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mdr-1 SNPs in Ovarian Cancer Tissue – G2677T/A Correlates with Response to Paclitaxel Chemotherapy

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Abstract

Purpose: P-glycoprotein, encoded by the mdr-1 gene, confers multi-drug resistance to a variety of antineoplastic agents, e.g. paclitaxel. Recently, different polymorphisms in the mdr-1 gene have been identified and their consequences for the function of P-glycoprotein as well as for the treatment response to P-glycoprotein substrates are being clarified. We analyzed the allelic frequencies at polymorphic sites G2677T/A and C3435T in ovarian cancer patients with good or poor response to treatment with paclitaxel in combination with carboplatin in order to evaluate their predictive values.

Experimental Design: Fifty-three patients were included in the study and 28 of them had been relapse-free for at least one year and 25 had progressive disease or relapsed within 12 months. A reference material consisting of 200 individuals was also analyzed. The genotypes of each SNP were determined using Pyrosequencing.

Results: The G2677T/A SNP was found to significantly correlate with treatment outcome. The probability of responding to paclitaxel treatment was higher in homozygously mutated patients (T/T or T/A) (Fisher's exact test $P < 0.05$). The frequency of the T or A alleles was also higher in the group of patients who had a good response ($P < 0.05$). There was also a dose-dependent influence of the number of mutated alleles on the response to paclitaxel treatment (Chi²-test for linear-by-linear association, $P = 0.03$). However, the C3435T SNP was not found to correlate to treatment outcome.

Conclusions: The mdr-1 polymorphism G2677T/A in exon 21 correlates with the paclitaxel response in ovarian cancer and may be important for the function of P-glycoprotein and resistance to paclitaxel and provide useful information for individualized therapy.

Introduction

Paclitaxel (Taxol[®]) has a broad activity spectrum and is clinically used to treat breast, ovarian and lung cancer (1). Paclitaxel was originally isolated from the stem bark of the Pacific yew tree, *Taxus Brevifolia* (2). Drug resistance is a major obstacle to successful treatment of cancer patients and several potential mechanisms have been reported to account for resistance to paclitaxel. These include decreased sensitivity to apoptosis-inducing stimuli (3), alterations in tubulin binding and microtubule dynamics (4) and overexpression of the transport protein P-glycoprotein (5). P-glycoprotein, encoded by the *mdr-1* gene, is a 170 kDa plasma membrane protein that functions as an ATP-driven drug export pump. The taxanes and other cytotoxic drugs of natural origin can be extruded by P-glycoprotein through the cell membranes and enhanced expression on tumor cells leads to a resistant phenotype (6, 7). A high expression of P-glycoprotein on tumor cells has been shown to correlate with a poor response to paclitaxel treatment (8, 9).

P-glycoprotein is also expressed in nonmalignant tissues, e.g. in the intestine and the blood-brain barrier, and influences the activity and distribution of different drugs. In the intestine, P-glycoprotein has proved to be a major determinant for the intestinal absorption of such drugs as protease inhibitors, β -blockers, cyclosporine A, and digoxin (10). At the blood-brain barrier, P-glycoprotein is important for the distribution of various substances to the CNS and therefore may not only be of importance for the therapeutic effect of psychopharmacological drugs, but also for the central neurotoxicity of chemotherapeutic agents and pesticides (11).

The first report on the polymorphisms of the *mdr-1* gene was presented in the late 1980s, and the sequence variant showed an altered resistance phenotype (12). However, it was not until 2000 when Hoffmeyer et al. systematically screened the *mdr-1* gene for sequence variations

that the functional importance of these polymorphisms was demonstrated. They indicated that the synonymous single nucleotide polymorphism (SNP) in exon 26, C3435T, correlated with the level of expression of P-glycoprotein in the intestine. Individuals homozygous for this SNP had lower P-glycoprotein expression and showed higher plasma levels of the P-glycoprotein substrate digoxin (13). Up to now, more than 25 SNPs have been reported for the mdr-1 gene, resulting in up to 20 coding region variants (14-16). However, most SNPs are present at very low frequencies and there are large interethnic variations (14). In Caucasians the SNPs G2677T/A and C3435T have been considered most interesting since they have been shown to correlate with the P-glycoprotein expression and phenotype (13, 17).

