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Research Article

High frequency of the *HRAS* oncogene codon 12 mutation in Macedonian patients with urinary bladder cancer

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Abstract

Point mutations at codon 12 of the *HRAS* (v-Ha-ras Harvey rat sarcoma viral oncogene homolog) oncogene are one of the best defined and widely studied molecular genetic events in transitional cell carcinoma (TCC) of the urinary bladder. The aim of this study was to use the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis of paraffin-embedded tissue-derived DNA to determine the frequency of the *HRAS* oncogene G →T codon 12 mutation in TCC patients being treated at the University Urology Clinic in Skopje, Republic of Macedonia. DNA isolated from paraffin-embedded tissue (PET) surgically removed TCC specimens of 62 (81.58%) out of 76 patients were successfully amplified, the remaining 14 (18.42%) showing compromised DNA integrity. The codon 12 mutation of the *HRAS* oncogene was found in 24 (38.71%) out of 62 successfully tested TCC urinary bladder samples. No significant relationship between the mutation frequency and the histopathological grade of tumor differentiation was detected (χ^2 = 0.044; p = 0.978). The relatively high frequency of mutations found in our study was comparable with some of the previously reported data obtained by this and/or other PCR-based methods. This highly sensitive and specific PCR-RFLP analysis was demonstrated to be a suitable method for the detection of mutations at codon 12 of the *HRAS* oncogene in PET samples of urinary bladder TCC.

Key words: HRAS, oncogene, bladder cancer, PCR-RFLP.

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Introduction

Transitional cell carcinoma (TCC) of the urinary bladder is the most common urothelial neoplasm in the urinary tract and one of the most frequent malignancies found in the human population (Steiner *et al.*, 1997, Ozen, 1998). The molecular genetics of TCC has been extensively studied and reviewed (Levesque *et al.*, 1993 Hovey *et al.*, 1998, Knowles, 1999, Jung and Messing, 2000, Knowles, 2001). One of the best-defined molecular events in TCC and in some other neoplasms is point mutation at the 'hot-spot' positions of the *HRAS* (v-Ha-ras Harvey rat sarcoma viral oncogene homolog) oncogene (Saito, 1992, Ooi *et al.*, 1994, Reznikoff *et al.*, 1996, Ross *et al.*, 1996, Saito *et al.*, 1997, Pfister *et al.*, 2000). The G →T substitution at the codon 12 of the *HRAS* oncogene leads to a glycine to valine change and consequently to an activated protein p21, in a permanently guanosine 5'-triphosphate (GTP)-bound state, possessing dominant oncogenic potential (Jung and Messing, 2000). About twenty years ago, Taparowsky *et al.* (1982) elegantly demonstrated the transforming potential of the mutant *HRAS* oncogene by transfection of the NIH 3T3 cell line with a human bladder cancer-derived clone of the *HRAS* oncogene. Further research on the genetics of urothelial malignant transformation, including sequencing analyses (Caponet *et al.*, 1983, Willumsen *et al.*, 1986, Knowles *et al.*, 1993, Levesque *et al.*, 1993, Burchil *et al.*, 1994), has found that codon 12 mutations are the most frequently affected single 'hot-spot' position in the *HRAS* oncogene (Czerniak *et al.*, 1990, Fitzgerald *et al.*, 1995, Cristaudo *et al.*, 1997, Jung and Messing, 2000).

The amplification power of methods based on the polymerase chain reaction (PCR) has greatly improved the understanding of molecular abnormalities associated with

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urothelial malignant transformation (Ross *et al.*, 1996, Steiner *et al.*, 1997, Bonnal *et al.*, 2000, Christensen *et al.*, 2000, Friedrich *et al.*, 2000, Phillips *et al.*, 2000). Retrospective investigations of archived paraffin-embedded tissue (PET) from patients with a known clinical outcome can be useful in evaluation of the prognostic significance of defined molecular events, such as *HRAS* mutations in TCC patients (Czerniak *et al.*, 1990). However, histopathological processing of surgical samples leads to various degrees of DNA degradation and consequently compromises the quality and quantity of DNA isolates (Greer *et al.*, 1995). This type of DNA source requires the careful selection of highly sensitive detection method to avoid the possibility of misdetecting mutations. Restriction fragment length polymorphism (RFLP) analysis of PCR-amplified DNA has proven to be a very sensitive method for the detection of known mutations, with up to 100% specificity being reported under optimized conditions (Nollau and Wagener, 1997).

