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C-*myc* Proto-oncogene Amplification Detected by Polymerase Chain Reaction in Archival Human Ovarian Carcinomas

Gilbert Schreiber and Louis Dubeau

From the Kenneth Norris Jr. Comprehensive Cancer Center, University of Southern California, Los Angeles, California

Polymerase chain reaction (PCR) technology was used to examine the state of amplification of the proto-oncogene c-myc in archival ovarian carcinomas. Sequences from the c-myc gene and from a control gene were amplified simultaneously by PCR and the ratios of the two products measured. The results provided an accurate measurement of the relative number of copies of the two genes in each tumor genome if the control and test sequences amplified by PCR were of equal lengths. The results were not affected by the number of PCR cycles used. This technique should facilitate gene amplification studies in clinical medicine. Increased c-myc copy number was found in 17% of the 30 cases examined when a control from the same chromosome as c-myc was used, but in 37% of cases if a control from another chromosome was used. This underlines the importance of the genetic location of the selected control genes for such studies. (Am J Pathol 1990, 137:653–658)

Genetic amplification is a common mechanism of activation for several cellular proto-oncogenes. Such molecular alterations are relevant to our understanding of tumor genetics and, at least in certain cases, can provide prognostic information for affected patients.[1,2] The multidrug resistance locus[3,4] is another example of a gene commonly amplified in malignant cells, which may be important in altering tumor sensitivities to various chemotherapeutic agents.

The currently favored approaches to detect gene amplification involve Southern or slot blot analyses of genomic DNA using DNA probes for the potentially amplified genes. Those techniques depend on the availability of relatively large quantities of genomic DNA in a form suitable for hybridization studies. These restrictions limit the applicability of molecular knowledge to clinical medicine because adequate amounts of such DNA often are not available from clinical tissue specimens. Although DNA may be recovered from the virtually unlimited number of formalin-fixed archival human tissue specimens kept in hospital centers around the world, these techniques largely depend on the quality of fixation and success rates vary significantly between different academic centers.[5,6]

The recent development of polymerase chain reaction (PCR) technology[7,8] literally revolutionized the use of molecular techniques for genetic analyses. Not only does this technique require only minute amounts of DNA[9] but it is also readily applicable to the partially degraded and chemically altered DNA commonly present in crude preparations from archival clinical material.[10] Until now the use of such technology for quantitative analyses, however, has been limited because the quantities of PCR products obtained can be markedly altered by slight fluctuations in variables such as incubation temperatures and reagent concentrations. In the present study, we eliminated these difficulties by simultaneously performing PCR on test and control genes in the same PCR mix. The results were similar to those obtained from Southern blot analyses and were not significantly affected by the number of cycles used for the reaction. We used this approach to examine the state of amplification of the proto-oncogene c-*myc* in archival human ovarian carcinomas. Our results showed increased c-*myc*-to-control ratios in 17% of the tumors examined when our control gene was from the same chromosome as c-*myc*. This percentage was increased to 37% when a control from another chromosome was used. These differences underscore the importance of selecting control genes from the same chromosome as test genes for gene amplification studies, as previously emphasized by other authors.[2]

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Dr. Schreiber's present address is Universitäts-Frauenklinik Frankfurt, Abteilung für Gynäkologie und Gynäkologische Onkologie, Theodor-Stern-Kai 7, 6000 Frankfurt/Main 70, FRG.

Address reprint requests to Louis Dubeau, MD, PhD, Kenneth Norris Jr. Comprehensive Cancer Center, 1441 Eastlake Ave., Los Angeles, CA 90033.

