ABSTRACT

Purpose: 9p21 is a major target in the pathogenesis of human urinary bladder cancer. The locus harbors the CDKN2A/ARF tumor suppressor gene, which encodes two cell cycle regulatory proteins cyclin dependent kinase 2A (p16INK4a) and alternate reading frame (p14ARF). We have designed a real-time quantitative PCR (QPCR) application to study homozygous deletion (HD) of CDKN2A/ARF human urinary bladder cancer. The locus harbors the CDKN2A/p16INK4a and ARF/p14ARF genes, respectively, and that simultaneous inactivation of ARF and p53 occurs.

INTRODUCTION

The CDKN2A/ARF gene, inactivated in a number of human tumors, is located at 9p21, and encodes two functionally unrelated proteins, CDKN2A/p16INK4a and ARF/p14ARF (hereafter referred to as CDKN2A2 and ARF; Refs. 1, 2). CDKN2A and ARF are transcribed from separate promoters, have unique first exons (exon 1a and 1b) but share exons 2 and 3, are translated from alternative reading frames and bear no amino acid homology (3). Both ARF and CDKN2A are cell cycle regulators involved in the p53 and the retinoblastoma pathways of tumor suppression. ARF interacts with MDM2 thereby inhibiting degradation of p53. CDKN2A maintains retinoblastoma in its active, hypophosphorylated, growth-suppressive form by disrupting the CDK4/6-cyclin D complex (1, 4, 5). Deletion of the ARF and CDKN2A genes, individually lead to tumorigenesis, the overlapping and cooperating functions of these cell cycle regulators are evidenced in increased tumorigenesis in mice with complete loss of Cdkn2a together with loss of one Arf allele. In this context, Arf in mice with the Cdkn2a null background has been suggested to be haploinsufficient for tumor suppression (6, 7).

Genes encoding multiple proteins from overlapping reading frames are common in viruses but probably rare in eukaryotes. The unusual genomic arrangement of the CDKN2A and ARF suggests possible coregulation of expression. Studies on animal models have shown that although loss of Cdkn2a and Arf individually lead to tumorigenesis, the overlapping and cooperating functions of these cell cycle regulators are evidenced in increased tumorigenesis in mice with complete loss of Cdkn2a together with loss of one Arf allele. In this context, Arf in mice with the Cdkn2a null background has been suggested to be haploinsufficient for tumor suppression (6, 7).

LOH on chromosome 9 has been observed in >50% of urinary bladder cancers of all stages and grades (8–10). The area around 9p21 where the CDKN2A/ARF gene is located is one of the major sites for deletions (11, 12). Point mutations and other small changes in the gene are rare (13–15). The predominant mechanism of inactivation at this locus is by HD (3). Gene

Received 2/6/02; revised 8/12/02; accepted 8/13/02.

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2. The abbreviations used are: CDKN2A, cyclin dependent kinase 2A; QPCR, quantitative PCR; ARF, alternate reading frame; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LOH, loss of heterozygosity; FISH, fluorescence in situ hybridization; HD, homozygous deletion; HET, heterozygote normal; DUP, duplication; WT, wild-type.
silencing by promoter methylation together with LOH has also been shown for many cancers, although reported frequencies for urinary bladder cancer vary considerably (16–20).

Methods to study genetic changes affecting one or more specific genes or loci are numerous, e.g., FISH, Southern blot, LOH analysis using microsatellite markers, and QPCR (21–23). All of these methods encounter technical problems associated with background contamination by normal tissue. FISH, Southern blot, and QPCR can be performed without requirements of heterozygosity between alleles (24). Yet, Southern blot require large amounts of DNA and like FISH, it is time consuming. Real-time QPCR is a relatively new technique that was designed primarily to study mRNA expression (25). The method has many advantages: precise studies targeted throughout the genome can be made using a small amount of DNA (in the range of nanograms), it is a closed tube system that does not require post-PCR handling, and the method has a high sample throughput. In this study we have developed a real-time QPCR application, specifically targeted to ARF exon 1β, to analyze aberrations in the CDKN2A/ARF gene in urinary bladder cancer patients.

