Ha-ras-1 restriction fragment length polymorphism and susceptibility to colon adenocarcinoma

L. Ceccherini-Nelli, V. De Re, A. Viel, G. Molaro, L. Zilli, C. Clemente, and M. Boiocchi

Summary

It is not yet clear whether some polymorphic variants of the Ha-ras-1 gene confer genetic predisposition to cancer. However, recent data on myelodysplasia and lung cancer are controversial. To clarify this point, 62 colorectal adenocarcinoma patients were examined for the Ha-ras-1 gene restriction fragment length polymorphism and results were compared with those of 108 healthy blood donors. No Ha-ras-1 polymorphic variants specifically associated with the cancer patients were detected. However, a specific genotype was significantly more frequent in the healthy donors than in the cancer patients (16% versus 5%), suggesting an interaction between the two alleles of the gene.

Materials and methods

Subjects

Colorectal adenocarcinoma tissue samples and peripheral blood leucocytes (PBL) were obtained during surgery from 62 CCP. Normal PBL were obtained from 108 HBD at the Transfusion Center of the Pordenone Hospital and used as controls to determine frequency of the Ha-ras-1 alleles in a normal population.

DNA extraction

DNA was extracted from carcinoma samples, from PBL of CCP, and from PBL of HBD according to the method of Wong-Staal et al. (1979).

Probe DNAs

Plasmid p344 carrying the normal Ha-ras-1 human gene (Pulciani et al., 1982) was grown and purified by standard methods. Two fragments were used to study the polymorphism of the VTR region: (a) the BamHI 6.6 kilobase pair (kb) fragment encoding the complete Ha-ras-1 sequence and the VTR region and (b) the 1 kb Mspl and HpalII fragment encoding the VTR region. The p344 fragments were purified by preparative agarose electrophoresis and the low melting agarose procedure. Both probes were 32P labelled by nick translation at specific activity >10⁶ cpm μg⁻¹.

Southern analysis

Genomic DNAs (10 μg) were digested to completion with the appropriate restriction enzymes, as specified by the commercial supplier. Digested DNAs were subjected to electrophoresis on horizontal 0.7% or 1.0% (w/v) agarose gels in 40 mM Tris acetate, 20 mM Na acetate and 2 mM EDTA buffer pH 7.6. Denatured DNA fragments were blotted onto a Gene Screen Plus (New England Nuclear, Firenze, Italy) following standard procedures, as described by Southern (1975).

Hybridization, washing and autoradiography were carried out as described by Ceccherini-Nelli et al. (1982) HindIII-digested 2DNA and HaelII-digested φX 174 DNA were used as size markers.
Results

Variation in the length of the VTR region generates RFLP of the Ha-ras-1 gene

In human genomic DNA, BamHI restriction enzyme generates restriction fragments containing the complete functional Ha-ras-1 gene. These fragments range in size from 6.6 to 8.2 Kb (Figure 1a) owing to the variation in length of the VTR region of the gene. This is clearly demonstrated by digestion of the human genomic DNAs by the two isoschizomers Mspl and HpaII, which cleave the Ha-ras-1 gene just outside the VTR region and generate a restriction fragment from each Ha-ras-1 allele, whose length is proportional to that of the corresponding BamHI restriction fragment (data not shown).

Sequential digestions of genomic DNAs, first with the Mspl and HpaII restriction enzymes and subsequently with the TaqI restriction enzyme, followed by hybridization with the 1Kb VTR-specific probe showed two different behaviours of the VTR region from different Ha-ras-1 alleles: some Mspl and HpaII VTR-containing fragments were only slightly reduced in size by TaqI digestion (Figure 2, samples I-II-III-IV and the 2 Kb fragment of sample V) whereas other Mspl and HpaII VTR-containing fragments were cleaved by the TaqI enzyme into small fragments of 800 and 650 bp (Figure 2, sample V fragment of 2.5 Kb). Presumably, three fragments were produced, two of which comigrated in our gels. Results therefore indicated that human Ha-ras-1 alleles could be divided into three main types, as depicted in Figure 3. Type A represents the molecular asset of the more abundant Ha-ras-1 allele characterized by the shorter VTR region; type B includes those alleles characterized by amplification of the VTR region; type C is characterized by unique amplification of the VTR region with concomitant reiteration of TaqI sites inside the VTR amplified region.

Figure 1 Southern analysis of representative genomic DNAs from colonic adenocarcinoma patients and healthy blood donors digested with (a) BamHI and (b) TaqI restriction enzymes, probed with the p344 Ha-ras-1 gene.

Figure 2 Southern analysis of representative genomic DNAs from colonic adenocarcinoma and healthy blood donors digested with HpaII and Mspl, lane 1, and HpaII and Mspl plus TaqI, lane 2, probed with the 1Kb VTR-specific fragment of the p344 Ha-ras-1 gene.

Genotype and allele distribution in patient and control group

RFLP was generated in normal PBL DNAs and in tumoral DNAs by digestion with BamHI and TaqI restriction enzymes and resolved by molecular hybridization with the BamHI fragment of the p344 plasmid. Digestion by BamHI gave fragments which ranged in size from 6.6 to 8.2 Kb and which we separated into four classes of 6.6, 7.2, 7.8 and 8.2 Kb. Despite some heterogeneity, we did not separate BamHI alleles into more classes due to difficulty in ascertaining whether variations in the range of 0.1-0.2 Kb were due to effective differences in dimensions or to experimental variability. However, BamHI mapping was useful to indicate homozygosity or heterozygosity of the genotypes and to confirm TaqI restriction maps (Table 1). TaqI digestion of the Ha-ras-1 gene generated invariable and variable fragments (Figure 1b). The variable fragments that contained the VTR region ranged in size from 2.3 to 4.2 Kb. The VTR-containing fragment of 2.3 Kb comigrated with the major invariable fragment of 2.3 Kb (Figure 1b, lanes f, h, i). However, it could be identified by its association with two small variable fragments of 0.8 and 0.65 Kb and by the presence of a fragment of 7.8 Kb in the corresponding BamHI restriction maps (Table 1).

