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Immunological Implications of Alterations in the c-Ki-ras and p53 Genes in the Stepwise Progression of Colorectal Cancer: Indications for the Improvement of Prognosis, Biotherapy Treatment and Tumor Biology Understanding

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Alterations in gene structure and functions involving the c-Ki-ras and p53 genes have been shown to play an important role in the various stages of human colorectal carcinogenesis. However, how these gene alterations cooperate with tumoral mechanisms at an immunological level is not known. To this aim an immunological study of a group of healthy subjects, patients with p53 gene deletions (53D), with c-Ki-ras mutations (KrM) and no gene alterations (53D-KrM-) have made. In a previous study we found that a disregulation between TH1/Th2 cell functions seems to be implicated in the establishment and progression of colorectal cancer disease and that soluble interleukin (IL)-2Receptor (sIL-2R) serum level is involved in this. On this basis we investigated the immunological implications of p53 and c.Ki-ras gene alterations, evaluating the relationhips in the immune network between sIL-2R levels in the serum and immunological parameters (IL-2, IL-4 serum levels; CD3,CD16 and CD19 expression on the surface of peripheral blood mononuclear cells - PBMC). Our results suggest that, in the stepwise progression of colorectal cancer, the c-Ki-ras gene alteration is involved in a switch of the host immune response to a suppressive type which, as we have previously reported, may be a determining or concurrent cause of malignant transformation. Alteration in the p53 gene does not appear to ulteriorly impair the patients' immunological response. Our data supports the role of c-Ki-ras gene mutations and p53 deletions as prognostic markers in the passage of normal tissue to adenoma and adenoma to carcinoma respectively. Moreover, the evaluation of the mechanisms involved in the alterations of c-Ki-ras gene seems to be more important than that of p53 suppressor gene for the improvement of prevention, biotherapy treatment and tumor biology understanding.

Key words: Colorectal cancer, biotherapy, immunological parameters, c-Ki-Ras, p53.

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INTRODUCTION

Disturbances in gene structure and function have been shown to characterize the initiation, promotion, and progression of cancer in general,[1-6] and especially in colorectal cancer.[7-11] Point mutations, deletions, and chromosomal rearrangements involving specific oncogenes and tumor suppressor genes have been demonstrated to play an important role during the various stages of human colon carcinogenesis.[7-12] The gene most frequently altered is the c-Ki-ras gene which encodes a protein involved in the transduction of mitogenic signals across the cell membrane and mutations in this gene may therefore result in the establishment of a state of continuous stimulus for cell division.[13] Several groups have demonstrated that activated ras alleles are present in approximately 40% of cancers of the colon in the rectum and that whilst large adenomas contain activated ras oncogenes at a similar frequency (40%), smaller adenomas are less likely to contain such mutations (<20%),[14] indicating that ras activation is an early event occurring during the benign phase of the disease.

The most studied of the tumor suppressor genes is p53 which has been located on chromosome 17p[15] and encodes a protein which blocks the progression of cells through the cell cycle.[13,16] One of the documented roles of this protein on cell proliferation is the regulation of the transition from G1 to S phase of the cell cycle[17]; it can also cause cell death by apoptosis.[18] These regulatory functions may be mediated by the interaction of the p53 protein with specific DNA sequences[19,20] which in turn may allow regulation of other genes at the transcriptional level[21] or may have a role in initiating DNA replication.[22] DNA binding capacity, transcriptional activator function and initiation of DNA replication are altered when there is mutation and loss of heterozygosity in the p53 gene.[18,19,21,23] Point mutations and deletion of the p53 gene have been detected in several human tumors including colorectal carcinoma.[14,23,24] Previous studies have shown that the mutated form of the p53 is more stable than the wild type due, in part, to an increase in the half-life of the protein itself and the formation of stable complexes between the mutated protein and wild p53 proteins as well as other cellular proteins.[25,26] Loss of heterozygosity has been associated with an increase in the expression of the protein suggesting the presence of mutations in the remaining allele.[23,24] Moreover, the p53 tumor suppressor gene is recessive and so the overall effect of both alterations (gene mutations and loss of heterozygosity), is inactivation of p53 protein function. Loss of heterozygosity has been detected in 77% of colonic carcinomas but only in 6% of adenomas[14] showing that the loss of the p53 gene may be closely associated with the malignant transformation of adenomas.