Materials and Methods

Source and Grading of Tumor Specimens

Specimens of formalin-fixed, paraffin-embedded ovarian carcinomas were obtained from the pathology archives of the Kenneth Norris Jr. Cancer Hospital. Frozen tissue specimens were obtained from a tumor bank at the same hospital. Each tumor was reviewed histologically and assigned an histologic grade using the criteria of the International Federation of Gynecology and Obstetrics (FIGO). Briefly, lesions with a solid component accounting for less than 10% of the total tumor mass were assigned a grade 1, those that were 10% to 50% solid were assigned a grade 2, and those that were more than 50% solid were assigned a grade 3. NIH:OVCAR-3 and SK-OV-3 ovarian carcinoma cell lines were obtained from the American Type Culture Collection (ATCC HTB161 and HTB77, respectively). HEY and HOC-7 cells were obtained from Dr. Ronald Buick of the University of Toronto.[11]

Preparation of Archival Specimens for PCR

Sections from each tumor were first reviewed histologically to identify any area containing large amounts of nonneoplastic stroma and such areas were removed from the specimens with scalpel blades before being used for PCR studies. Formalin-fixed archival tissues were prepared for PCR using the technique of Shibata et al,[10] with minor modifications. Briefly, single 5-μ–thick tissue sections were obtained with a microtome and deparaffinized by two consecutive centrifugations in xylene. The sections were washed three times in absolute ethanol and resuspended in 50 μl of 100 mmol/l (millimolar) TRIS Cl (pH 8.0), 4 mmol/l EDTA, and 400 μg/ml proteinase K. The preparations were incubated overnight at 37°C and finally heated at 100°C for 7 minutes to denature the proteinase K. Aliquots of 5 μl were added directly to PCR mixes (see below).

Oligonucleotide Primers for PCR

Synthetic primers used for enzymatic amplification of c-*myc* sequences were: 5′-CTCGGAAGGACTATCCTGCTGCCAA-3′ and 5′-GGCGCTCCAAGACGTTGTGTGTTCG-3′. The former corresponds to nucleotides 6840 to 6865 of the published c-*myc* sequence[12] and the latter is complementary to nucleotides 6990 to 6965 of the same sequence. Primers used for enzymatic amplification of alpha-lactalbumin sequences were 5′-GGTTCTTGGGGGTAGCCAAAATGAG-3′ and 5′-CAGGCAAAGCGATGCCTCCATAACC-3′, which correspond, respectively, to positions 7 to 32 and 132 to 157 of the published lactalbumin sequence.[13] For tissue plasminogen activator (tPA) sequences we used oligomers 5′-CAAAGGAGGGCTCTTCGCCGACATC-3′ and 5′-TCCTGGAAGCAGTGGGCGGCAGAGA-3′. The first extends from nucleotides 1018 to 1043 and the second is complementary to nucleotides 1167 to 1142 of the published tPA sequence.[14] Each of the above set of primers resulted in the enzymatic amplification of sequences of a length of 150 bases. The c-*myc* sequence had a HinfI restriction endonuclease site 105 bases away from its 3′-end and the lactalbumin sequence had a site for HaeIII exactly in its center. All oligomers were synthesized using a model 380A DNA synthesizer (Applied Biosystems, Foster City, CA) at the Microchemical Core Facility of the Kenneth Norris Jr. Comprehensive Cancer Center.

Conditions for PCR

The composition of the PCR mix was essentially as published by Saiki et al,[8] using TaqI DNA polymerase (Perkin-Elmer-Cetus, Emeryville, CA) and an automatic DNA cycler (Perkins-Elmer-Cetus). Two different sets of primers, one for c-*myc* and the other for either tPA or alpha-lactalbumin, were present simultaneously in each reaction, as explained in the text. Machine settings for each cycle were 94°C for 30 seconds to denature the DNA, 55°C for 1 minute to anneal the primers, and 72°C for 30 seconds for chain elongation.

Digestion, Electrophoresis, Blotting, and Quantitation of PCR Products

After addition of 0.4 mol/l (molar) LiCl and 40 μg/ml glycogen to each PCR mix, the reaction products were precipitated with 2.5 volumes of absolute ethanol at −20°C overnight. The samples were then electrophoresed directly on gels containing 3% NuSieve and 1% Seakem agaroses (FMC, Rockland, ME) or first digested with either HaeIII or HinfI restriction enzymes obtained from Promega (Fisher Scientific Co., Santa Clara, CA). Enzyme digestion was done according to manufacturer's instructions using 40 units of enzyme per sample. After electrophoresis the DNA samples were transferred overnight to Zetaprobe nylon membranes (Bio-Rad, Richmond, CA) under alkaline conditions, as previously described,[6] and hybridized to the appropriate ^{32}P-labeled DNA probes. Conditions for hybridization and labeling of the probes have been described.[6] The membranes were autoradiographed and resulting signal intensities were measured using an ultrascan XL 2222-020 laser densitometer purchased from LKB (Bromma, Sweden).

