Multiplex Pyrosequencing of Two Polymorphisms in DNA Repair Gene *XRCC1*

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DNA repair enzymes play a pivotal role in platinum-based chemotherapy. Within the gene encoding for the base excision repair enzyme *XRCC1*, several nonsynonymous polymorphisms have been identified. It has been shown that the Arg399Gln single-nucleotide polymorphism results in a polymorphic enzyme that is less capable of initiating DNA repair. We developed a multiplex pyrosequence assay to simultaneously detect two nonsynonymous polymorphisms within the *XRCC1* gene. Both of these polymorphisms resulted in amino acid changes: G/A in codon 399 changes Arg into Gln, and deletion of A in the second position of codon 576 results in a stopcodon. We established the frequency of these mutations in 270 patients suffering from colorectal cancer. Allele frequencies of G in second position of codon 399 and A in the second position codon 576 are 61.1 and 99.6%, respectively, in these patients. This fast and reliable method allows for simultaneous detection of the infrequent mutant C or CT alleles instead of the A deletion at codon 576. The method may be used in pharmacogenetic studies of platinum-based chemotherapy. (*J Mol Diagn* 2006, 8:444–448; DOI: 10.2353/jmoldx.2006.060018)

In the Western world, colorectal cancer is the second most common cancer-related cause of death. In the United States, an estimated 147,500 new cases of colorectal cancer are diagnosed per year, whereas in the Netherlands, 9000 new cases of colorectal cancer are diagnosed every year, and ~4000 patients die as a consequence of this disease.¹

In recent years, the pharmacotherapeutic options for the treatment of metastatic disease has expanded and includes drugs such as fluorouracil, irinotecan, oxaliplatin, and bevacizumab.² Despite the availability of new drugs, the responsiveness to chemotherapy is relatively low (20 to 40%),³ and the median survival of patients with metastatic colorectal cancer is only 20 months.² Moreover, besides considerable interindividual variability in

drug efficacy, drug-related toxicity has shown to vary from patient to patient as well.

There are clear indications that genetic variability (ie, presence of single-nucleotide polymorphisms [SNPs]) between patients may, at least, contribute to differences in drug responsiveness.⁴ DNA repair enzymes play an important role in the pharmacology of platinum-based drugs such as oxaliplatin. Oxaliplatin prevents DNA synthesis by incorporating into the chromosomal DNA, consequently resulting in apoptotic cell death.⁵ A naturally active DNA repair system will remove oxaliplatin from the DNA thereby rescuing the cell.⁶ Consequently, the effect of oxaliplatin treatment is dependent on the activity of the so-called nucleotide excision repair (NER) and the base excision repair (BER) systems. In contrast to BER, NER can only recognize lesions that distort the DNA helix. This is an important difference because some lesions, such as U paired with A, do not distort the helix and are not repaired by NER, whereas they are by BER. The DNA repair enzyme *XRCC1* belongs to the BER system and its encoding gene has shown to be polymorphic.^{7–9} *XRCC1* contains a domain that functions as a protein-protein interface that interacts with poly(adenosine diphosphate-ribose) polymerase, a zinc-finger containing enzyme that detects strand breaks and subsequently removes proteins from the DNA helix, which in turn becomes more accessible for DNA repair enzymes.^{10,11} Three mutations have been identified in the *XRCC1* gene, with the G to A substitution in the second position in codon 399 resulting in an amino acid change (Arg399Gln) in the poly(adenosine diphosphate-ribose) polymerase-binding domain.¹² As a result, this enzyme is less capable of initiating DNA repair due to altered binding characteristics. Indeed, it was shown that this polymorphism is associated with drug resistance to oxaliplatin/fluorouracil chemotherapy in advanced colorectal cancer.¹³ Moreover, in individuals with the amino acid glutamine instead of arginine at codon 399, increased DNA damage marker levels were found due to inadequate repair or increased damage tolerance.^{14–16} Patients with glutamine at position 399, have a more than a fivefold risk of combined oxaliplatin/fluorouracil chemotherapy failure, when compared with patients with wild-type allele.¹³ In contrast, the other mutations, at codon 194 and 280, appeared to be