Since establishment and progression of cancer is generally due to the malfunction of the host's immune response, we investigated the relationships between p53 gene deletion, c-Ki-ras mutations and the host's immune system in patients suffering from colorectal cancer.

Our previous results[27,28] have shown that the up/down regulation of the sIL-2R serum level within physiological ranges in healthy subjects has a role in the cytokine network between TH1 and TH2 cells and that sIL-2R levels in the peripheral blood of colorectal cancer patients may be the cause of the disregulation between TH1 and TH2 functions in the immune response.

So, we studied the relationship between the sIL-2R serum level and the IL-2 and IL-4 levels in the serum and CD3, CD16 and CD19 PBMC expression in a group of colorectal cancer patients and in healthy subjects. The statistical evaluation of the results was performed comparing the patient groups with the p53 deletion (53D) and the c-Ki-ras mutation (KrM), and then with the healthy subject group and patients with neither alterations (53D-KrM-).

MATERIALS AND METHODS

Patients and Healthy Subjects

Twenty-three patients (9 men and 14 women, aged from 46 to 85 years), who were diagnosed for the first time as having colorectal cancer and who had undergone colectomy were studied. Clinical diagnosis was confirmed histopathologically and patients were subtyped by pTNM classification according to the diagnostic criteria of the American Joint Committee on Cancer and the Committee of the International Union Against Cancer (Table 1).

Tumors varied from 2.5 to 9.0 cm in diameter. Seventy healthy subjects (40 men and 30 women, from 43 to 78 years of age) served as the control group. Age (N=36, 66±10 vs N=70, 64.5±9.1, p= 0.1) and sex (p=0.132) parameters were the same for both groups. Venous blood (donated by both patients and

Table 1

Clinical and histopathological data of patients with colorectal cancer

	53D		KrM		53D- KrM-	
Characteristic [a]	pTNM	Stage	pTNM	Stage	PTNM	Stage
Descending	T3N0M0	3			T4N1M0	2
	T3N0M0	2				
Ascending					T3N0M0	2
					T3N1M0	3
					T3N0M0	2
					T3N2M0	3
					T3N3M1	4
					T3N0M0	2
					T3N0M0	2
					T3N1M0	3
Rectum	T1N0M0	1	T3N0M0	2	T3N0M0	2
			T1N0M0	1	T3N1M0	3
Sigmoid			T2N0M0	2	T2N0M0	1
			T4N3M1	4	T2N0M0	1
					T3N1M1	4
					T4N0M1	4
Rectum/sigmoid			T2N0M0	1		

[a] Location of colorectal cancer

healthy subjects) was collected by venepuncture in syringes containing preservative-free heparin (Liquemin-Roche), 1 h prior to anaesthesia and surgery.

Tumor tissue and adjacent normal mucosa obtained during surgical exeresis were frozen in liquid nitrogen and stored at 80°C until analysis.

DNA Extraction

We analyzed 23 samples of colorectal cancer tissue and adjacent normal mucosa. High molecular weight DNA was obtained by the "salting-out" method and amplified by PCR amplification using specific primers for the first exon of the c-Ki-ras gene and for exon 4 of the p53 gene.

PCR Amplification

P53 Deletion

The fourth exon of the human p53 gene was amplified using the sense oligonucleotide 5'-AAT GGA TGA TTT GAT GCT GTC CC-3' and the antisense oligonucleotide 5'-CGT GCA AGT CAC AGA CTT GGC-3'. The amplification was performed for 32 cycles of denaturation at 95°C for 45 sec, annealing at 56°C –for 60 sec and extension at 72°C for 60 sec with a final extension at 72°C for 7 min.