**MATERIALS AND METHODS**

A real-time multiplex QPCR application was developed to measure HDs in the CDKN2A/ARF gene, especially targeted to ARF exon 1β. Target gene (ARF) amplification was compared with simultaneous amplification of an endogenous reference gene (GAPDH). The method was evaluated in three cell lines: IGR-1 (established from a melanoma metastasis; Ref. 26), SK-MEL-5 (established from a lymph node metastases; Ref. 27), and WM-266-4 (melanoma metastatic lesion; Ref. 28). SK-MEL-5 has selective HD of ARF exon 1β but intact CDKN2A exon 1α, whereas IGR-1 and WM-266-4 have HDs of both exon 1β and exon 1α (29). Samples were analyzed in duplicate, and a standard curve with normal DNA from 1–50 ng was included. All of the points in the standard curve represent 100% contamination, i.e., WT tissue only.

**Patients and Tissue.** Tumor tissues from almost all of the newly diagnosed cases of urinary bladder cancer during the years 1995 and 1996 were collected in the Stockholm area. We have ~600 cases, which comprise a large majority of cases that occurred in the area at the time. Of the total number of urinary bladder neoplasms collected, 186 cases were selected where corresponding normal tissue and etiological information were available. We have analyzed previously genetic changes in the p53 gene in tumors from these patients (30). None of the patients had been given treatment before analysis. Tumors were removed with transurethral resection. Four tissue samples were taken with cold cup biopsy before removal and snap frozen in –80°C. Frozen tissues were cut into ~5 µm-thick sections. The first and last sections were stained and examined for tumor contents by a pathologist. Only biopsies with >70% tumor cells were included in the present analysis. Tumor DNA was extracted by a method described previously (31). Tumor stage was assessed according to the modified TNM-system suggested by Hall and Prout (32), where all of muscle invasive tumors were analyzed together. Grading was done according to Bergkvist et al. (33) with a distinction between G2a and G2b tumors. Stage and grade for the 186 urinary bladder neoplasms were distributed as follows: Tis: 3 (2%), Ta: 105 (56%), T1: 29 (16%), T2: 49 (26%), G1: 10 (5%), G2a: 78 (42%), G2b: 32 (17%), G3: 63 (34%), and G4: 3 (2%).

**Primer Design.** Oligo 6.5 software (National Biosciences Inc.) was used to design two primer pairs and two probes, creating a 92-bp fragment of ARF exon 1β and a 97-bp intronic fragment of GAPDH. During primer design, care was taken to exclude frequently repeated sequences in the genome, and the priming efficiency of the two primer pairs was kept close to have similar efficiencies of the two amplification reactions. Because most pseudogenes lack introns (34), we chose to amplify an intronic sequence of the GAPDH gene to avoid
mispriming in shared exonic regions. We are not aware of any GAPDH pseudogenes with introns; however, to prevent false priming sites, all of the primers and probes were thoroughly analyzed with a BLAST search. The GAPDH gene was used as control gene because there are hardly any reports that implicate this locus in any tumor. Variations in expression levels of housekeeping genes, such as GAPDH, could make them less suitable for certain applications studying mRNA expression levels (35), although, in a quantitative study where the analysis is performed on genomic DNA, gene copy numbers and not expression levels, are used as reference. The ARF ex1β probe was labeled, at the 5’ end, with VIC (Applied Biosystems), the GAPDH probe was labeled, at the 5’ end, with FAM, and both probes had a quencher dye (6-carboxytetramethylrhodamine) at the 3’ end. Primer and probe sequences are given in Table 1.