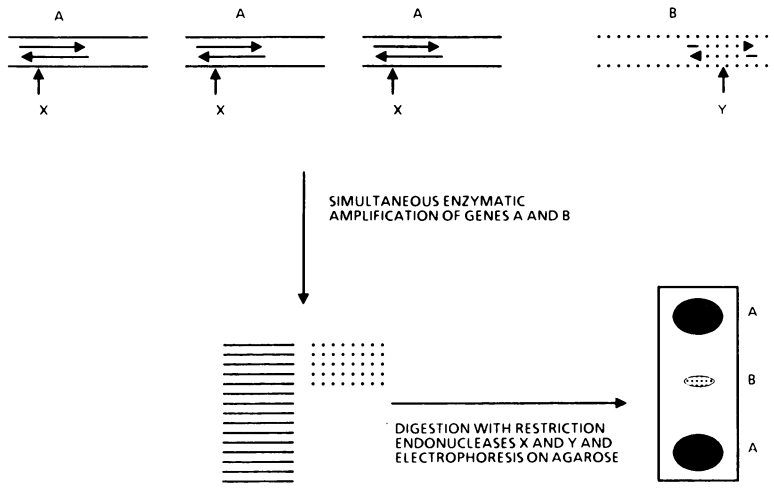

Figure 1. *Detection of gene amplification by PCR. Two genes, A and B, are represented. The number of copies of gene A is three times higher than gene B. Sequences from genes A and B are amplified simultaneously by PCR in the same reaction mix. After completion of PCR, there is three times more product from gene A than from gene B. In this example, the two products are distinguished from each other by taking advantage of different restriction endonuclease sites in the A and B sequences. The two products are separated electrophoretically after digestion with the appropriate enzymes and their relative amounts determined by densitometry.*

Source of DNA Probes

Probe phB-35[15] is a cDNA probe for human alpha-lactalbumin and was obtained from Professor R. K. Craig of the Middlesex Hospital Medical School, London. Probe pHSR-1 was obtained from the American Type Culture Collection (ATCC 41010). It is a genomic probe for the c-*myc* proto-oncogene. For our studies we used a Hind III/EcoRI fragment corresponding to the third exon of this gene. Our tPA probe was pPA01, which is a cDNA probe isolated by Ny et al[14] and provided to us by Dr. T. Ny of the University of Umea, Sweden.

Results

Strategy for Gene Amplification Studies by PCR

Our strategy for quantitative measurements of gene amplification using PCR technology is outlined in Figure 1. Represented are two different genes, A and B, with the number of copies of gene A three times as high as the number of copies of gene B. The two genes are amplified simultaneously and in the same reaction mix by PCR using gene-specific oligonucleotide primers. It is important that the two sets of primers are included in a single reaction mix, as opposed to two separate ones, to avoid variations in amplification efficiencies for the two genes due to slight fluctuations in incubation temperatures or reagent concentrations. In addition, the two amplified sequences should be of similar lengths because longer sequences have increased chances of being partially degraded and, in addition, are associated with decreased reaction efficiencies. Ideally the amplimers also should have similar G-C contents as well as similar degrees of secondary structure to avoid large differences in reaction efficiencies. If those rules are met, theoretically there should be three times as much product from gene A than from gene B after the reaction. A number of approaches then can be used to quantitate the two different products. In the example of Figure 1, we have taken advantage of the presence of different restriction enzyme sites within the two sequences to digest the two product species into fragments of different sizes that can be resolved by agarose gel electrophoresis and quantitated by densitometry.

