

Methodology article

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Characterization of mutations and loss of heterozygosity of p53 and K-ras2 in pancreatic cancer cell lines by immobilized polymerase chain reaction

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Abstract

Background: The identification of known mutations in a cell population is important for clinical applications and basic cancer research. In this work an immobilized form of the polymerase chain reaction, referred to as polony technology, was used to detect mutations as well as gene deletions, resulting in loss of heterozygosity (LOH), in cancer cell lines. Specifically, the mutational hotspots in p53, namely codons 175, 245, 248, 249, 273, and 282, and K-ras2, codons 12, 13 and 61, were genotyped in the pancreatic cell line, Panc-1. In addition LOH analysis was also performed for these same two genes in Panc-1 by quantifying the relative gene copy number of p53 and K-ras2.

Results: Using polony technology, Panc-1 was determined to possess only one copy of p53, which possessed a mutation in codon 273, and two copies of K-ras2, one wildtype and one with a mutation in codon 12. To further demonstrate the general approach of this method, polonies were also used to detect K-ras2 mutations in the pancreatic cell lines, AsPc-1 and CAPAN-1.

Conclusions: In conclusion, we have developed an assay that can detect mutations in hotspots of p53 and K-ras2 as well as diagnose LOH in these same genes.

Background

The advent of the polymerase chain reaction (PCR) played an important role in revolutionizing research in the field of molecular biology [1]. A recent adaptation of this technology, known as polymerase colonies, or "polonies", holds tremendous promise as well. Polony technology is a form of PCR in which the reaction is immobilized in a thin polyacrylamide gel attached to a microscope slide [2,3]. As the PCR proceeds, the PCR products diffuse radially within the gel from its immobilized template (e.g., genomic DNA), giving rise to a circular PCR product, or

polymerase colony. When the gel is stained with SybrGreen I and scanned with a microarray scanner, the polymerase colony resembles a colony on an agar plate, hence its name. Herein, we describe the applications of polony technology to cancer research with the goal of screening for: 1) mutations in key tumor suppressor and oncogenes; as well as 2) loss of heterozygosity.

One of the hallmarks of cancer includes the accumulation of mutations in key genes, namely DNA repair genes [4], tumor suppressor genes [5,6], and proto-oncogenes [5,6],

accompanied by the loss of the wildtype allele, resulting in loss of heterozygosity [LOH, see reviews by [7–9]]. Pancreatic cancer, which results in the death of approximately 30,000 Americans annually and is the fourth leading cause of cancer mortalities in the United States, exhibits this hallmark [10]. For example, in pancreatic cancer there is a high incidence of mutations (>50%) in the tumor suppressor genes p16, p53 and DPC4 and approximately 90% of cases coincide with a mutation in the oncogene *K-ras2* [see review by [11]]. Furthermore, a significant percentage of tumors harboring a mutated copy of DPC4 or p53 also lose the corresponding wildtype allele, resulting in LOH [see reviews by [11,12]].

Of particular importance to this study are the genes p53 and *K-ras2* since a significant proportion of the mutations are localized to a relatively small number of mutational hotspots [13–16]. In this paper we introduce a screen to detect the presence of pancreatic cancer by looking for somatic mutations in p53 and *K-ras2* using polony technology to microsequence mutational hotspots within these two genes. In addition polonies are used to diagnose LOH in these same genes.

Results

Previous work has shown that a significant percentage of mutations in p53 and *K-ras2* are localized to mutational hotspots, namely codons 175, 245, 248, 249, 273, and 282 in p53 and codons 12, 13, and 61 in *K-ras2* [reviewed in [14,17,18]]. Each of these mutational hotspots was sequenced in the genomic DNA of various pancreatic cell lines using polony technology as follows. Initially, each exon bearing a mutational hotspot was individually PCR amplified in a polyacrylamide gel giving rise to one polymerase colony, or polony, per copy of genomic p53 or *K-ras2* DNA. The non-acrydited strand of the polony was then stripped away after formamide treatment and electrophoresis. A sequencing primer was hybridized to the single-stranded copy of the PCR-amplified p53/*K-ras2* fragment and a single base extension with either a Cy-3 or Cy-5 labeled dNTP was performed prior to scanning on a microarray scanner. The process of formamide denaturation, hybridization, and extension was repeated 3 additional times in order to perform an extension with each of the four dNTPs and completely sequence each position.