**Optimization of Multiplex QPCR.** Optimal primer and probe concentrations were determined using optimization protocols from Applied Biosystems TaqMan universal PCR master mix manual. Reaction conditions for ARF ex1β and GAPDH were optimized separately, and then pooled in a multiplex PCR. Reactions were performed in 25-μl volumes containing 5–10 ng of DNA, 100 nM GAPDH forward primer, 300 nM GAPDH reverse primer, 300 nM each of ARF ex1β forward and reverse primer, 150 nM of each probe, and 1× TaqMan Universal PCR master mix (Applied Biosystems). The TaqMan universal PCR master mix contains AmpliTaq Gold DNA polymerase, AmpErase uracil-N-glycosylase, deoxygenucleotide triphosphates with dUTP (replacing dTTP), passive reference, and optimized buffer components. PCR conditions were: an initial 2 min 50°C incubation (activating the uracil-N-glycosylase enzyme, which can prevent the reamplification of carryover PCR products by removing any uracil incorporated into the DNA in previous experiments), followed by 95°C for 10 min (activates the AmpliTaq Gold DNA polymerase), and then 40 cycles of 95°C for 15 s and 60°C for 1 min. Samples were analyzed in triplicate, first as duplicate in one run and then as singletons in an additional run. Each run contained a standard curve of human normal DNA from 1.25 to 20 ng in duplicate. Two template-free blanks were included in each experiment.

**Real-Time QPCR Analysis.** The test parameter, Ct value, (minimum threshold cycle, the cycle where the amount of product is significantly separated from the background baseline) generated by the ABI PRISM 7700 SDS software version 1.7, was analyzed and then exported to an excel sheet (Microsoft Office 2000 Excel) where equations from the standard curve were generated, one for ARF and one for GAPDH. Using the Ct values, initial concentrations of ARF and GAPDH were calculated from the equations. The amount of ARF was divided by the amount of GAPDH in each sample. Theoretically a normal sample has two copies of each gene and therefore the result is 1, by the same principle for a sample with a HD the result is 0 (no copy of ARF, two copies of GAPDH), for a hemizygous deletion (LOH) the result is 0.5 (one copy of ARF, two copies of GAPDH), for a one copy DUP the result is 1.5 (three copies of ARF, two copies of GAPDH), and so on. However, exact figures are valid for cell lines only, because human tumor tissue samples are invariably contaminated with normal cells and/or there can be some degree of tumor mosaicism/heterogeneity or losses at the location of the control gene, which makes analysis parameters less exact. On the basis of the low variation in results from different runs (Fig. 4) ARF:GAPDH ratios were scored as follows: HD 0–0.4, LOH >0.4–0.7, WT >0.8–1.2, DUP >1.3–1.7, 2× DUP >1.8–2.2, and so forth. Because these definitions are exact boundaries on continuous results, the data have to be interpreted with caution as discussed later. In between each group higher than LOH, a “borderline group” was inserted where no definite interpretation was done e.g., LOH/WT >0.7–0.8. No borderline group was inserted between HD and LOH because a theoretical value for LOH is 0.5, and to allow for a small margin of error the limit was set to 0.4. Because of the relatively low contents of normal cells in tumor tissues analyzed, any value <0.4 should be a HD.

**D9S942, D9S1748, and D12S99 Analysis.** The microsatellite markers D9S942, D12S99, and for some cases D9S1748 were used to study LOH using the primer sequences given in the Genome Data Base. Methods for D9S942 and D9S1748 amplification have been described previously (36). PCR for D12S99 was performed in a total volume of 10 μl containing 10 ng genomic DNA, 2 pmol each of forward and reverse primers, 1× Tris-HCl PCR buffer, 1.5 mM MgCl2, 0.11 mM deoxyribonucleotide triphosphates, 10% DMSO, and 0.5 units of Platinum Taq polymerase (Life Technologies, Inc.). Annealing temperatures were 64°C/63°C. PCR was carried out for 40 cycles, 5 cycles with the higher annealing temperature and 35 cycles with the