Restriction fragment length polymorphism (RFLP) analysis

The amplified DNA was digested for 2 hours at 37°C using the restriction enzyme Mvn I (5U) in a 20 µl final volume of buffer solution.

Mvn I enzyme is able to define biallelic polymorphism (C or G) recognizing the CG↓CG palindrome in codon 72 (exon 4) of the p53 gene: A1=259 bp, A2=160+99 bp.

DNA fragments were separated by electrophoresis through a 2% agarose gel in TBE 1x. The presence of a p53 deletion was determined by RFLP analysis and loss of a heterozygous pattern.[23]

c-Ki-ras

The analysis of mutations in codons 12 and 13 of the c-Ki-ras gene was carried out using an enzymatic amplification (PCR) of a nucleotidic fragment of 110 pb with the following pair of primers (Clontech Laboratories):

5'-ATGACTGAATATAAACTTGT-3';
5'-CTCTATTGTTGGATCATATT-3'

in a 100 l of final volume and for 35 cycles of denaturation (94°C x 45 sec), annealing (49°C x 60 sec) and extension (72°C x 60 sec) with a final extension at 72°C for 7 min. As in PCR positive controls we used amplification products with degenerate primers, specific for various substitutions (Gly, Ser, Cys, Arg, Asp, Ala, Val).

Hybridization by ^{32}P-SSOs

110 bp PCR fragments were hybridized with fourteen ^{32}P sequence-specific oligos (SSO) complementary to the normal and mutant c-Ki-ras codons 12-13.

Filters were first washed at room temperature, and then at the adequate washing temperature in SSPE 0.1X, SDS 0.1% solution, and were exposed overnight to X-OMAT-AR (Kodak) at 80°C.

Detection of Cytokines and Soluble Molecules in the Serum Level

Sera from patients and the control group, was centrifugated at 250 g within an hour of withdrawal

together with cell free supernatant obtained *in vitro* and stored in aliquots at 80°C until use. ELISA assays were employed. This method has been described in detail elsewhere.[29] The sensitivities of these ELISA assays were as follows: sIL-2R <50U/ml, (T Cell Diagnostics-Cambridge,USA); IL-2 <5pg/ml, IL-4<1pg/ml (Endogen, Cambridge USA).

PBMC Preparation

PBMC cells were separated by centrifugation over a Ficoll/Hypaque gradient (20 min, 1000g), and washed with RPMI-1640 medium (Gibco). Isolated cells were cultured at a concentration of $1x10^6$cells/ml in RPMI-1640 complete medium (supplemented with 10% heat-inactivated human serum or foetal calf serum, L-glutamine 0.2 nM, penicillin 50 UI/ml, streptomycin 50 g/ml; Sigma).

Phenotypic Antigen Analysis

The expression of phenotypic antigens on the surface of PBMC was determined using monoclonal antibodies of CD3, CD16, CD19 receptors (Becton Dickinson). Briefly, the cells were washed twice in PBS and resuspended at $5x10^6$ cells/ml. 100 l was utilized for each marker. A 20 l sample of fluorescein-isothiocyanate-conjugated antibody was added, vortexed and incubated in ice for 30 min. The sample was then washed twice and resuspended in 0.5 ml cold PBS for subsequent fluorescent-activated flow cytometer analysis. (Cytofluorograf-IIs -Ortho Diagnostic Sytem Inc.)

Statistical Analysis

The results were expressed as means ± standard deviation and the differences between groups were assessed by the §Mann-Whitney U test, the *two-tailed Student's t-test and the Chi-square test as appropriate. The level of significance was set at $P< 0.05$.

To study the correlations between the sIl-2R level and other immunological parameters we performed a multivariate statistical analysis.

We used Covariance analysis which allowed us to discriminate the networks' positive and negative relationships between the sIL-2R serum level and other immunological parameters. The relationship between the sIL-2R serum level and each parameter is positive (according to the covariance method) if both levels fall above or below their means at the same time. However, the Covariance is negative if the sIL-2R level in the serum increases whilst the level of the immunological parameter decreases (and vice versa) at the same time. The Principal Components (PC) analysis indicates the degree of correlation between the sIL-2R serum level and other parameters within the network. This method plots the vectors of parameter weights in healthy and patient networks. The length of each vector is proportional to its network contribution and the angle between any two is inversely proportional to the correlation between them.

