

## Genetic analysis of H-ras intron-1 polymorphic and variable tandem repeat regions in human breast cancer

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**ABSTRACT:** H-ras is a member of the *ras* superfamily of genes. This gene encodes for a 21 kDa protein (p21) which is located on the inner surface of the plasma membrane. *Ras* genes are involved in a wide variety of human tumors, and there is a known correlation between H-ras activation and breast carcinogenesis. H-ras contains a polymorphic region, a repeated hexanucleotide -GGGCCT- located in intron 1 close to the 5' of the gene (*HRM* region). Three alleles of this region, P1, P2 and P3, have been identified that contain two, three and four repeats of the hexanucleotide, respectively. H-ras possesses a minisatellite DNA of the variable tandem repeat (*VTR*) which is located 1000 bp downstream of the gene displaying linkage disequilibrium with *HRM*. The purpose of this study was to estimate the frequency of P1, P2 and P3 in the normal population and in patients with breast cancer. We studied 56 biopsy specimens from patients with breast cancer, 61 normal blood samples, and 30 pairs of normal and tumoral breast tissues for *VTR* analysis. There was a difference in the distribution of P1, P2 and P3 alleles between normal and breast cancer samples. The frequency of P1 homozygosity was shown to be almost twice as high in women with breast cancer compared to healthy women (72% versus 39%). These results suggest that P1 homozygosity may be considered as a potential risk factor in breast carcinogenesis. In *VTR* analysis one sample presented a shift in mobility, but no polymorphism in the *Bst*NI pattern of the 28 bp repetition core was observed. (Int J Biol Markers 2003; 18: 195-9)

**Key words:** H-ras, Intron 1, *VTR*, Breast cancer

### INTRODUCTION

The incidence of breast cancer is almost one in nine women, and current therapies for the disease are inadequate once it has metastasized. The disease is characterized by high morbidity and mortality. Breast cancer seems to progress as hyperplastic ductal or lobular epithelial growth, acquiring progressive genetic changes (including those of oncogenes and tumor suppressor genes) leading to clonal outgrowths of progressively malignant cells (1).

The three forms of *ras*, K-*ras*, H-*ras* and N-*ras*, encode for 21 kDa proteins (p21) located on the inner plasma membrane. *Ras* proteins are membrane-bound GTPases that are active in the GTP-bound state. The result of p21 activation is the induction of other genes which stimulate cell proliferation (2, 3). H-*ras* is a member of the *ras* superfamily of genes and its activation has been reported to be correlated with breast carcinogenesis.

The first intron of the human H-*ras* gene (*HRM*) possesses a hexanucleotide polymorphic site (4), which is

often used as a marker in mapping analysis of the 11p15.5 chromosomal region (5-10). This intron 1 microsatellite region is located 83 bp away from the 5' of the gene (4). Iwahana and colleagues (4) first described two alleles, P1 and P2, which consisted of four and two repetitions, respectively, of the hexanucleotide consensus -GGGCCT. This repetition results in an H-*ras* length polymorphism. P1 and P2 alleles were initially described as the *Pst*I RFLP site (4). A subsequent study by Tanci and associates (5) revealed the existence of a third allele, P3, consisting of three repeats of the hexanucleotide, based on its electrophoretic mobility. The *HRM* site provides a system with sufficient heterozygosity for detection of genetic alterations, such as loss of heterozygosity (LOH) and shifts in length of constitutive alleles.

This H-*ras* microsatellite region was found to display linkage disequilibrium with the other known *VTR* region at the 3' end of the H-*ras* gene (4, 6). The linkage between these two sites was suggested to reflect the functional relationship with cell survival (4-6). It has been employed in a variety of tumors such as renal (11), head

and neck (7) and lung (10) cancer.

We evaluated the frequency of P1, P2 and P3 microsatellite alleles of H-ras and examined whether they could represent a risk factor for patients with malignant breast lesions with respect to a normal control group. We also investigated whether genetic instability is a detectable phenomenon in the polymorphic VTR region of the H-ras oncogene. Alterations of this minisatellite were investigated either at the level of the repetition number of the 28 bp core element, or its sequence creating a detectable restriction fragment length polymorphism (RFLP). Breast lesions were found to carry P1 homozygosity in 70% of the samples whereas this genotype appeared in only 38% of the normal blood samples. Therefore, it is suggested that P1 homozygosity could represent a potential risk factor for tumorigenesis of the breast.

## MATERIALS AND METHODS

### H-ras Intron 1 (HRM) region

The study population included 56 patients with breast cancer from Greece. Directly after dissection the specimens were stored at -70°C until DNA extraction. Peripheral blood was obtained from 61 healthy women aged  $\geq 55$  years with no known breast lesions.

### VTR region

Specimens from 30 patients with sporadic invasive breast carcinomas were obtained immediately after surgery and stored at -70°C. Samples consisted of tumor tissues and histologically normal counterparts located distantly from the tumor site.