A non-functional P-glycoprotein could affect the pharmacokinetics and pharmacodynamics of paclitaxel in several ways. Cancer cells with an ineffective P-glycoprotein efflux should be more sensitive to the drug. Secondly, paclitaxel is given intravenously and excreted via the feces, so ineffective transport of paclitaxel from the blood circulation to the intestine would increase the systemic exposure of the drug. We therefore designed this study to evaluate the effect of the mdr-1 sequence variants G2677T/A and C3435T on the response to paclitaxel treatment in ovarian cancer.

Material and Methods

The allele frequencies of the mdr-1 SNPs G2677T/A and C3435T were investigated in a Swedish population. DNA samples (n=200) were taken from a regional DNA bank consisting of genomic DNA isolated from randomly selected individuals in the southeastern part of Sweden after obtaining their informed consent.

To evaluate the relationship between the mdr-1 genotype and the response to paclitaxel treatment, we identified the SNPs in 51 epithelial ovarian tumors and 2 fallopian tube carcinomas from two groups of patients. After primary surgery all patients had been treated with paclitaxel at a dose of 175 mg/m² or 135 mg/m² (n=5) in combination with carboplatin for at least four cycles. The patients were treated according to the same treatment protocol at Linköping University Hospital or Sahlgrenska University Hospital, Gothenburg, in the southern part of Sweden. In the first group, the patients had a complete response and were relapse free for at least one year (defined as a good response). In the other group, the patients had progressive disease during treatment or had a relapse within 12 months (defined as a poor response). The patient and tumor characteristics are presented in Table 1.

Eleven tumors were collected from paraffin embedded tissues stored at the Division of Molecular and Immunological Pathology, Linköping University, and 42 tumors were fresh-frozen and obtained from a bio-bank at the Department of Oncology, Sahlgrenska University Hospital, Gothenburg. The local ethics committee approved the study.

DNA Isolation and PCR

After tumor collection, genomic DNA was isolated using QIAamp[®] DNA mini kits (VWR International, Stockholm, Sweden) according to the manufacturer's protocol. The amount of DNA extracted was quantified by absorbance spectroscopy (260 and 280 nm) and diluted to 10 ng/μl for working solutions. The isolated DNA was stored at -70°C and the working solutions were stored at -20°C.

The sequence of the PCR primers (Table 2) for amplifications of exon 21 and exon 26 was designed using primer 0.5 free-software and checked for specificity using the NCBI BLAST

server (<http://www.ncbi.nlm.nih.gov/blast/>). One primer for each PCR product was biotinylated in its 5'-end for purification of single-stranded DNA. All primers were obtained from Invitrogen (Paisley, U.K).

The PCR reactions were based on the HotStarTaq master mixture (VWR International) and carried out on a Mastercycler gradient (Eppendorf) in a total volume of 25 µl. The reactions were optimized for annealing temperature (58°C) and MgCl₂ concentration (1.5 mM). Each PCR primer was used at a concentration of 0.4 µM and each reaction used 25 ng of human genomic DNA as template. The amplification was performed with the following temperature cycles: 1 cycle at 95°C for 15 min; 50 cycles at 95°C for 30 s, at 58°C for 30 s, and at 72°C for 30 s; followed by 1 cycle at 72°C for 10 min.

All PCR products were sequenced using both forward and reverse primers on a MegaBACE 1000 (Amersham Biosciences, Uppsala, Sweden) and the sequences were consistent with mdr-1 gene in the GenBank sequence AC005068.