RESULTS

Analysis of KrM and 53D Gene Alterations

The tumoral tissue from 5 (22%) patients was found to contain mutant c-Ki-ras alleles; 3 (13%) patients showed loss of heterozygosity of the p53 gene and no alteration were observed in either gene in 15 (65%) patients.

Four of the 5 patients with ras gene mutations included 4 (80%) patients with mutations in codon 12: 2 (50%) from glycine to valine; 2 (50%) from glycine to aspartic acid. The mutation at codon 13 (20%) was a substitution of glycine with aspartic acid.

Immunological Parameters Determination

We previously found that statistical differences in immunological parameter in patients depended on the stage of the disease.[27,29] However in this study the statistical evaluation was independent of the disease stages parameter because the distribution of the stages in the various groups counterbalanced.

The values of sIL-2R, Il-2, IL-4, CD3, CD16 and CD19 are shown in Table 2.

The sIL-2R and IL-4 levels were significantly higher in 53D- KrM- group compared to the group of healthy subjects, while the IL-2 level was lower which supports our previously reported results. [27,28]

Moreover, the values of immunological parameters in the 53D group were not significantly different to those of healthy subjects, whilst an increase of sIL-2R and CD19 PBMC expression was found in the KrM- group.

When comparing the 53D and KrM groups with the 53D- KrM- group we found that 53D parameter values did not differ statistically whilst IL-2 serum level (*p=0.022) and CD19 PBMC expression (*p=0.015) KrM parameter values were higher.

TABLE 2

Immunological parameters in the group of patients and in the control group.

*Cytokine serum level (pg/ml) ** Phenotypic antigens (%)	Healthy Subjects		53D–KrM–		53D		KrM	
sIL-2R*	104	314.5±168	14	475.2±252 §p=0.006	2	465±288	4	621±113.3 §p=0.005
IL-2*	64	27.9±17.8	12	9.3±7.5 §p=0.00005	2	17.9±11.2	4	19.9±5.4
IL-4*	76	17.3±8.2	12	249±291 §p=0.014	3	26±30	4	33.7±22.5
CD3**	42	60.5±12.1	9	5.1±16	2	51±13.7	2	63±12
CD16**	39	11.6±7.9	7	9.1±7.9	2	12.4±0.85	3	15.2±12
CD19**	18	5.8±2.6	8	5.7±2	2	5.25±5.6	3	10.4±3 *p=0.013

§ * The statistical evaluation was performed comparing the 53D and KrM patient groups with each other and with the healthy subject group and 53D–KrM– patients. This was done using the most appropriate statistical test depending on the population distribution: * Student's t-test, §the Mann-Whitney U-test.

There were no significant differences in parameter values between the 53D and KrM groups (Table 2).

Correlations Between sIL-2R and Immunological Parameters

Covariance analysis of the sIL-2R serum level and other immunological parameters (Table 3) showed that in both healthy subject and KrM groups the sIL-2R level in the serum is positively related to IL-2 serum level and negatively to the IL-4 serum level.

However, the sIL-2R serum level in healthy subjects showed a positive relationship with CD16 PBMC expression, whilst this was not so far the KrM patient group.

However the relationship between the sIL-2R serum level and other immunological parameters was the same in the 53D and 53D-KrM- groups. There was also a negative relationship between the sIL-2R level and the IL-2 level and a positive relationship with the IL-4 level which is the opposite to what was seen in the healthy subjects and the KrM groups. Moreover there was a positive relationship between the sIL-2R level and CD16 PBMC expression which also existed in the healthy subject group but not however in the KrM group.

This situation was in concordance with the results of the Principal Components analysis showing that in both 53D and 53D-KrM- groups (Figure 1) the sIL-2R level in the serum has the same relationship with IL-2 and IL-4 serum levels and with CD3, CD16 and CD19 PBMC expression.