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Table 1. PCR and Sequence Primers

Primer	Sequence	Modification
PCR		
XRCC1-Arg399Gln-fb	5'-GTGGGTGCTGGACTGTC-3'	5'-Biotine
XRCC1-Arg399Gln-r	5'-CAGGGTTGGCGTGTGAGG-3'	
XRCC1-Tyr576*-f	5'-GGGGACAGCCATTGAGAGTG-3'	
XRCC1-Tyr576-rb	5'-ATCCCATTCCTGTGCTGTGA-3'	5'-Biotine
Sequence		
XRCC1-Arg399Gln-rs	5'-CGTGTGAGGCCTTACC-3'-'	
XRCC1-Tyr576-fs	5'-GGGAGCTCGAGGACT-3--'	

nonfunctional and do not correlate with increased levels of DNA damage *in vivo*.⁷ Until now, there are no reports concerning the deletion of A in the second position of codon 576, despite the fact that this deletion causes a stop codon (*) and consequently results in a truncated protein. These genetic variations in the *XRCC1* gene may contribute to the different responsiveness among colorectal cancer patients treated with oxaliplatin containing regimens. We developed a method that enables identification of both mutations (G to A substitution in codon 399; rs25487 and deletion of A in codon 576; rs2307177) in a single reaction using the pyrosequencing technology. Moreover, as this method is based on "real-time" sequencing, it also determines a substitution of A for C or CT in codon 576, which is a rare genetic variant in *XRCC1* occurring in a frequency <1%. In addition, we assessed the genotype frequencies in 270 patients with colorectal cancer.

Materials and Methods

Blood samples were obtained from 270 patients with colorectal cancer. DNA was isolated with the total MagnaPure Total Nucleic Acid Isolation Kit I on the MagnaPure LC (Roche Diagnostics, Mannheim, Germany). Chromosomal DNA was quantified using Nanodrop (Isogen, IJsselstein, The Netherlands) and diluted to a concentration of 10 ng/ μ l. Polymerase chain reaction (PCR) primers (listed in Table 1) and Pyrosequence kit were purchased from Isogen Life Science (Maarssen, The Netherlands). Sepharose beads were from Amersham (Uppsala, Sweden). Hotstart PCR mastermix was obtained from Qiagen (Hilden, Germany), and PCR reactions were performed on the MyCycler (Biorad, Veenendaal, The Netherlands). Samples were genotyped on a Pyrosequencer 96 MA (Biotage, Uppsala, Sweden).

PCR reactions for both SNPs were performed as a duplex PCR, and conditions were as follows: each tube contained 10 ng of DNA, and 5 pmol of each PCR primer in a total of 12 μ l. Cycle conditions were denaturation initially for 15 minutes at 95°C, 35 cycles of 95°C, -55°C, and -72°C each for 30 seconds, ended by 10 minutes at 72°C. The pyrosequence reactions were performed in accordance with the manufacturers' protocols. Pyrosequencing is a real-time-based sequence method. Each nucleotide that is expected to be incorporated is separately dispensated for. Incorporation of nucleotides results in a release of ATP, the amount of which is related to the amount of incorporated nucleotides. The ATP release

on its turn results in a detectable signal because luciferase and luciferine are present in the enzyme mixture. This would result in a false positive signal when deoxyadenosine triphosphate (dATP) is dispensated as a nucleotide to be incorporated. Therefore, alfa-thio triphosphates (dATPS) are used as a substitute for the natural dATP, because dATPS is not recognized by luciferase. However, the increased signal after incorporation of dATPS, as compared to the regular nucleotides, can be explained by the fact that dATPS is used more efficiently by the DNA polymerase.¹⁷ As a result, incorporation of nucleotides is depicted as peaks in the so-called pyrogram. The peak heights are thus representative of the amount of incorporated nucleotides. Nonincorporated nucleotides, on the other hand, are degraded by the enzyme apyrase and do not contribute to the peaks of next dispensated nucleotides in the pyrogram.