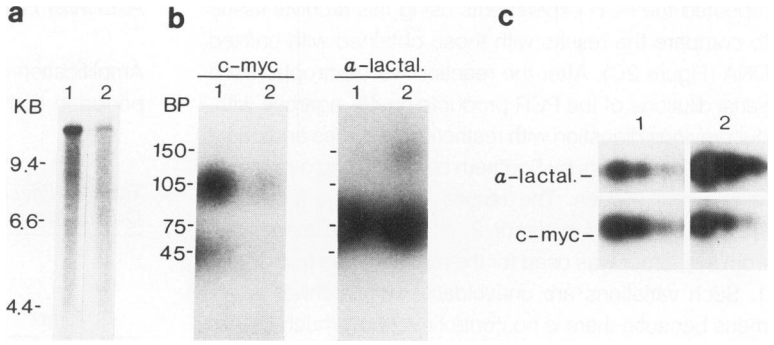

Figure 2. *Comparison of* c-myc *amplification obtained by either Southern blotting or PCR in unfixed and fixed ovarian carcinoma specimens.* **A:** *DNAs were extracted from frozen specimens of two different ovarian carcinomas, digested with EcoRI, and analyzed by Southern blotting using a radiolabeled* c-myc *DNA probe.* **B:** *The same two DNAs were amplified by PCR using* c-myc *and lactalbumin primers, double digested with HinfI and HaeIII, electrophoresed on agarose, transferred to a nylon membrane, and probed first for* c-myc *and then for lactalbumin products.* **C:** *Formalin-fixed specimens of tumors 1 and 2 were obtained from pathology archives and analyzed by PCR. Serial dilutions of the PCR products were electrophoresed on agarose without previous digestion with restriction enzymes. DNAs were then transferred to a nylon membrane and hybridized first with alpha-lactalbumin and then with* c-myc *probes.*

Table 1. *Quantitation of Relative c-myc Gene Copy Numbers in Ovarian Tumor Genomes by Southern Blotting and PCR Studies*

Tumor number or cell line	Southern blotting	PCR
1	4–5*	4–5*
2	1	1
19	1	1
31	1	1
HEY	2	2
NIH:OVCAR-3	3	3
HOC-7	3–4	3–4
SK-OV-3	2	2

* The relative number of copies of the c-*myc* gene was determined in DNA extracted from either frozen tumor tissue or cultured cell lines using alpha-lactalbumin as the control.

In Figure 2 we examined the feasibility of using the above approach for the quantitation of the relative number of copies of the c-*myc* proto-oncogene in two different human ovarian carcinomas. Shown in Figure 2A is a Southern blot of DNA extracted from two frozen tumors and analyzed with a probe for the c-*myc* gene. The results show a four- to fivefold increase in c-*myc* signal intensity in tumor 1 compared to tumor 2. Reprobing the same blot with a cDNA probe for human alpha-lactalbumin confirmed that those results were not due to inequalities in amounts of DNA loaded in each lane (results not shown). Then we studied the same two DNAs by PCR using specific oligonucleotide primers for c-*myc* and lactalbumin sequences. Both sequences were amplified simultaneously in the same reaction mix. The enzymatically amplified c-*myc* and lactalbumin fragments were both 150 bases long. The former had a HinfI restriction site 45 bases away from its 5'-end and the latter had a HaeIII site in its center. The two DNA products were digested with those two restriction enzymes, electrophoresed on 4% agarose, and analyzed by Southern blotting using c-*myc* and alpha-lactalbumin probes (Figure 2B). The results showed a four- to fivefold increase of the c-*myc* signal in tumor 1, although the lactalbumin signals were similar for both tumors. Then we retrieved formalin-fixed specimens of the same two tumors from our pathology archives and repeated the PCR experiments using this archival tissue to compare the results with those obtained with unfixed DNA (Figure 2C). After the reaction we electrophoresed serial dilutions of the PCR products on 4% agarose without previous digestion with restriction enzymes and quantitated the products by Southern blotting using c-*myc* and lactalbumin probes. The results showed an increased lactalbumin signal in tumor 2, implying that more DNA from this tumor was used for the reaction than from tumor 1. Such variations are unavoidable with archival specimens because there is no control over how much DNA is present in each sample. However, when the lactalbumin results were used as averaging signals to determine the relative amounts of c-*myc* in both tumors, again we found that tumor 1 had four to five times more copies of this gene than did tumor 2.