The sIL-2R level was more closely related to the IL-2 level than to the IL-4 level in the healthy subject group and the KrM group (Figure 2); in the KrM group however, the sIL-2R and IL-4 levels were closer than in healthy subjects. CD16 expression was related to the same degree to the sIL-2R level and CD3 in the healthy subject immune network whilst CD16 expression was principally linked to CD19 expression in the KrM group.

TABLE 3

Covariance analysis

*Cytokine serum level (pg/ml) ** Phenotypic antigens (%)	sIL-2R*				IL-2*				IL-4*				CD3**				CD16**			
	h	°	°°	°°°	H	°	°°	°°°	h	°	°°	°°°	H	°	°°	°°°	h	°	°°	°°°
CD19**	−	−	−	−	+	−	+	+	+	+	−	−	+	+	+	+	−	+	−	−
CD16**	+	−	+	+	−	−	−	−	+	+	+	+	−	+	−	−				
CD3**	−	−	−	−	+	−	+	+	+	+	−	−								
IL-4*	−	−	+	+	+	−	−	−												
IL-2*	+	+	−	−																

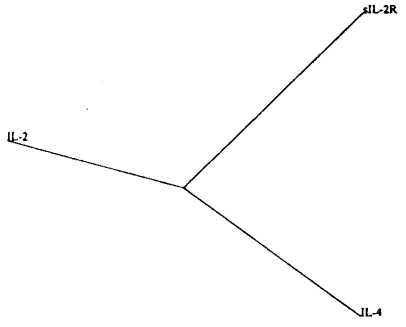
[h] Healthy subjects °KrM patients °°53D patients °°°53D− KrM− patients

DISCUSSION

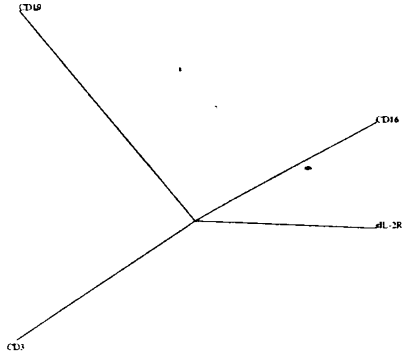
A number of stages of colorectal cancer are recognised, starting from normal mucosa and progressing in a stepwise fashion through to hyperproliferation, adenoma, primary carcinoma and ultimately metastasis.[13] Two classes of genes are essentially involved in the evolution of colorectal cancer: oncogenes and suppressor genes. The K-ras and p53 are, respectively, the oncogene and the tumor suppressor gene most