### DNA extraction from breast tissue and blood samples

DNA extraction was performed under a standard protocol using organic detergents (6). Genomic DNA was extracted from blood and tissue using proteinase K, followed by phenol extraction and ethanol precipitation according to standard procedures. DNA was resuspended in 50  $\mu$ L of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Working stocks were prepared by 10-fold dilution in double-distilled H<sub>2</sub>O.

### PCR amplification of the 5' hexanucleotide polymorphic region of H-ras

The polymorphic region of the H-ras gene was PCR-amplified from the genomic DNA of both breast tissues and blood samples for amplification of the P1, P2 and P3 alleles using primer pairs 5' CTGTGGGTTTCCCCTTCA-GA 3' and 5' CTCCTACAGGGTCTCCTGCC 3'

In each PCR reaction two blank samples were used

as negative controls to ensure that no contaminants were introduced. PCR was performed in a 25  $\mu$ L reaction volume containing 200 ng genomic DNA, 1  $\mu$ M of each primer, 250  $\mu$ M dNTPs, 10% DMSO, 2.5  $\mu$ L of 10x buffer and 1 unit of Taq DNA polymerase (Invitrogen). The mixture was heated for 1 min at 95°C and samples were subjected to 30 cycles of amplification at 94°C for 40 sec, 63°C for 35 sec, and 72°C for 40 sec. Elongation was at 72°C for 5 min. PCR products were analyzed on a 10% acrylamide gel and photographed on a light transilluminator.

### VTR analysis

The primers used for amplification of the VTR alleles were 5'GAGCTAGCAGGGCATGCCGC 3' and 5'AG-CACGGTGTGGAAGGAGCC 3'. PCR was performed in a 50  $\mu$ L reaction volume containing 200 ng genomic DNA, 1  $\mu$ M of each primer, 250  $\mu$ M dNTPs, 10% DMSO, 5  $\mu$ L of 10x buffer and 1 unit of Taq DNA polymerase. In every PCR reaction two blank samples were used as negative controls to ensure that no contaminants were introduced. The mixture was heated for 1 min at 95°C and samples were subjected to 30 cycles of amplification at 94°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec. Elongation was at 72°C for 5 min.

In order to detect alterations at the 28 bp core repetition unit, 15  $\mu$ L of the PCR product was electrophoresed on 2% agarose gel and stained with ethidium bromide. The generation of novel VTR alleles was interpreted by comparison of the amplification patterns of the pair for each specimen.

Polymorphisms within the 28 bp core were detected by comparison of the RFLP pattern, after digestion with *Bst*NI endonuclease (New England Biolabs), which recognizes one site within the core element. The PCR product (20  $\mu$ L) was digested with 15 U of enzyme in a buffer recommended by the supplier. RFLP products were electrophoresed in a 10% polyacrylamide gel, stained with silver and visualized on a light transilluminator.

## RESULTS

The presence of amplifiable DNA, using primers for a fragment of the  $\beta$ -globin gene, was confirmed in all tissue and blood samples (data not shown).

### Analysis of the hexanucleotide locus in normal samples

To analyze the H-ras intron-1 polymorphism, we used a PCR-based assay that specifically detects P1, P2 and P3 alleles. The primer pair gives a PCR product of 106 bp for the P2 allele, 112 bp for the P3 allele and 118 bp for the P1 allele (Fig. 1) (5, 6). The results obtained from the 61 normal blood samples are shown in Tables I

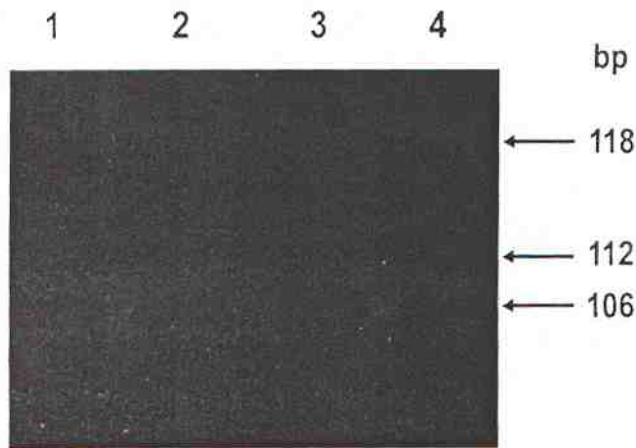


Fig. 1 - *H-ras* intron-1 allele amplification products (106, 112, 118 bp) obtained with PCR. PCR products were electrophoresed through a 10% acrylamide gel. Lane 1: P1/P2, lanes 2 and 3: P2/P2, lane 4: P2/P3.

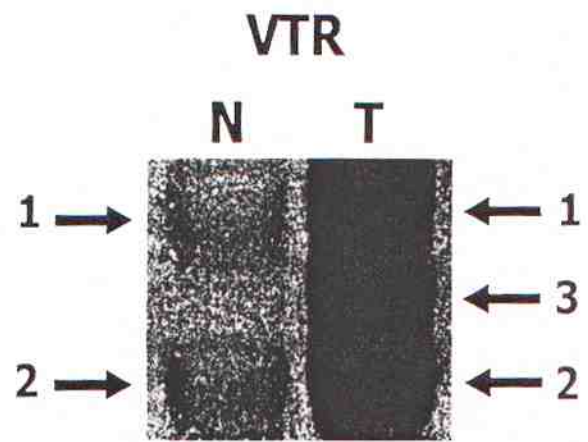


Fig. 2 - Example of specimen exhibiting microsatellite instability for VTR. N = normal DNA, T = breast cancer sample. Black arrows and numbers 1, 2 indicate each allelic band. Black arrow and number 3 indicate the novel generated allele.