When all the mutational hotspots were sequenced in the Panc-1 cell line (results of sequencing in Table 3), it was determined that *K-ras2* was heterozygous (i.e. one mutant and one wild type allele) at the second position of codon 12 (Figure 1) and p53 harbored a mutation at the second position of codon 273 (Figure 2). In addition to the cell line Panc-1, *K-ras2* mutations in the second position of codon 12 were also shown to be present in the cell lines AsPC-1 (G → A; data not shown) and CAPAN-1 (G → T;

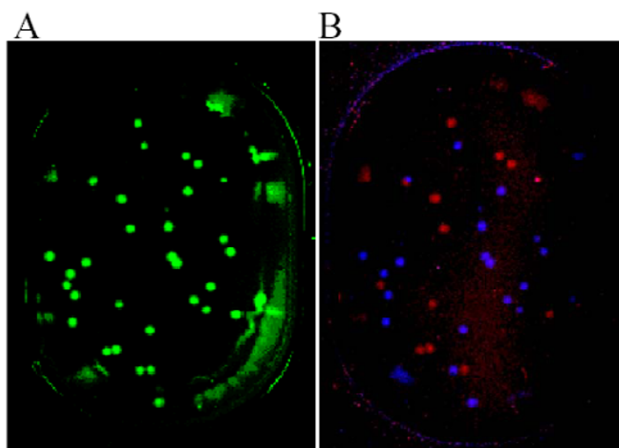


Figure 1

The second position of codon 12 in *K-ras2* of Panc-1 genomic DNA is heterozygous with roughly half the alleles being wildtype {G} and with the other half being mutant {A}. (A) All *K-ras2* exon I polonies are shown following Sybr Green I staining. (B) After the polonies were made single-stranded, a sequencing primer was hybridized to the polony and the position was shown to be heterozygous by performing independent single base extensions with Cy-5 labeled: dATP (blue) and dGTP (red). Extensions with Cy-5 labeled dCTP and dUTP did not yield any significant signal (data not shown). Colors are artificial in both images.

data not shown). These results are in agreement with previously published data concerning the genotype of these cell lines [19–21].

One hallmark of cancer that can be readily observed using polonies is loss of heterozygosity (LOH). Polony technology provides this capability due to its "digital" nature, where one DNA molecule gives rise to one polony. As a result, polonies can be used to detect changes in gene copy number that arise due to either deletions or multiplications. For example, previous studies have shown that Panc-1 has two copies of *K-ras2*, but only one copy of p53, due to LOH [20]. When equivalent quantities of Panc-1 genomic DNA were polony amplified, there were approximately twice as many *K-ras2* polonies as there were p53 polonies (Figure 3). These results, taken in conjunction with the *in situ* genotyping shown previously in Figures 2 and 3, clearly demonstrate the ability of polonies to detect LOH. It should be noted that polony amplification of genomic DNA from strains with equal p53 and *K-ras2* copy numbers, such as CAPAN-1, yielded equivalent numbers of p53 and *K-RAS2* polonies (data not shown). This eliminates the role of primer bias contributing to the distinct number of p53 and *K-ras2* polonies amplified in Panc-1 genomic DNA.

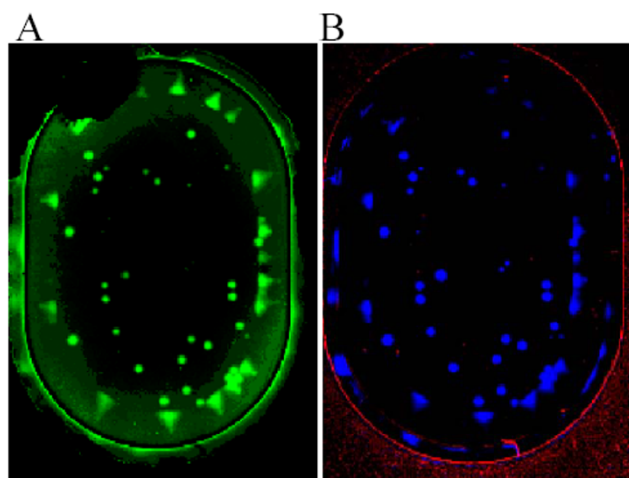


Figure 2

The second position of codon 273 in p53 of Panc-1 is mutated, possessing an "A" instead of a "G". (A) All p53 exon 8 polonies are shown following Sybr Green I staining. (B) The position was shown to harbor a G → A mutation by performing independent single base extensions with Cy-5 labeled: dATP (blue) and dGTP (red). Extensions with Cy-5 labeled dCTP and dUTP did not yield any significant signal (data not shown). Colors are artificial in both images.