FIGURE 1 Principal component analysis plots of the immunological parameters

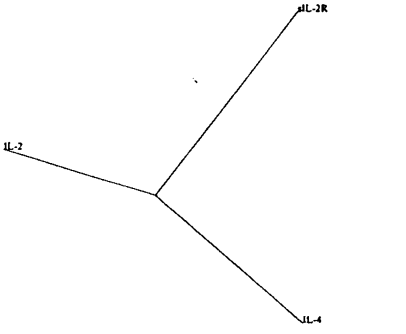
Cytokine serum levels of the 53D group



Expression of phenotypic antigens of the 53D group



Cytokine serum levels of the 53D−KrM− group



Expression of phenotypic antigens of the 53D−KrM− group

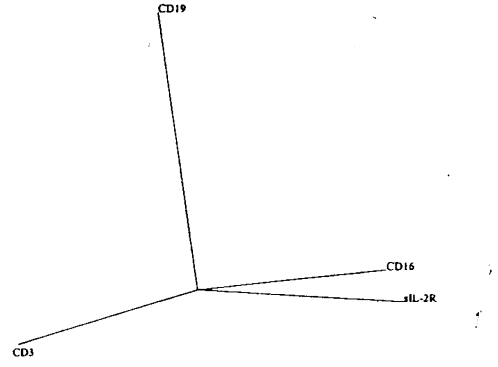
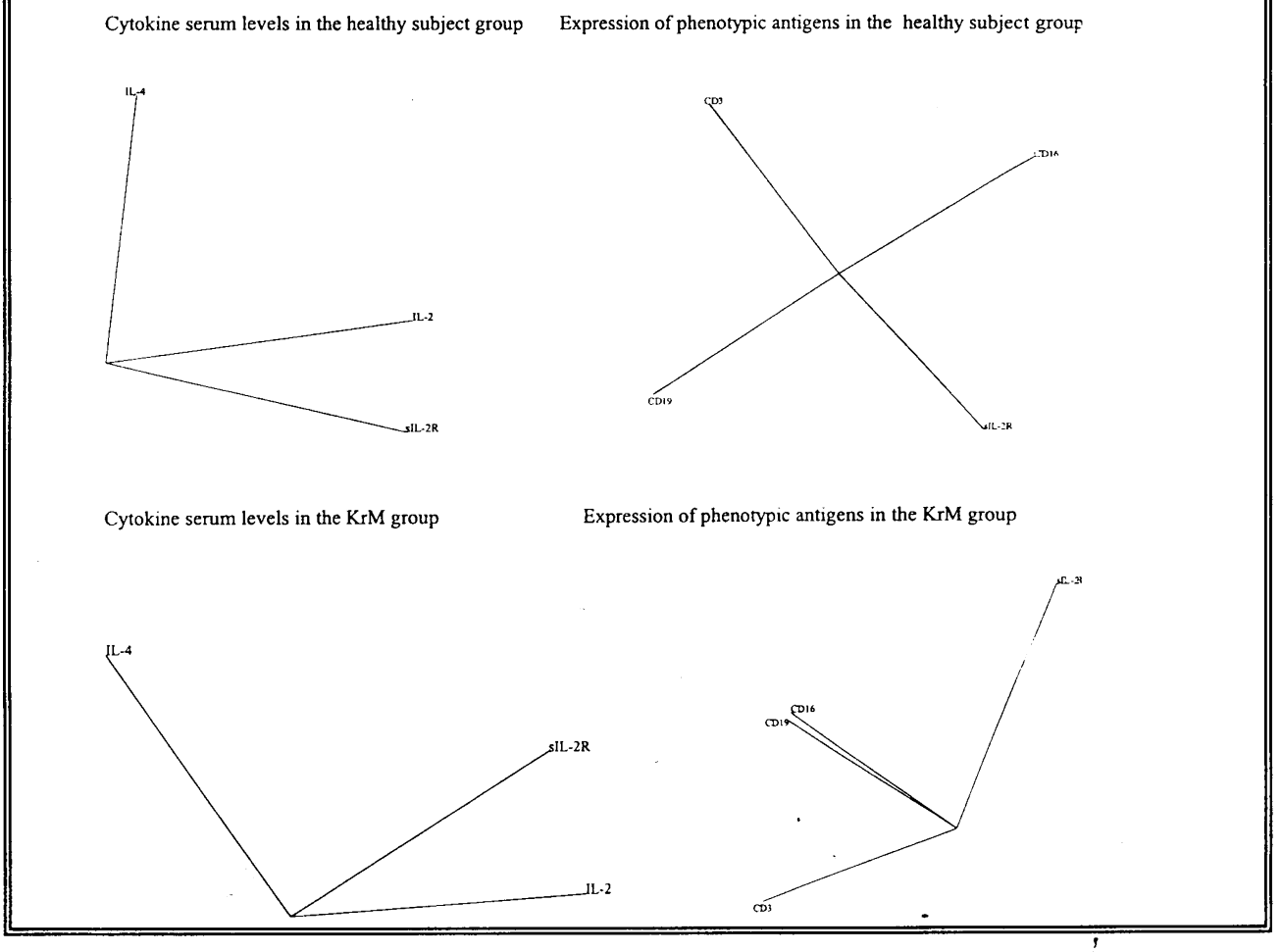


FIGURE 2 Principal component analysis plots of the immunological parameters



frequently altered in colorectal cancer but how these gene alterations cooperate with tumoral mechanisms at an immunological level is not known. In order to evaluate this an immunological study of healthy subjects and 53D, KrM and 53D-KrM- groups was made.