The sequence to analyze for rs2307177 was 5'-[A]TATGAGTGA-3' and 5'-TCC/TGGGAGGGCAGCCG-3' for rs25487. The calculated dispensation order was 5'-gAgTCTcGAGCTGAG-3'. The lower case nucleotides are negative controls; therefore, these nucleotides will not be incorporated in the target DNA and consequently should not appear in the pyrogram.

Results

To detect both SNPs in one single reaction, we located the sequence primers in such a manner that the nucleotide-dispensation order was able to discriminate between the two SNPs, and that separate reference peaks exist. Once an assay has been developed, the pyrosequence software (PSQ 96MA 2.1; Biotage, Uppsala, Sweden) predicts the programs for each possible genotype (Figure 1). Within the dispensation order there are negative controls in the form of nucleotides that cannot be incorporated. The second dispensated nucleotide (A) will be incorporated in both chromosomes in case of a wild-type genotype (A at second position in codon 576). The theoretical peak height will be 1 (Figure 1A). In case of heterozygous A/-, the theoretical peak height will be 0.5 (Figure 1D), whereas homozygous deletion of A at codon 576 will show no peak at all. Depending on the amount of PCR product that was used, the peak height will vary, therefore reference peaks are a useful tool to compare peak heights. Dispensated nucleotides 12 and 13 are reference peaks for the A deletion. The peak height of both reference peaks should be equal and as

Figure 1. Histograms of XRCC1 duplex pyrosequence assay as predicted by pyrosequence software. G399A (rs25487) is shown as blue bars and A576delA (rs2307177) is shown as red bars. The depicted genotypes are G399G and A576A (A), G399A and A576A (B), A399A and A576A (C), G399A and A576delA (D). The yellow areas indicate the nucleotides of interest (SNP).

high as that of the AA genotype. The peak height of an A/– genotype, should be 50% of that of the reference peaks. The fifth and sixth nucleotides show the genotype of Arg399Gln. Because the nucleotide adjacent to the SNP is a C, the peak-height of the fifth dispensated nucleotide (C) will be two in case of a CC genotype (Figure 1A), and 1.5 or 1 in case of C/T (Figure 1B) or TT (Figure 1C), respectively. Figure 1D depicts the theoretical output for the heterozygous deletion of A in codon 576 and Arg399Gln. The pyrosequencing assay identified clearly both genotypes (Figure 2) in a single run and this was confirmed by conventional sequencing (sequence of rs2307177 is shown in Figure 3). The genotype frequencies of the 270 patients with colorectal cancer are depicted in Table 2. Within the 270 samples tested, all but two showed the wild-type genotype (99.3%) for rs2307177, which is Tyr576Tyr. The pyrogram of the two patient samples that are not AA, was not consistent with the expected pyrogram for A/– or –/–. Consequently, the pyrosequence software did not judge the result as passed. Further review of the results showed the pyrogram indicated the rare A/C genotype. We confirmed this genotype (Figure 4) in both patient samples by changing the target sequence information (5'-A/CTATGAGTGA-3') and subsequent dispensation of nucleotides. The derived dispensation order allows the detection of a C or an A at position 576. Of the Arg399Gln SNP, we found 93 samples with genotype GG (34.5%), 144 samples with GA genotype (53.3%), and 33 samples that were genotyped as AA (12.2%).

Figure 2. Representative pyrogram of XRCC1 duplex pyrosequence. **A:** The upper pyrogram shows G399G and A576A, middle pyrogram shows G399A and A576A, and the lower pyrogram shows A399A and A576A. Note that C399T is sequenced with antisense primer, and therefore the complementary nucleotides are shown. **B:** Pyrogram of A576C (and G399A) genotype that, due to the dispensation order, is genotyped as A576delA. The shaded nucleotides indicate the SNP of interest.

