A Comparison between Microsatellite and Quantitative PCR Analyses to Detect Frequent *p16* Copy Number Changes in Primary Bladder Tumors[1]

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ABSTRACT

We tested 70 primary bladder tumors for altered copy number of *p16* (*D9S1752*) by microsatellite analysis and by a quantitative PCR (QPCR) assay. These two approaches were fully concordant for 53 tumors, including all 39 tumors in which microsatellite analysis detected loss. In addition, the QPCR method detected useful anomalies in 17 additional cases, including those in which *D9S1752* was uninformative. QPCR was abnormal in 56 of 70 (80%) cases, whereas microsatellite analysis was abnormal in 39 of 70 (56%) cases. Although QPCR uses more DNA than microsatellite analysis, it represents a rapid, informative technique that can readily detect both chromosome 9p21 deletions and amplifications in primary bladder tumors without the need for electrophoretic separation.

INTRODUCTION

We have developed a QPCR-based[4] method to detect gene copy number variation (1).[5] Unlike microsatellite analysis, QPCR does not require polymorphism, so that any set of unique markers (including intragenic markers from the target of interest) may be used. Deletions and amplifications are scored at the same time, using the same mode of detection, so that both types of genetic instability in cancerous cells are detected in the same assay. This is especially valuable, because anomalies of copy number in cancer include deletions, small amplifications, and large amplifications.[5] As a result, QPCR is more informative than microsatellite analysis, which requires polymorphism and cannot detect amplifications. However, although a smaller amount of sample is required for QPCR than for Southern blotting, QPCR requires more DNA than microsatellite analysis. To compare QPCR and microsatellite analysis in cancer identification, we performed each procedure on the same set of 70 bladder tumors.

MATERIALS AND METHODS

QPCR Assay Procedure. The principle of this quantitative method was described previously (1). The asymmetric PCR profile for both *IGF-I* and *D9S1752* was 95°C for 25 s, 58°C for 30 s, and 72°C for 30 s for 19 cycles. After the asymmetric PCR, 5 μl of AmpliSensor (1 ng/μl) in reaction buffer were added to the reactions (except the blank reaction), and the PCR was continued with the AmpliSensor profile. The AmpliSensor profile for *IGF-I* and *D9S1752* was 95°C for 25 s, 62°C for 30 s, and 72°C for 30 s. The reaction was followed dynamically by monitoring fluorescence after the addition of the AmpliSensor. The sequences of PCR primers are as follows: *D9S1752*, limiting (5′-TCTGATGTGTCCTACTCCAC-3′), excess (5′-GCAAGTCATAAGGGGATTTC-3′), and AmpliSensor (5′-GTTACAATTGCTCTCACTCCACTCC-3′); and *IGF-I*, limiting (5′-GATGAGGCAAAGACTATGCCG-3′), excess (5′-CCCAGGTACCCTTCTCCCAGAGTGG-3′), and AmpliSensor (5′-TACTAGGCTGCCTGTCACTGTC-3′).

Quantitative analysis requires reproducibility, so that each reaction was repeated multiple times. Only H_2O was added to the blank well. For the apex well, everything except Taq DNA polymerase was added. For the negative well, everything except genomic DNA was added. The first PCR reaction (asymmetric PCR profile) was accomplished. This was required to generate sufficient template for the second PCR reaction; use of a nested PCR system also serves to increase the fidelity of the PCR reaction. The second (nested) PCR reaction was then initiated by adding a nested third, partially duplex primer carrying an AmpliSensor oligonucleotide duplex (1) with fluorescein on one strand and Texas Red on the other strand. The initial asymmetric PCR was performed in 10 μl of 50 mM Tris-HCl (pH 8.7), 50 mM KCl, 5 mM NH_4Cl, 5 mM $MgCl_2$, 1 mM DTT, 0.1% Triton X-100, 0.2 units of Taq DNA polymerase, 90 ng of excess primer, and 12 ng of limiting primer. The genomic DNAs were in the range of 5×10^2 to 1×10^4 copies per μl of the DNA sequence to be amplified, using 50 ng/μl yeast tRNA as the dilution solution. An aliquot of 2.5 μl from each dilution was used for PCR. The asymmetric PCR profile for the reaction was followed dynamically by monitoring fluorescence after the addition of AmpliSensor. The reaction was performed as two sequential nested PCR amplifications to increase the specificity