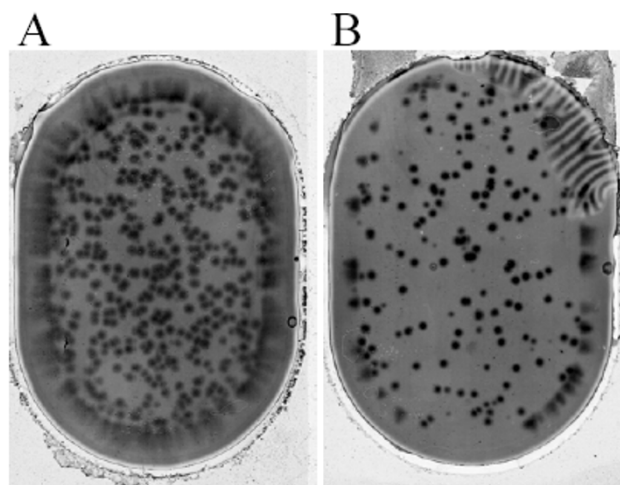


Figure 3

Loss of heterozygosity analysis of *K-ras2* and p53 in Panc-1. There are approximately twice as many (A) *K-ras2* polonies than (B) p53 polonies following the polony amplification of equal amounts of genomic DNA, likely indicating that one of two p53 alleles was lost. These results, coupled with the sequencing of mutational hotspots in these same genes, demonstrate that p53 experienced LOH.

Discussion

We have shown that polony technology is an improved method to study, and potentially diagnosis, cancer [22]. Specifically, polony technology was successfully applied to both detect intragenic mutations in well-defined mutational hotspots in key cancer genes as well as determine if loss of heterozygosity of these same genes had occurred. For example, Panc-1 was determined to possess only one copy of p53, which possessed an intragenic mutation in codon 273, and two copies of *K-ras2*, one wildtype and one with an intragenic mutation in codon 12. These results are consistent with findings from previous work [20,21].

The approach described herein was applied to a clonal cell line. However, this approach could be used to detect rare somatic mutations and to diagnosis cancer with genomic DNA collected from patient samples or biopsies [23–27] in a manner similar to Digital PCR developed by Vogelstein and Kinzler [22]. In the case of analyzing pancreatic juice for p53 and *K-ras2* mutations, it seems likely that only a small percentage of DNA harbors mutant DNA. However, polonies seem well suited for this type of analysis since several hundred to several million [2] polonies for a particular gene can be analyzed on a single slide, thereby increasing the likelihood of detecting a rare event

[22]. Additionally, the frequency of mutations can be readily determined using this approach due to the "digital" nature of polonies.

Finally, it should be noted that this approach is not without limitations. For example, sequencing each mutational hotspot is laborious and detecting mutations outside the hotspots is not feasible. Adherence to mutational hotspots was directly responsible for the inability to detect the p53 mutations in the CAPAN and AsPc-1 cell lines [19,21]. The best approach for detecting mutations outside the hotspots will involve direct sequencing of the polony PCR products [2,13,14].

Conclusions

We have shown that polony technology is an improved method to study, and potentially diagnosis, cancer [22]. Specifically, polony technology was successfully applied to both detect intragenic mutations in well-defined mutational hotspots in key cancer genes as well as determine if loss of heterozygosity of these same genes had occurred. These results are consistent with findings from previous work [20,21].

Table 1: Primers used to polony amplify p53 and K-ras 2 exons bearing mutational hotspots from pancreatic cancer cell line genomic DNA.