In the present study, we used PCR-RFLP to retrospectively evaluated the frequency of point mutation at codon 12 of the *HRAS* oncogene in PET surgical samples taken from patients with urinary bladder TCC.

Materials and Methods

Methodology

The investigation was performed on archived PET material from 76 patients with urinary bladder TCC (61 male, 15 female, mean age 64.27 ±30.5 years), 85.5% of the patients being ethnic Macedonians, 13.2% Albanians and 1.3% Serbs. Surgical samples had been obtained by transuretral resection, biopsy and/or partial or total cystectomy (performed at the University Urology Clinic in Skopje, Republic of Macedonia) and had been formalin-fixed and paraffin-embedded using the routine procedures in place at the histopathology laboratory of the same hospital. The archived PET samples used in our study were all less than 2 years old and were selected from the histopathology laboratory PET archive and assessed by clinical pathologists according the degree of histological differentiation of the tissues (Bane *et al.*, 1996), lower levels of differentiation indicating more advanced TCC. The pathologists divided the samples into three TCC groups: TCC grade 1, containing the most differentiated samples; TCC grade 2, containing samples with an intermediate level of differentiation; and TCC grade 3, containing the least differentiated samples. Genomic DNA isolated from a T24 human bladder tumor cell line was used as a positive control for the presence of point mutation at codon 12 of the *HRAS* oncogene (Taparowsky *et al.*, 1982; Capon *et al.*, 1983). Human leukocyte DNA from a healthy subject was used as a negative control (Steiner *et al.*, 1997). This study was approved by the Ethical Committee of the Medical Council of the Republic of Macedonia.

Isolation of DNA

Depending on tissue area, 10 to 35 mm sections were taken from each paraffin block, de-paraffinized with xylene, washed in absolute ethanol and air-dried; all under strict conditions designed to reduce cross-contamination of the samples (Greer *et al.*, 1995). Protein digestion was performed by incubating the dried specimens overnight at 55 °C with lysis buffer (50 mM Tris·HCl, pH 8.5; 1 mM EDTA; 0.5% Tween 20; 0.01% Proteinase K) using 100 mL of buffer per 10-20 mg of dried tissue. DNA was isolated using lithium chloride/chloroform extraction followed by ethanol precipitation (Gemmell and Akiyama, 1996, Wolff and Gemmil, 1997). The concentration of DNA and of contaminating protein was determined by UV-spectrophotometry and DNA integrity evaluated by agarose gel-electrophoresis.

PCR amplification

The amplification of a 123-bp sequence which included codon 12 of the *HRAS* oncogene (Levesque *et al.*, 1993, Burchil *et al.*, 1994) was performed using the HPLC-purified oligonucleotide primer pair H12A/H12B (sense: 5'-atgacggaatataagctggt-3'; antisense: 5'-cgccaggctcacctctata-3') (MycroSynth, Balgach, Switzerland). Each amplification mix was assembled in a final volume of 25 µL of PCR buffer (50 mM KCl; 10 mM Tris·HCl, pH 9.0 at 25 °C; 1.5 mM $MgCl_2$; 0.1% Triton® X-100) containing: 200 µM dNTP mix; 1 µM each of sense and antisense primer; 0.5 U Taq polymerase and 200 ng of template DNA. The reaction mixtures were amplified in a GeneAmp PCR System 2400 thermocycler (Perkin Elmer, Norwalk, CT) using the following cycling profile: initial denaturation (5 min at 95 °C); followed by 30 cycles of three steps each: denaturation (15 s at 95 °C), annealing (30 s at 56 °C) and extension (30 sec at 72 °C). After completing the last cycle, terminal extension was applied (7 min at 72 °C). All PCR reagents were obtained from Promega, (Madison, WI).