Discussion

In this report we describe a rapid assay that allows the simultaneous determination of SNPs in the *XRCC1* gene:

Figure 3. Electropherogram of conventional sequence analysis of the observed genotypes. PCR product of a patient heterozygous for XRCC1 A576C was subcloned in pGEM-Teasy (Promega, Leiden, The Netherlands); several clones were taken for sequence analysis. The **arrows** indicate the nucleotide at codon-position 576 in the *XRCC1* gene, **upper graph** 576C and **lower graph** 576A.

Arg399Gln and Tyr576*, resulting in amino acid substitution of Arg to Gln and in a premature stopcodon, respectively. This gene is known to have more SNPs, such as T194C (rs1799782) and A280G (rs25489), but we chose to determine the nonsynonymous SNP (Arg399Gln), which has a high allele frequency^{7,18} and has been proven to be functional.^{7,18} Next, we investigated frequency of the deletion of A in codon 576, which until now had not been described. Obviously, these SNPs could successfully be included in future pharmacogenetic studies. The observed frequencies of Arg399Gln differ considerably from those published elsewhere. We found frequencies of 34.5, 53.3, and 12.2% for G/G, A/G, and A/A, whereas others found frequencies of 52, 40.2, and 7.8%, respectively (National Center for Biotechnology Information, <http://www.ncbi.nlm.nih.gov>). One reason for this discrepancy could be that frequencies are different

Table 2. Genotype Frequencies in 270 Patients

Genotype	XRCC1-Arg399Gln (rs25487)		
	GG 34.5%	GA 53.3%	AA 12.2%
Genotype	XRCC1-Tyr576* (rs2307177)		
	AA 99.3%	AC 0.7%	Other 0.0%

Figure 4. Pyrogram of XRCC1 duplex pyrosequence with adapted sequence to analyze to detect A/C substitution at position 576. The C-nucleotide is dispensed after the A-nucleotide, allowing discrimination between A576A, A576delA, delA576delA, A576C, or C576C. The shaded nucleotides indicate the SNP of interest.

in patients with colorectal cancer when compared with those in a healthy population. Therefore, we tested the frequency of Arg399Gln in 84 healthy individuals. Frequency of the observed genotypes (47.6, 42.9, and 9.5% for G/G, A/G, and A/A, respectively) was not significantly different with the National Center for Biotechnology Information frequencies. The different genotype frequency in colorectal cancer patients when compared to that of healthy subjects may indicate that XRCC1 genotype is associated with the risk of cancer development. This hypothesis is strengthened by the fact that our data are more consistent with those published by Stoeckelmacher et al¹³ (39, 52, and 9% for G/G, A/G, and A/A), which were also established in patients with colorectal cancer. The frequency of the deletion of A in codon 576 as shown on the National Center for Biotechnology Information website indicates that deletion of A is quite common (allele frequencies: (–)0.459, (A)0.531, (C)0.01, and (CT)0.001). We established the frequency of the A deletion in codon 576 in 270 patients and did not find the possible A deletion, the A nucleotide at the second position in codon 576 was present in all but two patients tested. Instead, we detected an A/C substitution at a frequency of 0.7% that causes an amino acid substitution of Tyr to Ser of codon 576. Therefore, we could state that codon 576 is formed by TAT (encodes for tyrosine). In only 0.7% of the patients tested, codon 576 is formed by TCT (encodes for serine). The not observed A deletion or the CT substitution would have resulted in a frame shift, thereby forming a stopcodon at the next or third codon respectively.

The advantage of pyrosequencing lies in the possibility of detecting unexpected mutations that are located near the sequence of interest. This is well illustrated in this study. In two cases the program differs from the theoretic output because bases different from the expected ones were incorporated. In this way, it is possible to determine additional base substitutions in the sequence that is analyzed. Indeed, we screened for the A deletion at codon 576 but were also able to determine a rare C substitution. It also would be possible to determine the rare CT substitution at codon 576; however, this genotype was not found among the 270 colorectal cancer patients studied for this article.

In conclusion, we developed a reliable and fast method, enabling genotyping within 3 hours after genomic DNA isolation, for the simultaneous determination of the *XRCC1* SNPs Arg399Gln and Tyr576Ser. Pharmacogenetic studies in patients treated with platinum-based chemotherapy may also benefit from this method.

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