons in cancer cells (2,42,43), and points to the involvement of extrachromosomal intermediates in the amplification process (44). It is usually assumed that DMs reflect a transient state of the amplified sequences and precedes their stable integration in HSRs (8). A minor subline of SNU-16 indeed shows several HSRs (19). It should be of interest to see whether the amplified sequences are located in the HSRs in this subline. It should be born in mind that additional regions on chromosome 10 might be involved in the formation of the HSR on chromosome 11. Such 'recruitment' of DNA sequences from different parts of the short arm of chromosome 2 to form an HSR on chromosome 1 was previously demonstrated in the neuroblastoma cell line IMR-32 (43). Further isolation of PERT clones and their localization should be carried out in order to address this possibility. Isolation of large parts of the amplicon should also allow structural analysis of the amplified sequences, which might supply clues to the nature of the amplification process. Particularly interesting is the internal structure and arrangement of the repeated units and the nature of the sequences involved in the formation of novel joints (39,45,46). It should be noted that 10 of the first 21 amplified PERT fragments detected rearrangements representing novel joints in KATO III DNA. This indicates a high extent of structural alterations which occurred during the formation of the KATO III amplicon. Identification of the sequences involved in these joints might reveal sequences which are 'hot spots' of genomic instability.

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