Pyrosequencing

For the real-time sequencing of the PCR products and SNP analysis, Pyrosequencing PSQ96MA (Pyrosequencing AB, Uppsala, Sweden) was used. Sequence-specific primers (Table 2) were designed with the software provided by Pyrosequencing AB (<http://www.pyrosequencing.com>). Pyrosequencing was performed according to the manufacturer's protocol. In brief, for each genotype, single-stranded DNA was isolated from the PCR reactions using the Pyrosequencing Vacuum Prep Workstation (Pyrosequencing AB). Streptavidin SepharoseTM High Performance beads (Amersham Biosciences) were dissolved in BW buffer (10 mM Tris-HCL, 2 M NaCl, 1 mM EDTA, 1 mL/L Tween, pH 7.6,

Sigma, Stockholm, Sweden) and added to the PCR reactions and mixed (>1300 rpm) for 5 min at room temperature. The beads with the captured DNA were washed in ethanol (70%, Kemetyl, Stockholm, Sweden), transferred to 0.2 M NaOH (Sigma) and flushed with washing buffer (10 mM Tris-acetate, 5 mM magnesium acetate, pH 7.6, Sigma). The beads were then released into a 96 well plate containing annealing buffer (10 mM Tris-acetate, 5 mM magnesium acetate, pH 7.6, Sigma) and, for each genotype, the specific sequencing primer (Table 2). Annealing was performed by heating the sample at 80°C for 2 min and then allowing it to cool to room temperature. The plate was then transferred to the PSQ96MA where the real time sequencing took place. The dispensation order for each genotype is presented in Table 2.

Statistical Analysis

The statistical analysis was performed with the SPSS software package version 11.5.1 (SPSS Inc. Chicago, IL, USA). The significance of differences in allele frequencies and genotypes between good and poor responders was calculated using generalized Fisher's exact test (the P-values for the 2-sided exact significance are presented). For differences where $P < 0.05$, the relative risk (RR) of responding to treatment with a 95% CI was calculated when applicable. The χ^2 test for linear-by-linear association was used to analyze the significance of trends in different tables (the P-values for the 2-sided exact significance are presented). For these calculations, the wild type genotypes were denoted as 0, the heterozygous as 1 and the homozygous as 2.

Results

The PCR amplification of exon 21 and exon 26 of the mdr-1 gene resulted in single products of expected sizes (as judged by agarose gel electrophoresis) and the sequences of the products

were consistent with that of the mdr-1 gene. The pyrograms during real-time sequencing showed peaks corresponding to each genotype as shown in Fig. 1 and 2. All allele combinations, except the homozygous A/A at position 2677, were found in the individuals studied.

The frequencies of G2677T/A and C3435T were investigated in individual DNA samples from the reference population (Table 3). The frequencies of the altered sequences were high at both locations. We also found a correlation between the two SNPs, indicating that they are linked to the same haplotype in certain individuals (Table 4, $P < 0.001$). In the ovarian cancer patients, the frequencies of C3435T and G2677T/A were comparable to the percentages found in the reference population (Table 3). There was no significant difference in patient and tumor characteristics between good and poor responders.

The distributions of the mdr-1 SNPs in good and poor responders are shown in Table 4. The missense SNP, G2677T/A, correlated with the outcome of paclitaxel treatment. We compared the wild type and heterozygotes (G/G & G/T) with the homozygously mutated (T/T & T/A) patients and their relation to treatment outcome (Fig. 3A). A statistically significant correlation was found between homozygously mutated patients and successful treatment with paclitaxel ($P < 0.05$, Fisher's exact test). Nine of the 28 cases with a good response were homozygously mutated compared to 2 of the 25 cases with a poor response. The frequency of the T and A alleles in the group of patients with a good response was also significantly higher than in poor responders (32/56 compared to 18/50, $P < 0.05$, Fisher's exact test) as shown in Fig. 3B. The effect of increasing number of mutated alleles (G/G < G/T < T/T or T/A) on the treatment outcome was also found to be significant ($P = 0.03$, Fig. 3C, Chi²-test for linear-by-linear association). This shows that homozygously mutated patients are more likely to respond

to treatment than heterozygous ones, who in turn have a better prognosis than individuals carrying the G/G genotype. The allelic variants of the C3435T SNP did not, however, correlate with the outcome of paclitaxel treatment in ovarian cancer patients.