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[4] The abbreviations used are: QPCR, quantitative PCR; LOH, loss of heterozygosity; HD, homozygous deletion; FISH, fluorescence *in situ* hybridization; IGF-I, insulin-like growth factor I.
[5] P-W. Chiang, S. Ramamoorthy, D. G. Beer, C. N. Wang, and D. M. Kurnit. Facile detection by fluorescent-PCR of quantitative anomalies that occur in the plastic cancer genome, submitted for publication.

Table 1 Comparison of QPCR and microsatellite analyses

Normal WBC (control) DNA samples were analyzed by QPCR for each of five probe pairs, with the number of individuals tested given in parentheses. The averages, SDs, and ranges are given for each probe pair. Seventy bladder tumor DNAs were analyzed by both QPCR (for *D9S1752/IGF-I*) and LOH (for *D9S1752*). The results of the analyses are given below. The QPCR measurements for each probe result from replicate values for each of three different dilutions. The data demonstrate that QPCR can detect deletions below and amplifications above a normal range of 0.8–1.3.

	Probe pairs (no. of cases)[a]	QPCR average	QPCR SD	QPCR range
Control samples				
	D9S1752/IGF-I (8)	0.93	0.15	0.8–1.2
	p53/IGF-I (10)	0.94	0.15	0.8–1.3
	BRCA1/IGF-I (9)	0.96	0.1	0.8–1.1
	erbB-2/IGF-I (9)	1.03	0.14	0.8–1.2
	CYCLIN D1/IGF-I (9)	1.07	0.1	0.9–1.2
Tumor samples				
	QPCR low/MA HD (22)	0.26	0.12	0.1–0.5
	QPCR low/MA LOH (17)	0.42	0.14	0.1–0.7
	QPCR NL/MA NL (14)	1.03	0.15	0.8–1.3
	QPCR high/MA NL (2)	1.85	0.21	1.7–2.0
	QPCR high/MA UNINF (1)	3.8		
	QPCR low/MA NL (4)	0.38	0.13	0.2–0.5
	QPCR low/MA UNINF (10)	0.24	0.1	0.1–0.4

[a] MA, microsatellite analysis; NL, normal; UNINF, uninformative.

and to enable enough sequence to accumulate in the first reaction for QPCR to proceed efficiently on adding the fluorescent AmpliSensor. Each reported QPCR value represents six measurements for each of the two markers (*D9S1752* and *IGF-I*), representing duplicates of undiluted, 1:1 diluted, and 1:3 diluted samples. In this manner, we ensure both that the readings are reproducible, and that an appropriate straight line is obtained from serial dilutions. Due to error inherent in the technique (Table 1), values ≥ 0.8 and ≤ 1.3 are considered within normal limits. In all, ~300 ng of genomic DNA are required to yield information from six measurements for each of two probes.

Microsatellite Analysis. Normal and tumor DNA was analyzed for LOH after PCR amplification of polymorphic dinucleotide repeat sequences (2). Oligonucleotide primers for microsatellite PCR analysis were obtained from Research Genetics (Huntsville, AL). The primers were labeled with [^{32}P]ATP using T4 polynucleotide kinase (New England Biolabs). Fifty ng of genomic DNA were subjected to 30–35 cycles of PCR amplification as described previously (3). PCR products were separated by electrophoresis in denaturing 7% urea-polyacrylamide-formamide gels followed by autoradiography (4–6). For informative cases, allelic loss was scored if the intensity of one allele was at least 50% reduced in the tumor as compared with the normal DNA (4–6). HDs were scored when one or more closely spaced markers demonstrated apparent retention when flanked by markers demonstrating clear LOH (4). All primer sequences are available from Research Genetics (*D9S162*, IFNα, *D9S171*, *D9S736*, *D9S1748*, and *D9S1752*) or from the Genome Database (Johns Hopkins University).