Thus these experiments demonstrate the validity of our approach for quantitative gene amplification studies. In the example of Figure 2, the results of the PCR studies were similar to those obtained by conventional Southern blotting experiments and were essentially similar for both unfixed and fixed tumor specimens. In another set of experiments, we examined the state of amplification of the c-*myc* gene in four different frozen ovarian tumor specimens and in four different ovarian carcinoma cell lines both by Southern blotting and PCR technologies to extend our comparison of the two approaches (Table 1). Five of those eight tumors contained an increased c-*myc* copy number relative to lactalbumin. Again the results showed no appreciable differences between the two techniques (Table 1).

Effect of the Number of PCR Cycles Used on Results Reproducibility

The efficiency of PCR can show large variations, depending on the number of cycles used.[8] Although enzymatic amplification may be nearly logarithmic during the first cycles, the reaction eventually reaches a plateau due to reagent depletion and loss of enzyme activity. Therefore it was important to determine if results obtained using our above mentioned approach would be affected by the number of PCR cycles. The results of Table 2, however, showed no difference in c-*myc* amplification values obtained with two different tumors when we used either 25 or 50 PCR cycles. Thus although the enzymatic amplification reaction had undoubtedly exceeded the logarithmic phase after 50 cycles, the c-*myc* and lactalbumin sequences studied were affected equally and therefore the results were independent of the number of cycles used under these conditions.

Detection of c-myc Gene Amplification in Archival Human Ovarian Carcinomas

Amplification of the c-*myc* proto-oncogene has been reported in human ovarian carcinoma cell lines.[16] In addi-

Table 2. *Effect of the Number of PCR Cycles on Quantitative Gene Amplification Data*

	Relative c-*myc* gene copy number*	
Tumor	25 cycles	50 cycles
A	1.7 ± 0.2	1.8 ± 0.2
B	4	4

* The control gene used for these experiments was alpha-lactalbumin.

tion, Zhou et al.[17] reported similar amplification in 3 of 12 ovarian tumors. Increased c-*myc* expression was demonstrated by Watson et al.[18] in serous carcinomas of the ovary. Abnormalities involving this proto-oncogene therefore may be important in ovarian carcinogenesis. We have used the above approach to examine the state of amplification of the c-*myc* gene in 30 human ovarian tumors obtained from pathology archives. The results (Table 3) showed that this genetic abnormality was indeed detected in 5 (17%) of the 30 tumors when human tPA was used as our control gene. Although this number clearly is too small to allow any conclusions regarding correlations between c-*myc* gene amplification and tumor clinical behavior, it may be significant that only one of our five cases with such amplification was classified as grade 1 according to the FIGO criteria (Table 3). However there was similarly only one case with c-*myc* amplification that was classified as grade 3 and therefore additional studies are necessary.

Table 3 also shows results when a sequence from the alpha-lactalbumin gene was used as control instead of tPA. Our reasons for repeating the experiments with the latter were that although tPA and c-*myc* are located on the same chromosome, alpha-lactalbumin is not. It has been emphasized[2] that control genes from the same chromosome should always be used as test genes because chromosomal duplications, which are frequent in malignant tumors, otherwise would be impossible to distinguish from true gene amplifications. To demonstrate the extent of this phenomenon, we have compared the results obtained with both tPA and alpha-lactalbumin controls. The results showed an increase in the c-*myc* to control ratios in 11 tumors when we used the latter control, as compared to only five with the tPA control (Table 3). These differences were not due to experimental variabilities because some of our experiments were done three times and in each of these cases the standard deviations were small. It is interesting that there seemed to be significant correlation between increased c-*myc*-to-lactalbumin ratio and tumor grade in those samples. Although such molecular changes were seen in only two of nine grade 1 tumors (23%), they were present in 6 of 11 grade 3 lesions (55%) (Table 2). These results may be a reflection of increased random chromosomal abnormalities that are associated with more anaplastic tumors in general and therefore may be not directly related to the c-*myc* locus. Thus failure to use controls from the same chromosome as test genes can significantly affect the results and lead to major interpretation errors.