The establishment and progression of cancer is generally possible due to a malfunction of the host immune response. In a previous study we found that a disregulation between TH1 and TH2 cell functions was implicated in this malfunction[27] and that the sIL-2R serum level was involved in this disregulation. The sIL-2R serum level in healthy subjects seems to contribute to the operation of the cytokine network between TH1 and TH2 cell functions.[28]

On this basis, we investigated the immunological implications of p53 deletion and c-Ki-ras gene mutations, evaluating the immune network relationships between the sIL-2R level in the serum and IL-2, IL-4 serum levels and CD3, CD16, CD19 PBMC expression.

Our results show that the sIL-2R and IL-4 levels in the serum were higher in the 53D-KrM- group than in healthy subjects, whilst the IL-2 level was lower, supporting our previously reported data on sIL-2R and TH1/TH2 cell function disregulation in 53D-KrM- patients.

In addition, the immunological parameter values in the 53D group were not significantly different to those of the 53D-KrM- group suggesting that a gene alteration at this level does not ulteriorely impair TH1 and TH2 functions and T, NK and B cell number relationships in the host immune response network.

There were higher IL-2 level values in the KrM group than in the healthy subject group; CD19 PBMC expression was also greater (Table 2), and these differences were statistically significant compared to those of the 53D-KrM- group. These results indicate

that a mutation in the K-ras gene results in a switch from the immune response in a healthy subject to a suppressive response. The reason for this is that a phenotypical increase of B cells in the immune response seems to be detrimental to the cell-mediated immune response and so to TH1 function.[31]

This situation is supported by the results of our multivariate statistical analysis. In fact, the covariance study (Table3) showed that the parameters in the immunological network of 53D and 53D-KrM- groups had the same weighting. Whereas, the positive relationship between sIL-2R and IL-2 serum levels in the KrM group seems to have been re-established as in healthy subject groups but the relationships between the sIL-2R level and T, NK and B cells were all negative suggesting an immunosuppressive situation. The Principal Component analysis clarified these results underlining that in both 53D and 53D-KrM- groups (Figure 1) the sIL-2R serum level showed the same degree of relationship with all the immunological parameters. In healthy subjects and KrM patients (Figure 2) on the other hand, the Principal Component analysis indicated a similar close relationship between sIL-2R and IL-2 levels in the immune network; CD16 expression in the former group was related in the same manner to sIL-2R and CD3 expression, and in the latter group principally to CD19 suggesting an immune suppressive situation. In fact, in the immune system network the B cells are involved in a suppressive type of the response.[30]

On the basis of the above considerations the results of our immunological study suggest that in the stepwise progression of colorectal cancer the c-Ki-ras gene alteration is involved in a switch of the host immune response to a suppressive type which, as we previously reported [27,28] may be a determining or concurrent cause of malignant trasformation. p53 alterations on the other hand do not seem to further immunologically impair patient response. These results agree with data indicating that c-Ki-ras gene alterations occur during the adenoma phase of the disease and the p53 deletion in the invasive transformation of adenomas.[14] In fact, from our results it would appear that c-Ki-ras gene alteration is implicated in the switch of the host immune response toward a suppressive type which would allow adenoma growth; whilst, p53 gene function impairment would appear to result in loss of control of cellular growth related to the progression in the invasive phase but not to immunological impairment. Considering the research data on cell lines[32] which show that p53 gene alteration can contribute to cell malignant transformation in the context of ras oncogenes but when transfected alone the cell lines do not have transformed morphology, our results suggest that the immune suppressive situation concurrent with a c-Ki-ras mutation could be a determining factor in the carcinogenesis process.

To conclude, the results of our study support the role of c-Ki-ras and p53 alterations as prognostic markers in normal tissue to adenoma and adenoma to carcinoma passages respectively. However, a study of the mechanisms involved in c-Ki-ras gene alteration would appear to be more important than those of the p53 gene for both the improvement of prevention and biotherapeutic treatment.

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