RESULTS

We compared the QPCR results with conventional microsatellite analysis in 70 primary bladder tumors to determine the relationship between these techniques. The strategy of the QPCR assay was described previously (1). Briefly, this approach assesses the ratio of quantitative copy number between two probes by monitoring the loss of real-time fluorescence energy transfer during PCR. A more detailed explanation is given in Ref. 1. Simultaneous microsatellite analysis enabled us to compare the sensitivity and accuracy of the two techniques.

Previously, a panel of markers at chromosome band 9p21, flanking *p16*, was shown to be frequently hemizygously and homozygously deleted in primary bladder tumors (4, 5). A fluorescent primer derived from the polymorphic marker, *D9S1752*, 10 kb centromeric to p16, was synthesized and used for the QPCR assay. We used the same *D9S1752* probe for the microsatellite analysis to permit a direct comparison of the two techniques. The reference marker in the QPCR analysis was derived from *IGF-I* on chromosome 12. This marker has worked efficiently for other copy number analyses,[6,7] has been widely used with a number of probes (Table 1), and was not known to be affected in bladder cancers. Furthermore, chromosome 12q represents one of the least aberrant regions in bladder cancer, making it unlikely that this region is amplified or deleted *in toto*. The relative ratio of gene copy number between *D9S1752* and *IGF-I*, which is 1 in normal cells (Table 1), was measured by QPCR. The QPCR value was compared with standard LOH analysis involving PCR amplification of the *D9S1752* probe (containing a dinucleotide repeat). If necessary, markers that flank *D9S1752* were used to accomplish microsatellite analysis in both normal (germ-line) and tumor DNA (Table 1).

Thirty-nine of 59 (66%) informative tumors (11 cases were not informative by microsatellite analysis) demonstrated a deletion at D9S1752 by conventional microsatellite analysis (22 HD cases and 17 LOH cases). HD represents cases in which the marker in question manifests both alleles, but LOH is observed for both nearby proximal and distal markers (4, 5). In such a case, HD is inferred at the given locus, with the obtained biallelic signal arising from the normal cellular contamination that occurs in tumor samples. Fig. 1 demonstrates examples of HD and LOH for *D9S1752*.

We found that in 53 of 59 informative bladder tumors, there was concordance between QPCR and microsatellite analysis. The two techniques agreed on all 39 samples in which standard microsatellite analysis showed either HD or LOH. In 14 samples, both QPCR and microsatellite analysis were normal. Seventeen samples were normal or uninformative by microsatellite analysis but abnormal by QPCR. Of these 17 samples: (*a*) 11 tumors did not show polymorphism at *D9S1752* and could not be evaluated by LOH analysis. However, the QPCR analysis does not require polymorphism, and we were able to evaluate these 11 tumors. As outlined in Table 1, in all cases the QPCR analysis detected an anomaly, with 10 decreases and 1 increase of the *D9S1752:IGF-I* ratio observed; (*b*) two tumors

[6] C. N. Wang, personal communication.
[7] Unpublished data.