RFLP analysis

An aliquot of each PCR-amplified sample was subjected to restriction digestion at 37 °C for 4 h in a final volume of 20 µL of digestion buffer (25 mM Tris-acetate, pH 7.8; 100 mM potassium acetate; 1 mM dithiothreitol) containing: 10 µL unpurified amplified DNA; 100 µg/mL acetylated bovine serum albumin (BSA); 10 U *MspI* (Promega, Madison, WI). Both undigested and *MspI*-digested aliquots of each amplified sample, along with positive (T24 cell line DNA) and negative (human leukocyte DNA) control samples and a DNA size marker (20-1000 bp molecular ruler (Bio-Rad, Hercules, CA) were subjected to electrophoreses on non-denaturing 10% polyacrylamide gels and visualized under UV-light after ethidium bromide staining.

Results and Discussion

The DNA isolated from PET specimens of 62 (81.58%) out of 76 patients were successfully amplified and, as expected, yielded 123 bp PCR products. The remaining 14 (18.42%) of isolates showed extensive DNA degradation after control agarose gel electrophoresis and were not included in the investigation.

Digestion of non-mutated (wild-type) 123 bp amplicons with restriction endonuclease *MspI* results in two bands (one for a 91 bp fragment and one for a 32 bp fragment) while amplicons harboring the codon 12 G →T mutation are digestion-resistant and produce just one band representing the intact 123 bp product. Because tumor cells are diploid they are, theoretically, heterozygous for the codon 12 G →T mutation and PCR-RFLP analysis of a pure population of malignant cells should result in bands for both the undigested 123 bp PCR product and two digestion fragments (one of 91 and another of 32 bp) with the intensity of the UV-induced ethidium bromide fluorescence of the undigested electrophoretic band being equal to the sum of the fluorescence intensities of the two digestion products.

The results obtained by our PCR-RFLP analysis of the *HRAS* oncogene codon 12 G →T mutation are shown in Figure 1. In our experiments, the intensity of the fluorescence of the bands corresponding to undigested and digested PCR products differed considerably between samples. In most of the samples harboring the codon 12 G →T mutation the fluorescence of the 91 bp and 32 bp bands (corresponding to the digested PCR products representing the unmutated wild-type gene) was more intense than that of the undigested 123 bp band (Figure 1, line 6).

In contrast to non-neoplastic normal cells, neoplastic tissue (especially established cancer cell lines) contain multiple and often uncharacterized chromosomal abnormalities (amplifications, aneuploidy, polyploidy, translocations, inversions, deletions, etc.) that change the proportion of normal and mutant alleles. Since we performed no micro-dissection on our PET samples the proportion of neoplastic to normal tissue in each sample remained as it was (*i.e.* undetermined) in the original tumor, leading to different amounts of mutant *HRAS* alleles in samples from different patients and, consequently, to the observed differences in the intensity of fluorescence between the non-mutant and mutant digested (wilde-type) PCR products. Since our restriction digestion was performed to completion under optimized conditions, the appearance of even only a light band of undigested PCR products would be indicative of the presence of mutant DNA in the original sample.

The PCR-RFLP analysis of the DNA isolated from the T24 cell line (homozygous for the codon 12 G →T mutation) used as a positive control was completely digestion-resistant and only one 123 bp electrophoretic band appeared on the gel (Figure 1, lines 1 and 2).