Discussion

Ovarian cancer patients who are homozygously mutated for the G2677T/A mdr-1 SNP are more likely to respond to paclitaxel treatment and the presence of two mutated alleles can be considered a predictive factor for successful treatment. One explanation could be that the G2677T/A polymorphism has a functional consequence on P-glycoprotein mediated paclitaxel transport. The better response might be due to a reduced efflux of paclitaxel from the tumor cells or a reduced elimination from the body, giving higher plasma concentrations. However, in our study there was no indication of increased adverse drug reactions in patients with mutations. The C3435T variant did not affect treatment outcome.

There are several confounders, which could contribute to the results. A larger number of tumors with a low FIGO stage were actually selected in the group of good responders (Table 1). However, when excluding patients with FIGO stage I or II from the material, the correlations presented here still maintain the same level of significance ($P < 0.05$). To ensure that the total drug dose did not affect the correlation we only included patients who received at least four cycles of paclitaxel treatment and the mean number of cycles in the two groups was similar (good response, mean = 7.1 cycles, and poor response, mean = 7.8 cycles). Five patients received a lower dose of paclitaxel (135 mg/m^2). Two of these patients were poor responders and three were good responders. A larger number of patients in the group with a good response had no macroscopic tumor left after surgery or the result of surgery was not known. Five of these 11 patients had a late relapse indicating that they still had some tumor

residues after surgery. As for the other 6 patients we cannot be sure whether the treatment outcome is due to the chemotherapy or to the surgery. However, excluding these patients from the material the correlation between the response and the G2677T/A SNPs is still significant ($P < 0.05$).

Several drug-resistance-associated genes have been described and characterized in cell lines but their precise roles in clinical resistance still remain to be clarified. This is also true for the extensively studied mdr-1 gene and its product P-glycoprotein. Although P-glycoprotein, MRP1, MRP2, MRP3, MRP6, and MRP7 are able to confer resistance to natural products in cell lines, P-glycoprotein and MRP-7 are the only two that cause efflux of paclitaxel (18, 19). In the treatment of ovarian cancer with paclitaxel, it has been shown that P-glycoprotein expression in tumors correlates with a poor response (8, 9).

Several groups have investigated the effects of the mdr-1 polymorphisms on the development of drug resistance and relapse after chemotherapy as well as on the pharmacokinetics of anti-neoplastic agents. Illmer et al. (2002) found that acute myeloid leukemia patients with the wild type variant of C1236T, G2677T/A and C3435T had a higher risk of relapse than the other haplotypes (20). Our results support these findings since ovarian cancer patients with the wild type variant and the heterozygous patients did not respond to paclitaxel treatment as well as individuals homozygously mutated at position 2677. Several other groups have found that the wild type of the silent C3435T variant is associated with poor response (20-22). Although we could not detect an effect of the C3435T SNP on the outcome, others and we have shown that there is a linkage between the two SNPs (15, 16, 23). It has been proposed that the effect observed when studying one of the SNPs might be due to the other one (23). Considering this linkage the effect on treatment outcome in ovarian cancer is also supported

by findings by Jamroziak et al. (2004) showing that children with acute lymphoblastic leukemia and the C/C genotype at position 3435 have a worse prognosis (21). When treating breast cancer patients with anthracyclines alone or in combination with taxanes, Kafka et al. (2003) found that the T/T variant at position 3435 correlated with a complete clinical response (22). All of these studies including ours indicate that patients having one or more wild type alleles of the SNPs are at higher risk of not responding to treatment. On the other hand, Isla et al. (2004) did not find any effect of the C3435T mdr-1 polymorphism on the outcome of docetaxel-cisplatin treatment of non-small-cell lung cancer patients (24). In contrast to our results, the haplotype of 2677T/T and 3435T/T has also been shown to be at highest risk of drug resistance in lymphoproliferative diseases (25). Since most studies have been done on the silent mutation, C3435T, the discrepancies in the results might be due to the association of this SNP with different haplotypes in different populations. The contradictions might also be explained by the consideration that the different amino acids at position 893 (Ala, Ser or Thr, nucleotide position 2677) might have different effects on different drugs.