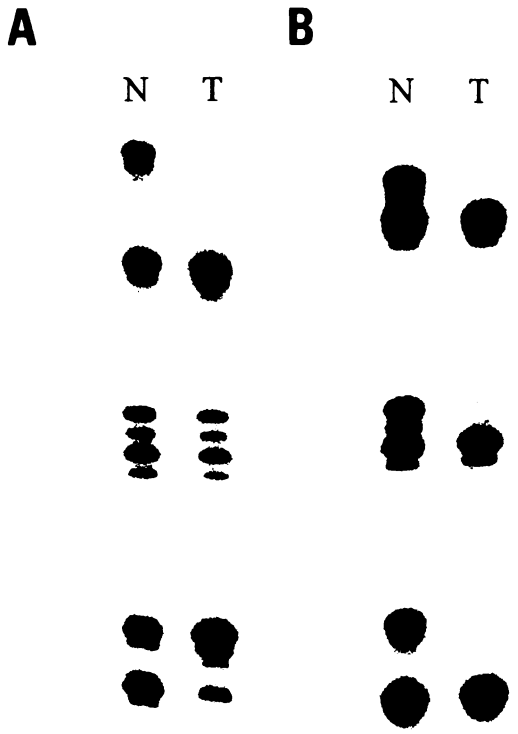

Fig. 1 *A*, bladder tumor 133 showing apparent retention of heterozygosity indicating HD at *D9S1752* (*middle*), flanked by LOH indicated by loss of the upper allele at *D9S162* (*top*) and lower allele at *D9S171* (*bottom*). *N*, normal; *T*, tumor. *B*, bladder tumor 239 showing LOH indicated by loss of the upper allele at *D9S162* (*top*), *D9S1752* (*middle*), and *D9S171* (*bottom*).

showed amplification of *D9S1752:IGF-1* without manifesting LOH for *D9S1752* (Table 1). One tumor showed amplification of *D9S1752/IGF-1* but was not informative in LOH studies; this tumor also was included in the category described in (*a*); and (*c*) in four tumors, the QPCR *D9S1752:IGF-1* ratio was in the deleted range, but microsatellite analysis for *D9S1752* showed two alleles. In these cases, use of another standard also yielded an abnormal ratio (data not shown), so that a copy number anomaly indeed occurred in these cases. In total, we detected copy number changes in 39 of 70 cases by microsatellite analysis and in 56 of 70 cases by QPCR.

DISCUSSION

Bladder cancer accounts for 2% of all cancers, with an incidence of approximately 50,000 new cases each year in the United States. Hemizygous deletions and HDs at 9p21 occur in more than 70% of all primary bladder tumors (4). These deletions usually extend into and inactivate the tumor suppressor gene *p16* (CDKN2/MTS-1/INK4A). Early bladder lesions, including papillary tumors and carcinoma *in situ* (5), also display these changes. Thus, *p16* inactivation represents the earliest and most common genetic event yet described in primary bladder cancer (4, 5).

In cancer, deletion of tumor suppressor genes (7) or amplification of oncogenes (8) is found frequently in primary tumors. Currently, detection of such gene copy number variation can be accomplished by several methods:

(*a*) Rapid detection of LOH is based on informative sequence variation, either different restriction enzyme cutting sites for RFLPs (9) or microsatellite analysis based on the detection of differing sizes of small repeat units in the genome (10). These LOH analyses are restricted by the need for polymorphic markers that may be uninformative in a given case and are often at some distance from the locus of interest. Furthermore, these techniques to analyze LOH do not detect amplifications that are usually detected by hybridization-based analyses.

(*b*) HD results in the absence of signal from the tumor cells. As a result, heterozygosity is observed, due to signal from the normal cells that contaminate a primary tumor sample. Microsatellite analysis using a panel of informative markers can detect HD as apparent retention of heterozygosity for a target locus surrounded by LOH at closely flanking markers. (The unlikely alternative to this situation would be for the locus in question to be normal, with cross-overs between that locus and each of the two surrounding markers. Because these markers are within 300–500 kb of each other, the probability of such a double cross-over is remote.) In addition, analysis by FISH confirmed a high frequency of HD for p16 in primary bladder tumors (4, 5).

Other approaches are considerably more laborious but can detect both deletions and amplifications. These include quantitative Southern blotting (11) or FISH (12). Quantitative Southern blotting and FISH can detect either gain or loss of sequences without the heterozygosity requirements of the RFLP or PCR polymorphism methods of typing. However, these quantitative Southern blot or FISH methods are difficult to perform, time-consuming, use large amounts of material or specialized tissue samples, and may offer limited resolution. In contrast, QPCR has the advantage that both deletions and amplifications can be detected with a single rapid assay.