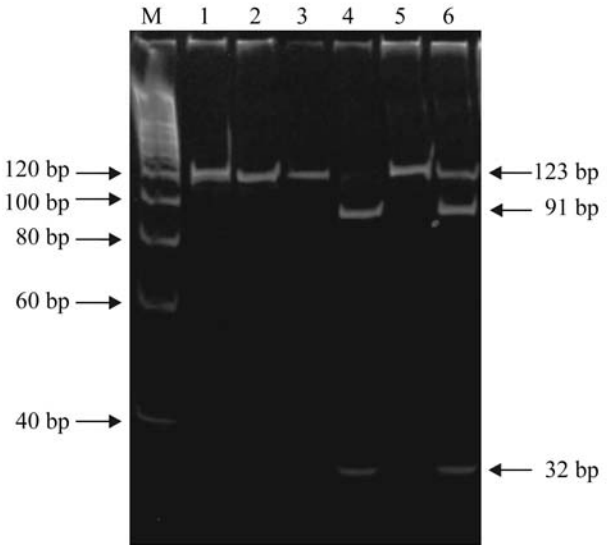

Figure 1 - Detection of the G →T mutation at codon 12 of *HRAS* oncogene by PCR-RFLP analysis. On a 10% polyacrylamide gel: line M, molecular weight marker; lanes 1 and 2, positive control (T24 cell line DNA), undigested and *Mst*I - digested, respectively; lanes 3 and 4, sample without the mutation, undigested and digested, respectively; lanes 5 and 6: sample with the mutation, undigested and digested, respectively.

PCR amplification and RFLP analysis revealed the 12 G →T point mutation at codon 12 of the *HRAS* oncogene in 24 (38.71%) out of 62 tested samples. Seven out of 19 patients (36.84%) with TCC grade 1, ten out of 26 (38.46%) with TCC grade 2, and seven out of 17 (41.18%) with TCC grade 3 were found to have the 12 G →T mutation (Table 1).

The point mutations of the *HRAS* oncogene is relatively common in various human neoplasms and usually affects 'hot-spot' positions at codons 12, 13, 59 and 63 (Manam and Nichols, 1991, Burchil *et al.*, 1994, Jung and Messing, 2000), but in this paper we will restrict our discussion to the G →T point mutation at codon 12 of the *HRAS* oncogene because the majority of previous reports indicate that this is the most frequent *HRAS* mutation in urinary bladder TCC (Czerniak *et al.*, 1990, Burchil *et al.*, 1991). However, the reported incidence of codon 12 *HRAS* codon

Table 1 - Distribution of patients according to G →T mutation at codon 12 of the *HRAS* oncogene and histopathological grade of transitional cell carcinoma (TCC) differentiation.

	Histopathological grade Showing number of patients in each grade (bold type) and percentage (%)		
HRAS G →T mutation	TCC 1	TCC2	TCC 3
Positive	7 (36.84)	10 (38.46)	7 (41.18)
Negative	12 (63.16)	16 (61.54)	10 (58.82)
Total (n = 62)	19 (30.64)	26 (41.94)	17 (27.42)

12 mutations in urinary bladder TCC display broad range between different studies (Burchil *et al.*, 1991, Saito, 1992, Levesque *et al.*, 1993, Burchil *et al.*, 1994, Hong *et al.*, 1996). For example, while Knowles and Williamson (1993) found a very low frequency of the *HRAS* mutation (2.63%) using Single-Strand Conformation Polymorphism (SSCP), RFLP and direct sequencing, Fitzgerland *et al.* (1995), also using SSCP analysis, reported a mutation frequency of 44% for exon 1 (which includes codon 12). In our study we found that the frequency of tumors with codon 12 mutations of the *HRAS* oncogene was relatively high at 38.71%, this being comparable to the 36.36% frequency reported by Czerniack *et al.* (1990) who used allele-specific oligonucleotide hybridization to detect this mutation. In addition, we have also detected a high prevalence of the *HRAS* codon 12 mutation in another study of patients with bladder cancer (Panov *et al.*, 2002).

The discrepancy in the reported incidence of mutations at codon 12 of the *HRAS* oncogene may be due to many different factors, including ethnic specificity, environmental exposure, exposure in the workplace, variability in the proportion of malignant to stromal and normal urothelial cells in the tissue specimen or to the degree of heterogeneity within the tumor cell population, as well as the type of methodology used to detect this type of mutation. In our research, different samples showed variation in the intensity of fluorescence of the electrophoretic bands corresponding to digested and undigested PCR products, probably due to both the different proportions of malignant versus stromal tissue and the presence of normal urothelial cells in the PET samples.