The functional consequences of the mdr-1 SNPs have not been extensively studied *in vitro*. Kimchi-Sarfaty et al. (2002) showed that the efflux of paclitaxel *in vitro* by the wild-type P-glycoprotein was slightly higher than the efflux seen with cells carrying a plasmid containing the 2677T variant (26). In contrast, for other substrates such as verapamil, vinblastine, calcein-AM, prazosin, bisantrene, forskolin, digoxin (0.1 μ M) and cyclosporin A the transport was not affected by known variants of P-glycoprotein, however, for each substrate only one concentration was tested (23, 26, 27). Unexpectedly an enhanced efflux has been reported for digoxin in a very high concentration (50 μ M) in cells expressing the mdr-1 Ser893 variant (2677T), indicating differences in substrate specificity for the different variants of P-glycoprotein (28).

The functionality of P-glycoprotein may not only affect the response of the tumor cells but also drug disposition. Sparreboom et al. (1997) showed that *mdr1a*(-/-) mice (lacking intestinal P-glycoprotein) have much higher plasma concentrations of paclitaxel and lower excretion via the bile compared to wild type mice, establishing an important role for P-glycoprotein in the transport of paclitaxel from the circulation to the intestinal lumen (29). In humans this is supported by findings showing that concomitant administration of P-glycoprotein blockers and paclitaxel, decreases the paclitaxel clearance and increases the exposure of paclitaxel (area under the curve) (30). The effect of the *mdr-1* polymorphism on paclitaxel pharmacokinetics has not yet been shown. In other studies, no significant differences in the clearance of docetaxel were observed between different *mdr-1* genotypes (31, 32) although the patients with C/C at position 3435 showed the highest docetaxel clearance (31). This suggests that fecal elimination is reduced by inhibiting P-glycoprotein-mediated transport in the gastrointestinal tract and that a nonfunctional P-glycoprotein may affect the pharmacokinetics of paclitaxel.

In conclusion our study shows that ovarian cancer patients who are homozygously mutated for the missense *mdr-1* SNP, G2677T/A, respond better to treatment with paclitaxel than those with at least one wild type allele. Obviously, further studies are needed before the role of the polymorphisms in the *mdr-1* gene can be defined conclusively. Studies that show a correlation of single nucleotide polymorphisms in the *mdr-1* gene and the function or expression of P-glycoprotein contribute to the pool of information on the genetic background that may be relevant to predicting the individual response to treatment.

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Tables

Table 1 Patient and tumor characteristics.

| | Good Response n=28 | Poor Response n=25 |
|-------------------------------|------------------------------|------------------------------|
| Median age (range) | 61 (43-72) | 59 (40-75) |
| Result of surgery | | |
| No macroscopic tumor | 7* | 1 |
| Macroscopic tumor left | 17 | 20 |
| Unknown | 4* | 4 |
| FIGO stage[§] | | |
| I | 1 | - |
| II | 4 | - |
| III | 20 | 20 |
| IV | 3 | 4 |
| Histology | | |
| Serous | 19 | 14 |
| Mucinous | 1 | 1 |
| Endometrioid | 5 | 2 |
| Undifferentiated | 1 | 3 |
| Unknown | 2 | 5 |
| Tumor grade (FIGO) | | |
| Well diff | 1 | - |
| Moderately diff | 8 | 4 |
| Poorly diff | 17 | 20 |
| Unknown | 2 | 1 |

NOTE: * Five of these 11 patients got a late relapse.

[§] In one patient the FIGO stage could not be determined.