**Table 1.** Primers and probes

<table>
<thead>
<tr>
<th>Primer/probe</th>
<th>Sequence 5’ &gt; 3’</th>
<th>Reporter</th>
<th>Quencher</th>
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</thead>
<tbody>
<tr>
<td>ARF ex1β sense</td>
<td>GGA GGC GGC GAG AAC AT</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>ARF ex1β antisense</td>
<td>TGA ACC CAC AAA ACC CTC ACT</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>ARF probe</td>
<td>TGC GCA GGT TCT TGG TGA CCC TCC</td>
<td>VIC</td>
<td>TAMRAα</td>
</tr>
<tr>
<td>GAPDH sense</td>
<td>CCA CTG GGC GCT CAC TGT TCT</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>GAPDH antisense</td>
<td>GCG AAC TCA CCC GTC GAC T</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>GAPDH probe</td>
<td>CTC CCT CCG CGC AGC CGA GC</td>
<td>FAM</td>
<td>TAMRA</td>
</tr>
</tbody>
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α TAMRA, 6-carboxytetramethylrhodamine.

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lower. Although the reactions were carefully optimized, a second PCR run for 25 cycles using 1 μl from the first PCR as template was required because of poor amplification. LOH was defined as a reduction of 40% of either the smaller or larger allele that could be reproduced in a separate PCR. All of the samples where LOH was found were reanalyzed from a new PCR.

**Single-Strand Conformational Polymorphism and Sequencing.** Mutations were screened for in the exons 1α, 1β, 2, and 3 of the CDKN2A/ARF locus using two different single-strand conformational polymorphism conditions and direct sequencing as described previously (36, 37). Identified mutations were confirmed subsequently with direct sequencing. WT tissues from corresponding mutated tumors were also sequenced to rule out/confirm polymorphisms.

**RESULTS**

**Deletions in Cell Lines Using Real-Time PCR.** All of the three cell lines (IGR-1, SK-MEL-5, and WM-266-4) amplified GAPDH but were completely negative for ARF amplification at 0% WT DNA contamination. An example of increasing ARF amplification at different rates of normal DNA contamination is given in Fig. 2. ARF amplification increased in a linear manner, which is shown in Fig. 3. With end-point PCR reading (Fig. 2A) there was little difference in amount of ARF product between cell lines with 10% and 100% contamination of WT tissue after 37 cycles of PCR, whereas with real-time PCR Ct values the difference was clear (Fig. 2B). At 0% contamination with WT tissue the result was 0.00 for all of the cell lines, for the WT sample (100% contamination) the average result was 1.06, and for cell lines with 50% contamination (equivalent to LOH) the average results were 0.51, 0.44, and 0.45 for the IGR-1, SK-MEL-5, and WM-266-4 cell lines, respectively. All of the values are shown in Table 2.

**Deletions in Urinary Bladder Cancer Patients.** Real-time PCR was used to measure quantitative alterations in the
LOH Analyzed with the Microsatellite Marker D9S942.

The microsatellite marker D9S942 was used to analyze 174 of the 186 urinary bladder cancer cases. WT tissue and tumor tissue did not match for 4 of the samples, and 1 sample failed amplification. Six tumor samples were borderline cases between HET and LOH, where losses were small (20–30%) but reproducible suggesting allelic imbalance or heavy normal tissue contamination. Of the remaining tumors from 163 cases, 139 (85%) were informative of which 30 had LOH (22%) compared with corresponding normal tissues. Twenty-four cases (15%) were not informative (HOM). No stage or grade association was found with either WT or LOH.

Results for the D9S942 marker were compared with real-time QPCR results, using the following comparison criteria: LOH and WT (HET) should be detected in both types of analysis, and HD detected with real-time QPCR could show up as heterozygote normal using microsatellite markers because of amplification of contaminating WT tissue. We found that the two analysis methods matched in 79% (81 of 103) of the cases (Table 3).