Primer Name	Sequence
p53 exon5 forward	tgccctgactttcaactctgtctccttctc
p53 exon5 reverse	ccagacctaaagagcaatcagtgaggatcagaggc
p53 exon7 forward	gttatctctaggtggctctgactgtacca
p53 exon7 reverse	gtggatgggtagtagtatggagaatcggt
p53 exon8 forward	ggtaggacctgatttccttactgcctcttgc
p53 exon8 reverse	gataaaaagtgaaatctgaggcataactgcacc
kras exon1 forward	tggaggagtatttgatagtgattaaccttatgtg
kras exon1 reverse	agagaacctttatctgatatacaagaatggctctg
kras exon2 forward	tgaagtaaaaggctcactgtaataatccagac
kras exon2 reverse	taatgtcagcttattattcaatttaaccacc

Table 2: Primers used to sequence codons in p53 and K-ras2 that experience a high incidence of mutation during carcinogenesis. The designations "for" and "rev" indicate whether the anti-sense or sense strand was sequenced, respectively.

Primer Name	Sequence
p53 c175 pos1 for	gcacatgacggaggtgtgagg
p53 c175 pos2 for	gcacatgacggaggtgtgaggc
p53 c175 pos3 for	gcacatgacggaggtgtgaggcg
p53 c175 pos3 rev	cagcctcatggtggggggca
p53 c175 pos2 rev	cagcctcatggtggggggcag
p53 c245 pos1 for	gtaacagttcctgcatgggc
p53 c245 pos2 for	gtaacagttcctgcatgggcg
p53 c245 pos3 for	gtaacagttcctgcatgggcgg
p53 c248 pos1 for	cctgcatgggcggcatgaac
p53 c248 pos2 for	cctgcatgggcggcatgaacc
p53 c248 pos3 for	cctgcatgggcggcatgaaccg
p53 c249 pos3 rev	gtgatgatggtgaggatggg
p53 c249 pos2 rev	gtgatgatggtgaggatgggc
p53 c249 pos1 rev	gtgatgatggtgaggatgggcc
p53 c273 pos1 for	gacggaacagctttgaggtg
p53 c273 pos2 for	gacggaacagctttgaggtgc
p53 c273 pos3 for	gacggaacagctttgaggtgcg
p53 c282 pos1 for	gtgcctgtcctgggagagac
p53 c282 pos2 for	gtgcctgtcctgggagagacc
p53 c282 pos3 for	gtgcctgtcctgggagagaccg
kras c12 pos1 for	aacttggtgtagttggagct
kras c12 pos2 for	aacttggtgtagttggagctg
kras c12 pos3 for	aacttggtgtagttggagctgg
kras c13 pos3 rev	gtcaaggcactcttcctac
kras c13 pos2 rev	gtcaaggcactcttcctacg
kras c13 pos1 rev	gtcaaggcactcttcctacgc
kras c61 pos1 for	atattctcgacacagcaggt
kras c61 pos2 for	atattctcgacacagcaggtc
kras c61 pos3 for	atattctcgacacagcaggtca

Methods

Preparation of Pancreatic Cell Line Genomic DNA

The pancreatic cell lines AsPc-1, CAPAN-1 and Panc-1 were purchased from the American Type Culture Collection (Manassas, VA) and were grown according to the

manufacturer's instructions [28–30]. Genomic DNA was harvested from these cells using a Qiagen (Alameda, CA) Blood and Cell Culture DNA Midi Kit.

Table 3: Results from sequencing p53 and K-ras 2 mutational hotspots in Panc-I genomic DNA.

K-ras				
	codon	strand sequenced	wt	Panc-I
	12	anti-sense	GGT	G G/A T
	13	sense	CCG	CCG
	61	anti-sense	CAA	CAA
p53				
	codon	strand sequenced	wt	Panc-I
	175	anti-sense	CGC	CGC
	245	anti-sense	GGC	GGC
	248	anti-sense	CGG	CGG
	249	sense	TCC	TCC
	273	anti-sense	CGT	CAT
	282	anti-sense	CGG	CGG