In this report, we sought to evaluate the efficacy and accuracy of a QPCR method for the detection of hemizygous deletions and HDs of p16 as compared to conventional PCR-based microsatellite analysis in primary bladder cancer. We detected deletions at *D9S1752* (*p16*) in 39 of 70 (66%) primary bladder cancers by conventional microsatellite analysis. This high frequency of p16 deletion agrees with our previous work (4–6). There was complete concordance between QPCR and microsatellite analysis for the 39 bladder tumor samples that showed HD or LOH (Table 1). This means that QPCR was sufficiently sensitive to detect all 39 deletions of *D9S1752* detected by microsatellite analysis.

Although QPCR did distinguish abnormality successfully, it did not distinguish fully between HD (0 copy number) and LOH (1 copy number) cases. Although the *D9S1752:IGF-1* ratio was indeed lower for the totality of HD cases (0.26 ± 0.12) than LOH cases (0.42 ± 0.14), overlap prevented distinction between HD and LOH in an individual case (Table 1). Thus, the ability to distinguish between HD and LOH still required microsatellite analysis. However, QPCR always distinguished abnormal cases successfully.

In our initial reports (4–6), we suggested that the 66% frequency of microsatellite alterations at *D9S1752* was likely to be an underestimate, because many cases could not be evaluated

due to a lack of informative markers near p16. Confirming this assertion, we have detected a higher number of quantitative anomalies, due to the increased sensitivity of QPCR:

(*a*) Polymorphism is not required for QPCR. QPCR demonstrated 10 additional deletions of *D9S1752/IGF-I* and 1 amplification of *D9S1752/IGF-I* that could not be detected by microsatellite analysis because the *D9S1752* locus was not polymorphic (Table 1). This is an advantage of the QPCR method, which does not require polymorphism.

(*b*) QPCR abnormalities can be detected in the absence of microsatellite anomalies. We observed both amplifications ($\geq$1.7) and deletions ($\leq$0.5) of *D9S1752/IGF-I* with retention of both alleles of *D9S1752* (Table 1). Because these QPCR changes do not occur in normal cells (Table 1), the quantitative abnormalities detected by QPCR are indicative of bladder cancer. Because the methodology depends only on the ratio between two probes, rather than on the absolute value of a given probe, we do not know if these anomalies result from deletions and/or amplifications of *D9S1752* and/or *IGF-I* in any given case. However, the existence of abnormality in a given bladder cancer case is substantiated both by the low SDs in these cases (Table 1) and the absence of overlap with QPCR on normal genomic DNAs (Table 1).

This substantial increase in informativeness provides the major justification for incorporating the QPCR method into analyses of bladder cancers. The increased informativeness stems from the abilities to evaluate samples without requiring polymorphism, to detect both deletions and amplifications of probes, and to ascertain abnormalities in the copy number ratio between two sequences rather than examining only one sequence at a time. In particular, we now study the ratio between an amplified and a deleted probe, which yields further increases in informativeness and discrimination between two probes than presented here for *D9S1752/IGF-I*. The major drawback of the QPCR technique is the requirement for ~300 ng of DNA to test a single marker. Although this increased need for DNA is not a problem for large tumor or urine sediment specimens, molecular analysis of small early tumors or of subregions within tumors (2) is not currently feasible by QPCR. Another potential drawback would be a normal QPCR value due to an equivalent copy number change of the two sequences used for QPCR.

Conventional microsatellite analysis has revolutionized our ability to detect deletions and/or allelic imbalance in minute amounts of DNA derived form primary tumors. However, this approach is limited to detecting deletions or, more rarely, novel bands. Detection of deletions requires that a given marker be informative. The availability of QPCR, a rapid, accurate, and sensitive method to assay sequence copy number in the genome (amplification or deletion of the ratio between specific probe pairs), can provide a generalized molecular tool to probe for critical genetic changes that lead to tumorigenesis.

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