LOH Analyzed with the Microsatellite Marker D9S1748. Sixty-two of the 186 urinary bladder cancer samples were analyzed with the microsatellite marker D9S1748, located close to exon 1β (Fig. 1). Of these samples, 14 were HOM, 25 were HET, 21 showed LOH, and 2 were borderline cases between HET and LOH. LOH frequency or stage/grade association were not calculated because only a selection of samples were analyzed based on real-time QPCR results and results from the D9S942 marker.

LOH Analyzed with the Microsatellite Marker D12S599. To evaluate the status of the reference GAPDH gene, located at 12p13, 169 of the 186 urinary bladder cancer cases were analyzed for LOH with the microsatellite marker D12S599. Three samples failed analysis. Two samples were reproducible borderline cases between HET and LOH (20–30% losses). Of the remaining 164 tumor cases, 94 (57%) were informative of which 14 showed LOH (15%) when compared with normal tissue. Seventy cases (43%) were not informative (HOM). Seven cases with LOH at the D12S599 marker showed clear HD at CDKN2A/
ARF with QPCR, which shows that HD can be detected even with loss of one allele at the reference locus. Only 2 of the 17 cases with single copy DUP were informative for the D12S99 marker. Both of these cases showed LOH, although 1 was unclear. Thus, whereas this method can reproducibly detect HDs, it does not seem to be suitable for picking up single copy deletions in Urinary Bladder Cancer.

**Mutations and Polymorphisms.** Four patients showed sequence changes in exon 2. One case (H12) had two alterations. The first was a G>A transition, silent Val106 for CDKN2A, Ala121Thr for ARF, and the second a known polymorphism affecting CDKN2A only, Ala148Thr (G>A; Ref. 38).

Both base changes were found in tumor as well as normal tissue. The three other sequence changes were found in tumor tissue only (Table 4). All of the mutations/polymorphisms have been described previously (3). All of the three cases with mutations in the CDKN2A/ARF gene also showed loss of WT allele at the D9S942 marker, although in 1 case (S100) the allelic loss was not unequivocal. Interestingly, one case (K15) with a Pro114Leu mutation, which is specific for CDKN2A (and silent for ARF), showed HD at exon 1β with QPCR, LOH at D9S942, and retention of heterozygosity at D9S1748. The D9S942 marker is close to exon 1α (~5kb upstream), whereas D9S1748 is adjacent to the 3’end of exon 1β. This observation is suggestive of separate and specific targeting of the CDKN2A and ARF genes.

In total, using information from microsatellite markers (D9S942 and D9S1748), CDKN2A/ARF mutation analysis, and real-time QPCR, 67 samples (36%) had detectable aberrations (mutations, HDs, hemizygous deletions, or multiple DUPs) in the CDKN2A/ARF gene. We have screened previously the same urinary bladder cancer cases for mutations and LOH in the p53 gene (30). Combining the p53 analysis with the present investigation of CDKN2A/ARF shows that 25 cases (13%) had detectable aberrations of both the CDKN2A/ARF gene and the p53 gene (mutation and/or LOH), and that 100 cases (54%) had detectable aberrations in the CDKN2A/ARF gene and/or in the p53 gene. Three cases with double missense mutations or mutation and LOH in the p53 gene (Table 4) also had HD of ARF.

**DISCUSSION**

We have developed an efficient real-time QPCR application for analysis of HDs in the CDKN2A/ARF gene in urinary bladder neoplasms. Evaluating the method in cell lines with known HDs showed that with end-point PCR reading there was little difference in amount of ARF product between cell lines with 10% and 100% contamination of WT tissue after 37 cycles of PCR. On the other hand, with real-time PCR the difference
was clear, showing that analysis in the exponential phase of the amplification reaction reduces the influence of contaminating WT tissue in determining the initial copy number of both target as well as the reference gene.