Table 2 PCR primers, sequencing primers and dispensation order for detecting the mdr-1 SNPs.

| SNP | G2677T/A | C3435T |
|------------------------------|---------------------------------------|--|
| Forward primer, 5'-3' | ^{bio} TAGCAATTGTACCCATCATTGC | GCAAAGAAATAAAGCGACTGAA |
| Reverse primer, 5'-3' | AAAAGATTGCTTTGAGGAATGG | ^{bio} TTGAAGAGAGACTTACATTAGGCAG |
| Sequencing primer | TTAGTTTGACTCACCTTCC | GTGGTGTACAGGAAGA |
| Dispensation order | GCCAGTCAGCTC | CGATCAGTG |
| Sequencing direction | Reverse | Forward |
| Product, bp | 230 | 213 |

NOTE: ^{bio} – biotinulated nucleotide

Table 3 The SNP frequencies in a Swedish population (n=200) and ovarian cancer patients. No significant difference could be found between the reference population and ovarian cancer patients (P≥0.4).

| | C3435T | | G2677T/A | | |
|--------------------------------|---------------|-----|-----------------|-----|----|
| | C | T | G | T | A |
| Swedish population | 45% | 55% | 56% | 42% | 2% |
| Ovarian cancer patients | 41% | 59% | 53% | 45% | 2% |

Table 4 mdr-1 genotypes in a general Swedish population and in ovarian cancer patients that had been successfully treated with paclitaxel and those that failed treatment. A significant positive correlation was found between the G2677T and C3435T SNPs in the general Swedish population (Chi²-test for linear-by-linear association, P<0.001).

| General Swedish population | | | | | | | |
|----------------------------|-----|-----|-----|-----|-----|---|-----|
| G2677T/A | | | | | | | |
| | G/G | G/T | T/T | G/A | T/A | Σ | |
| C3435T | C/C | 40 | 3 | 1 | 2 | 0 | 46 |
| | T/C | 21 | 60 | 0 | 3 | 4 | 88 |
| | T/T | 2 | 29 | 35 | 0 | 0 | 66 |
| | Σ | 63 | 92 | 36 | 5 | 4 | 200 |

| Ovarian cancer patients – Good response | | | | | | | |
|---|-----|-----|-----|-----|-----|---|----|
| G2677T/A | | | | | | | |
| | G/G | G/T | T/T | G/A | T/A | Σ | |
| C3435T | C/C | 2 | 1 | 0 | 0 | 0 | 3 |
| | T/C | 3 | 8 | 3 | 0 | 2 | 16 |
| | T/T | 0 | 5 | 4 | 0 | 0 | 9 |
| | Σ | 5 | 14 | 7 | 0 | 2 | 28 |

| Ovarian cancer patients – Poor response | | | | | | | |
|---|-----|-----|-----|-----|-----|---|----|
| G2677T/A | | | | | | | |
| | G/G | G/T | T/T | G/A | T/A | Σ | |
| C3435T | C/C | 5 | 0 | 0 | 0 | 0 | 5 |
| | T/C | 3 | 8 | 0 | 0 | 0 | 11 |
| | T/T | 1 | 6 | 2 | 0 | 0 | 9 |
| | Σ | 9 | 14 | 2 | 0 | 0 | 25 |

Figure Legends

Fig. 1 Representative pyrograms for genotyping the C3435T SNP, illustrating (A) an individual homozygous wild type (C/C), (B) a heterozygous (C/T) and (C) a T homozygous individual (T/T). The sequencing was performed on the forward strand.

Fig. 2 Representative pyrograms for the genotyping of the G2677T/A SNP illustrating (A) a homozygous wild type (G/G), (B) a heterozygous G/T, (C) a T homozygous individual (T/T), (D) a G/A heterozygous individual and (E) a T/A heterozygous. No homozygous A/A was found in the individuals studied. The sequencing was performed on the reverse strand.