TABLE I - FREQUENCY OF P1, P2, P3 ALLELES IN NORMAL BLOOD SAMPLES

Histological diagnosis	P2/P2	P2/P3	P2/P1	P3/P3	P3/P1	P1/P1
Blood samples	1	1	21	1	12	25
Total (%)	2	2	35	2	20	39

TABLE II - FREQUENCY OF P1, P2, P3 ALLELES IN SPORADIC BREAST CANCER SAMPLES

Histological diagnosis	P2/P2	P2/P3	P2/P1	P3/P3	P3/P1	P1/P1
Breast cancer samples	7	0	5	0	3	41
Total (%)	12	-	9	-	6	72

and III. Examination of the hexanucleotide locus revealed the presence of three alleles: P1 (68%), P2 (20%) and P3 (12%), which were amplified and isolated.

*Analysis of the hexanucleotide locus in breast cancer lesions*

In this study 56 samples of sporadic invasive breast carcinomas were also analyzed for the presence of genetic alterations of the *H-ras* HRM region. Examination of this locus showed the presence of all three alleles: P1 (80%), P2 (17%) and P3 (3%) (Tab. II, III).

P1, P2 and P3 allele distribution was different in women with sporadic breast cancer compared to control women. The allele frequency of P1/P1 homozygosity

TABLE III - FREQUENCY OF P1, P2, P3 ALLELES IN BLOOD SAMPLES FROM NORMAL WOMEN AND BREAST CANCER SPECIMENS

Alleles	Normal (%)	Breast cancer (%)
P1	68	80
P2	20	17
P3	12	3

was much higher in sporadic cancer lesions (72%) than in control samples (39%). The P3/P3 frequency was only 3% in the cancer group.

### VTR analysis

Alteration of the VTR minisatellite at the 3' end of the H-ras gene was detected in one among 30 cases (3.3%) (Fig. 2). In this case a shift in the mobility of the VTR alleles was observed after comparison of the amplification patterns of the VTR between normal and breast cancer tissue. Since changes in the repetition number of the 28 bp core, when occurring in a small number, cannot be detected in a 2% agarose gel, there may be an underestimation of the reported frequency of instability. Polymorphism of the BstNI pattern was not observed. Since BstNI recognizes only 5 bp among the 28 bp of the core, it could be assumed that the incidence of mutations of the VTR region might be higher. The case with the shift in the mobility of VTR allele was negative for BstNI, providing indirect evidence that such shifts should be attributed to alterations of the repetition number of the 28 bp core.

### DISCUSSION

Breast cancer is the most common cancer in women in Europe (11). A subset of molecular alterations has been associated with the development of the disease. H-ras is polymorphic at intron-1, so it may contain either a P1, P2 or P3 allele at this position. We have examined a hexanucleotide polymorphic region located in intron 1 of human H-ras (5, 6, 12, 13). The three alleles (P1, P2 and P3) were detected in a series of normal blood samples from 61 non-related individuals and 56 histologically malignant breast tissue specimens.

Our results confirm the difference in the distribution of P1, P2 and P3 alleles between breast lesions and controls as well as within each group (normal/sporadic breast cancer). In the control group, P1 was found in 68% and P3 in 12%. P1/P1 homozygosity and P2/P1 were the most frequent types (39% and 35%, respectively).

However, in breast cancer lesions P1 was also the most and P3 the least frequent allele (80% and 3%, respectively). P2/P3 and P3/P3 allele combinations were not observed and P1/P1 homozygosity was the most frequent (72%). There was a significant overrepresentation of P1 (72%) in breast cancer samples. Our results indicate that the presence of P1 homozygosity, an increase in P2/P2 and/or absence of P3/P3 in HRM analysis may represent potential risk factors for breast carcinogenesis.

Nevertheless, it should be noted that the HRM polymorphic region is linked with the H-ras VTR locus at the 3' end of the gene (4). This linkage could be the result of a low recombination frequency between the two sites due to the short genomic distance separating them (~4 kb). This linkage has been assumed to affect normal H-ras function (4, 6).

In the present study we investigated the presence of genetic instability in the VTR region of H-ras in 30 pairs of normal and breast cancer tissues. We analyzed for alterations and shifts of the mobility of the VTR alleles. Instability at the repetition number of the core was only found in one of 30 cases (3.3%). Although microsatellite instability was not a common event, it could be attributed to the activation mechanism for the H-ras oncogene in breast cancer. Polymorphism in the BstNI recognition site of the 28 bp consensus core element was not observed. Potential mutations could alter the affinity of transcription factors, thus dysregulating the expression of the H-ras proto-oncogene.

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