We found HDs to be facile to detect with real-time QPCR, but that hemizygous deletions, at least in some cases, proved to be difficult to distinguish from the diploid WT, a problem also observed by others (39). From our experience, we believe that it would be difficult in a separate tube system, to study hemizygous deletions, but that a multiplex system achieves higher reproducibility making analyses possible. Yet, many hemizygous deletions were borderline cases that were interpreted with caution. Although, to our knowledge there is no study indicating DUPs/deletions in the CDKN2A/ARF locus, detected by real-time QPCR results can show loss of one allele but amplification of the other allele, which could possibly explain why microsatellite analysis and QPCR give different results.

Our analysis of 186 urinary bladder cancer samples showed no association between homozygous and hemizygous deletions at the CDKN2A/ARF locus, detected by real-time QPCR results and microsatellite analysis, and tumor stage/grade. Deletions were found in both superficial papillary tumors (TaG1-G2a) and muscle invasive high-grade tumors (≥T2G2b+) supporting previous observations that inactivation of the CDKN2A/ARF locus is an early event in bladder tumorigenesis (11–15). The 3 cases with multiple amplifications at the ARF locus, detected in QPCR assay, were all muscle invasive (≥T2) and high-grade (G3) tumors. Although a small number, nevertheless, this observation suggests the nearby oncogenic location involved in latter events in urinary bladder cancer.

In this study we found 67 samples (36%) that had some form of detectable aberration (mutations, HDs, hemizygous deletions, or multiple DUPs) in CDKN2A/ARF. However, CDKN2A/ARF inactivation by hemizygous deletions together with promoter methylation has also been shown for urinary bladder cancer (17, 19), and it is therefore possible that 36% is an underestimate because we have not included methylation analysis. In urinary bladder cancer and other cancers, CDKN2A/ARF point mutations are rare (3). We found only 3 (2%) mutations in the CDKN2A/ARF gene, all in exon 2 and all described previously (3). This is in accordance with previous studies, which have shown that in urinary bladder cancer and other cancers, CDKN2A/ARF point mutations are rare (3).

Concordance between real-time QPCR and LOH analysis was 79% (81 of 103). Interestingly, exons1B of the CDKN2A/ARF gene is located ~20 kb upstream of exons1A (3, 40) and real-time QPCR was targeted at the sequence within exons1B whereas LOH analysis were performed using the marker D9S942, which is closer to exons1A (Fig. 1). We found a few cases with simultaneous HD at the ARF locus (with QPCR) and LOH (with the D9S942 marker), which perhaps are suggestive of separate and specific targeting of CDKN2A and ARF. In human tumors the deletions at the CDKN2A/ARF locus usually encompass larger segments including the CDKN2A/ARF, CDKN2B (p15INK4b), and neighboring genes. Fine mapping in tumors and cancer cell lines have shown that deletions affecting either CDKN2A or CDKN2B invariably include deletion of exon1B, suggesting that HDs specifically target ARF (29, 41, 42). The argument for specific HD of ARF is augmented by the reported mechanism involving illegitimate V(D)J recombinase activity in T-cell acute lymphoblastic leukemia (41, 43) and the mode of interaction of ARF with MDM2, which involves multiple domains of ARF rather than any specific residue (29, 41, 42, 44). One case where we detected HD of ARF and specific mutation in CDKN2A suggests that deletion and mutations at the locus could be unequal events.

We conclude that although expensive, the real-time QPCR method developed and applied in this study is fast, specific, and reproducible for the detection of HDs at the CDKN2A/ARF locus. Our results suggest that inactivation of CDKN2A/ARF is important and an early event in urinary bladder tumorigenesis. A combination of results from this study with previous results on p53 points toward a cooperative role of inactivation of both p53 and CDKN2A/ARF in tumors. Concomitant loss of p53 and ARF in some cases indicate that ARF has an additional role, independent of p53, in cell cycle regulation and tumorigenesis.

REFERENCES


Detecting Homozygous Deletions in the CDKN2A(p16INK4a)/ARF(p14ARF) Gene in Urinary Bladder Cancer Using Real-Time Quantitative PCR

Petra Berggren, Rajiv Kumar, Shigeru Sakano, et al.