Fig. 3 The effect of the mdr-1 SNP G2677T/A on the treatment outcome. (A) The treatment outcome, good response (white) and poor response (black), was evaluated for homozygously mutated individuals (T/T or T/A) and compared with the response of the individuals having G/G or G/T at position 2677. Patients who were homozygously mutated were significantly more likely to respond to paclitaxel treatment than patients carrying the other genotypes (Fisher's exact test $P=0.04$, relative risk = 1.81, 95% confidence interval for the relative risk: $1.17 < RR < 2.79$). (B) The nucleotide frequencies (2 x homozygous + heterozygous) in the two treatment groups were also compared using an exact test. A significantly higher frequency of T or A at position 2677 was found in the group of patients that had responded well to treatment compared to those who responded poorly (Fisher's exact test $P=0.03$, relative risk = 1.59, 95% confidence interval for the relative risk: $1.03 < RR < 2.45$). (C) The dose response effect of the allele variants on the success and failure of paclitaxel treatment was tested using the χ^2 test for linear-by-linear association and was found to be significant (χ^2 -test for linear-by-linear association, $P=0.03$).

Fig. 1

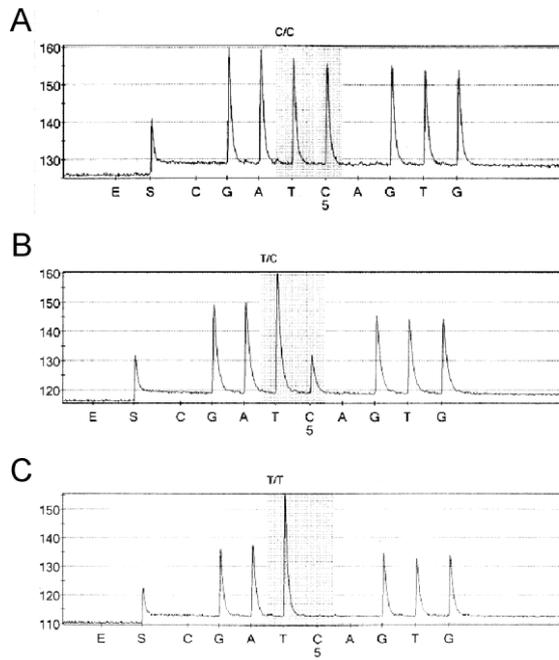


Fig. 2

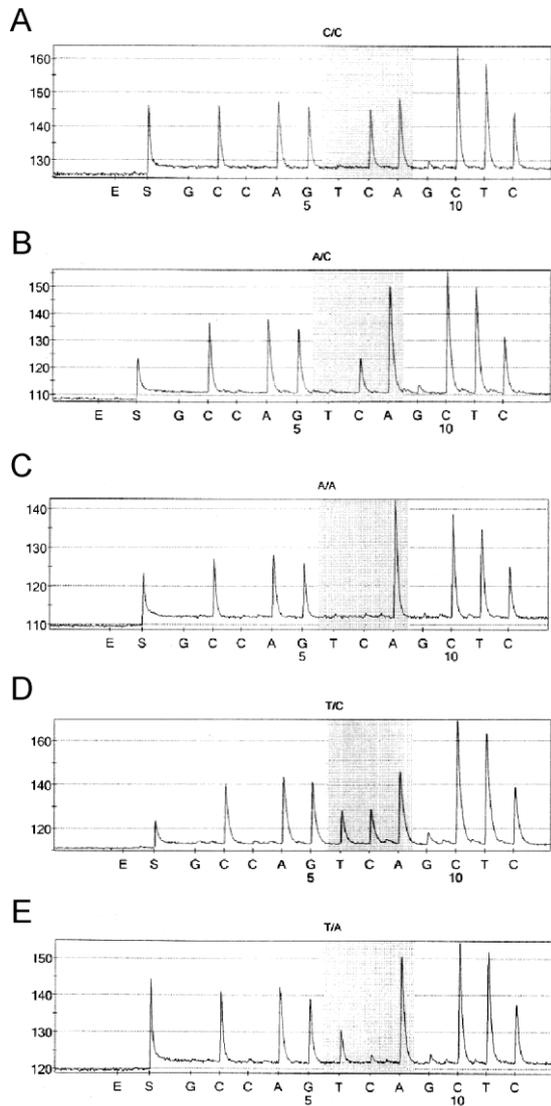


Fig 3.