As seen in Figure 1b, TaqI RFLP resolved at least 8 variable fragments: 2.3 (plus 0.8 and 0.65 Kb), 2.5, 2.7, 2.9, 3.0, 3.7, 4.0 and 4.2 Kb, with some degree of microheterogeneity within each class. TaqI restriction maps were much more resolvable than the ones obtained with BamHI. We
therefore classified Ha-ras-1 alleles in accordance with TaqI-generated fragments.

Table I shows that all the Ha-ras-1 genotypes, given by BamHI and TaqI restriction enzymes both in normal and patient populations, were in accordance with the previously depicted model based on the amplification of the VTR region as the origin of the different polymorphic variants of the gene (Figure 3). Only 5 out of 340 alleles analyzed generated restriction fragments that did not follow the proposed scheme. We supposed that mutations at the relevant restriction sites could generate the observed discrepancies.

Table II gives TaqI generated allele frequencies in CCP and HBD populations. Statistical analysis performed by the chi square test on allele frequencies shown in Table II did not indicate that any allele was significantly more frequent in CCP than in HBD. In contrast the genotype type IV (Table I) was significantly more frequent in HBD than in CCP; it appeared in 17/108 HBD versus 3/62 CCP $\chi^2=4.48$; $P=0.034$.

![Figure 3](image-url)
Finally, analysis of Ha-ras-1 RFLP in matched DNA samples derived from tumour and PBL of single patients showed identical restriction patterns by several restriction enzyme digestions.

In no case was evidence found of an allele lost in colon carcinoma tumoral DNA.

**Discussion**

The Ha-ras-1 protooncogene is highly polymorphic in a human population, mainly due to a hypervariability in the length of the VTR region localized at about 1.5 Kb from the the 3' terminus of the gene (Capon et al., 1983). No defined biological properties have been associated with the VTR regions. However, the characteristic of this region (that is 28 bp consensus sequence reiterated 29 times in the p34 gene) suggests that the VTR region could have an important function in the regulation of the expression of this gene. This hypothesis is supported by the report of Krontiris et al. (1985), who found that EJ-ras subclones lacking the VTR region are expressed 5- to 10-fold less than the original clone. Moreover, Ishi et al. (1986) reported that VTR acts as an enhancer element of the Ha-ras-1 gene and that specific conformations of this region have stronger enhancer activity.

Ha-ras-1 alleles are inherited in a Mendelian fashion. This circumstance has been utilized by different authors to ascertain genetic susceptibility to cancer diseases conferred by this gene.

Krontiris et al. (1985) described the association of rare Ha-ras-1 alleles with tumours of different histological types. Heinig et al. (1986) reported the lack of an association between Ha-ras-1 alleles and myelodysplasia. Heigl et al. (1986) found a significant association between a specific Ha-ras-1 allele and non-small cell carcinomas of the lung when compared to unaffected controls and small cell carcinoma patients.

In the present report we analyzed Ha-ras-1 gene RFLP in 62 CCP and 108 HBD. Since the median age of the control group was lower than the age at which colon cancer becomes clinically evident, we concluded that allele distribution in our control group represented that present in a general population. The data obtained from the present study indicated that no significant association exists between any Ha-ras-1 allele and predisposition to colorectal cancer. In fact, the observed frequencies of the four most abundant alleles were almost identical in patient and control groups. As regards the rare alleles, it is impossible, by this study, to define their influence in this pathology due to their very low frequency observed in the patient group. In contrast, our data seem to suggest that the genotypic asset at the Ha-ras-1 locus could have some influence in determining resistance to the development of colon cancer. In fact, the type IV genotype appears to be more frequent in the HBD than in the CCP group. The meaning of this finding is not yet clear. However, it could indicate that some interaction between the two alleles exists, with a consequent reduction in the frequency of colon carcinoma development in people carrying this genotype. If this situation is confirmed in a larger number of patients and in other tumour types, it could represent an important model to study at the molecular level.

More information will result from these studies when it is possible to link the gene structure analyzed here with defined biological properties; for the present, it is very important that investigators working on this topic decide on a standardized approach. In fact, the presently published studies are very heterogenous: Ha-ras-1 alleles range from 5 or 6 to more than 20 according to different authors, and this situation makes it very difficult to compare data obtained in different laboratories.

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**Table II** Ha-ras-1 allele frequencies

| Size of TaqI fragment (Kb) | Colon adenocarcinoma patients | Healthy blood donors | Total |
|---------------------------|-------------------------------|----------------------|-------|
| 2.5                       | 78 (63)*                      | 139 (64)             | 217 (64) |
| 2.3 + 0.8 + 0.65          | 19 (15)                       | 25 (12)              | 44 (13) |
| 3.7                       | 13 (10)                       | 24 (11)              | 37 (11) |
| 3.0                       | 10 (8)                        | 21 (10)              | 31 (9) |
| 2.7                       | 2 (2)                         | 3 (1)                | 5 (1) |
| 2.9                       | 1 (1)                         | 0 (0)                | 1 (0.3) |
| 4.2                       | 1 (1)                         | 3 (1)                | 4 (1) |
| 4.0                       | 0 (0)                         | 1 (0.4)              | 1 (0.3) |

The table refers to allele frequencies and not to individuals carrying the various alleles.

*Figures in parentheses are percentages of the totals.

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