Polony gels

Polony gels for genotyping mutational hotspots were cast on Teflon-printed microscope slides as described by Mitra et al [3]. The Teflon-printed, 24.4 × 16.7 mm oval slides (Grace Bio-Labs, Bend, OR) were treated with Bind Silane (Amersham, Piscataway, NJ) in accordance with the manufacturer's instructions. The slides were stored in a desiccator. To cast the polyacrylamide gels used for the immobilized PCR reaction, a master mix was first made for 12 polony gels (131.0 µL of filter-sterilized doubly deionized water, 25.5 µL of 10X JumpStart Taq Polymerase Reaction Buffer (Sigma, St. Louis, MO), 2.55 µL of dNTP (20 mM each), 1.5 µL of 30% BSA (Sigma), 2.55 µL 10% Tween 20, and 56.16 µL of degassed, filter-sterilized acrylamide). For each position within a mutational hotspot to be genotyped, 20 µL of master mix was combined with 1 µL of genomic DNA as well as 0.23 µL of each the forward and reverse primers (50 µM each; see Table 1) designed to polony amplify the portion of the exon bearing the mutational hotspot(s). Depending on whether the sense or anti-sense strand was to be sequenced, either the forward or reverse primer was modified with a 5' acrydite to covalently attach the PCR product to the acrylamide matrix (see below). Immediately prior to casting the polony gel, 1.38 µL of JumpStart Taq Polymerase (Sigma), 0.34 µL of 5% APS, and 0.34 µL of TEMED was added to the master mix/primer/DNA solution. The gel was polymerized for 10 min, a hybrid well cover (Grace Bio-labs) was placed on top of the gel and light mineral was pipeted into the hybrid well chamber. The slide was placed in a placed in an in situ PCR tower (MJ Research, MA) and thermalcycled (94 C for 2 min; 39 cycles of 94 C for 15 s, (T_m-3) C for 30 s and 72 C for 30 s; final extension at 72 C for 2 min). (Note: T_m is the melting temperature of the PCR primer with the lower melting temperature in a given primer pairing.)

Upon completion of the PCR reaction, the slide was immersed in clean hexane to remove oil prior to staining in 2X Sybr Green (Molecular Probes, Eugene, OR) for 15 minutes. The slides was then washed in TBE and scanned

with a ScanArray 5000 microarray scanner (Perkin Elmer, Wellesley, MA) with the FITC laser and filter set.

Denaturation and electrophoresis of polony gels

Prior to genotyping, the double stranded polonies were made single stranded by stripping away the non-acrydited strand in a two-step procedure [3]. The polony DNA was denatured by incubation in a formamide buffer (1x SSC, 70% formamide, 25% doubly deionized-water) at 70 C for 15 min. Immediately following denaturation, the gels were subjected to electrophoresis (42 % urea in 0.5x TBE) to remove the non-acrydited strand.

Hybridization and single base extension

After electrophoresis, the polony slides were washed 4× in wash 1E (0.1 M Tris-HCl, pH 7.5, 20 mM EDTA, 0.5 M KCl) to prepare for hybridization of the sequencing primer. 200 µL of annealing buffer (6x SSPE, 0.01% Triton X-100) containing 0.5 µM primer (Table 2) was then pipeted onto the gel and covered with a hybrid well chamber. The slide was then incubated for 2 min at 94 C followed by 20 min at (T_m-3) C to facilitate hybridization.

Genotyping of mutational hotspots was finally accomplished by performing single base extensions of the hybridized sequencing primer with fluorescently labeled deoxynucleotides. Following hybridization, the gels were washed 2× in Wash1E and then equilibrated in Klenow extension buffer (50 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 0.01% Triton X-100) for 1 minute. For each sample, 50 µL solution containing approximately 5 units of Klenow large fragment (New England Biolabs, Beverly, MA), 3 µg of single stranded binding protein (US Biochemicals, Cleveland, OH), and 0.5 µM Cy3- or Cy5-labeled dATP, dCTP, dGTP or dUTP (Perkin Elmer) was pipeted onto the gel. The single base extension was allowed to proceed for 2 min. The gels were then washed in Wash1E to reduce background fluorescence and scanned on the ScanArray5000 with the appropriate lasers and filters. The process of formamide denaturation, hybridization, extension, and scanning was repeated 3 additional times for

each primer in order to do a single base extension with each of the four labeled nucleotides.

Author's contributions

KD grew the cell lines used in experiments. JB designed and performed all of the experiments in this manuscript. JE and EW conceived this project. All authors read and approved the final manuscript.

Note

A PC-formatted CD-ROM containing this manuscript is included. The manuscript was created in Microsoft Word XP in Office XP for the PC.

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