Regarding the sensitivity of the currently available methodology, it has been demonstrated that direct sequencing can detect a mutant allele when at least 12.5% (Burchil *et al.*, 1994) to 20% (Knowles and Williamson, 1993) of cells in a tissue sample contain the mutation. Fitzgerald *et al.* (1995) reported that SSCP analysis required approximately 10% of mutated cells in order to be effective, while Knowles and Williamson (1993) found that mismatched primer extension techniques needed 6 to 10% of mutation-bearing cells in order to be effective. The extraordinary sensitivity of PCR-RFLP analysis has been demonstrated by Hong *et al.* (1996), who reported that even 0.1% of cells harboring mutated DNA mixed in normal DNA was detectable. Although, because of the amplification power of the PCR, extreme care must be taken to avoid cross-contamination of samples by previous PCR products. Under optimal conditions, including the use of a positive control, almost no false negative or positive results can be expected from PCR-RFLP analyses (Nollau and Wagener, 1997). In our study, we performed all DNA isolation and PCR assembling separately from the post-PCR manipulations using stringent rules for preventing cross-contamination of samples and also repeated analysis of randomly selected samples, these random replications always producing the same results. To ensure that there are no restriction endonuclease inhibitors present in the amplified DNA before digestion with *Msp*I enzyme, aliquots of the product of amplification can be digested with a different enzyme that recognizes a restriction site other than that of the expected mutation. The above data supports the usefulness of PCR-RFLP analysis as a sensitive, specific and convenient method for the detection of mutations at codon 12 of the *HRAS* oncogene in archived PET samples.

The use of PET in PCR-based molecular genetic studies has resulted in many exciting new insights into cancer research, molecular epidemiology and other related areas. Due to the intrinsic properties of this type of tissue source, the success of any PCR-based method involving PET material depends on several factors, including the type of fixative used in tissue processing, duration of fixation, the age of the paraffin block and the length of DNA fragment to be amplified. Greer *et al.* (1995) reported considerable variation in the quality of the DNA of PET specimens derived from different institutions. In the study, 62 isolates (81.58%) out of 76 PET specimens were successfully amplified. Our results gave a lower percentage of successful DNA isolation in comparison to that found by Greer *et al.* (1995) using PET samples of comparable age and an amplification product of a comparable length. The difference between our results and those of Greer *et al.* (1995) may have been due to delayed or prolonged tissue fixation or other tissue-processing factors used in the original preparation of our PET samples, such factors sometimes resulting in a significant decrease in the length of amplifiable DNA sequences. Even so, the proportion of amplifiable DNA isolates obtained in our investigation supports the view that PET samples are acceptable for use in methods involving the PCR-based detection of the *HRAS* oncogene codon 12 mutation in bladder TCC. This methodology apears to be an appropriate for detecting mutations and amplifying DNA sequences up to 200 bp in length (Greer *et al.*, 1995).

In the present study, no significant association was observed between mutation frequency and the histological grade of differentiation ($\chi^2 = 0.044$; $p = 0.978$). The almost even distribution of mutations in all three TCC grades support the assumption that, when present, point mutations at codon 12 in the *HRAS* oncogene are an early molecular genetic event in the pathogenesis of bladder tumors (Burchil *et al.*, 1994, Hong *et al.*, 1996). The role of the *ras* gene in uroepithelial oncogenesis has also been supported by immunochemical studies, such as that conducted by Fontana *et al.* (1996) who found a correlation between overexpression of the c-ras oncogene product (p21) and recurrence of the superficial bladder cancer samples. However, in our and other reported studies, the majority of urinary bladder neoplasms have no detectable mutations affecting the *HRAS* gene, implying that other or alternative molecular pathways may be involved in the events leading to the stimulation of cell proliferation in bladder cancer

(Levesque *et al.*, 1993, Cairns and Sidransky, 1998, Shields *et al.*, 2000). Such molecular events may activate the same RAS > RAF > MEK > MAPK circuitry or deregulate the parallel pathways leading to abnormal mitogenic growth signals and/or changes in normal gene expression (Hanahan and Weinberg 2000, Hahn and Weinberg, 2002).

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