Table 3. *Amplification and Relative Copy Number of c-myc Proto-oncogene in Archival Specimens of Human Ovarian Carcinomas*

Tumor	Histologic grade	c-*myc*/tPA*	c-*myc*/lactal*
1	3	1	4.3 ± 0.3†
2	1	1	1.3 ± 0.1
3	2	1	1.3 ± 0.1
4	3	1	5.2 ± 0.4
5	1	1	1.0 ± 0.1
6	3	1	4.5 ± 0.3
7	1	1	1
8	2	1	2
9	3	1	1
10	3	1	1
11	3	2	1
12	3	1	2
13	2	2	1
14	3	1	2
15	3	1	2
16	2	5	2
17	1	2	2
18	2	1	2
19	2	1	1
20	1	1	1
21	1	1	1
22	2	3	1
23	2	1	1
24	3	1	1
25	2	1	1
26	2	1	1
27	1	1	2
28	3	1	1
29	3	1	1
30	1	1	1

* The relative number of copies of the c-*myc* gene was determined using either tPA or alpha-lactalbumin as controls.

† Standard deviations are given for experiments performed at least three times.

Discussion

The results of our studies clearly show that PCR technology may be used for quantitative analyses of the state of amplification of specific genes. The results were similar to those obtained by Southern blotting analyses. According to this approach, two different genetic sequences are enzymatically amplified simultaneously, one corresponding to a control gene and the other to a test gene. The two sequences must be of equal lengths and be amplified together in the same reaction mix to ensure equal reaction efficiencies. Our results also demonstrated the importance of using control sequences from the same chromosome as the test genes for these studies, in accordance with other authors.[2] We were initially concerned that reaction efficiencies may be different for test and control sequences after a large number of PCR cycles because reaction kinetics may be affected by decreased reagent concentrations.[8] However the results of Table 2 showed no appreciable differences in the results when we used either 25 of 50 enzymatic amplification cycles.

The main advantages of using PCR technology rather than more conventional approaches for the detection of genetic amplification are that much smaller quantities of DNA material are needed, the technique is not dependent on the availability of pure intact DNA, and is readily applicable to formalin-fixed, paraffin-embedded archival speci-

mens. The latter is important because it markedly increases the number of cases available for retrospective studies. We have done such retrospective analysis of 30 archival human ovarian carcinoma specimens. We found various degrees of amplification of the c-*myc* proto-oncogene in five tumors (17%) when tPA, which is a gene located on the same chromosome as c-*myc*, was used as a control. These results confirm earlier findings[17] and give additional support to the notion that abnormalities involving the c-*myc* proto-oncogene may be important in the genesis of some ovarian tumors.

The number of cases that showed c-*myc* gene amplification in the present study was clearly too small to draw any conclusions regarding associations between such molecular changes and tumor clinical behavior. However a relationship between amplification of a specific oncogene and tumor clinical aggressiveness was reported by Slamon et al,[2] who examined HER-2/*neu* copy numbers in a large number of breast and ovarian tumors. Earlier Seeger et al.[1] reported similar findings for N-*myc* amplification in neuroblastomas. Therefore knowledge of the state of amplification of specific oncogenes may be useful in the clinical evaluation of cancer patients and the feasibility of obtaining such knowledge from PCR studies on formalin-fixed histologic sections is likely to contribute to the implementation of molecular tools in clinical medicine.

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Note Added in Proof

While our manuscript was being reviewed, Frye et al (Detection of amplified oncogenes by differential polymerase chain reaction. Oncogene 1989, 4:1153–1157) published a report on a similar technique for the detection of gene amplification in human tumors. Their results further substantiate the possibility of using PCR for gene amplification studies.